LEUCOCYTE & ENDOTHELIAL CELL BIOLOGY: THE MOLECULAR BASIS OF INFLAMMATION

A thesis submitted for the degree of

Doctor of Science

in

The Faculty of Science
of
The University of Adelaide

by
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ABSTRACT

This thesis describes a body of work that has significantly progressed and perhaps revolutionised the understanding of the cellular basis of inflammation. The author has been responsible for the description of the activation of endothelial cells by the cytokine TNF, a process that underlies the essential pathological events of leukocyte adhesion and transmigration, and also for the description of leukocyte activation, a process that intensifies the inflammatory reaction. More recent studies on the structure of cytokines, the regulation of their production and the signals they mediate has identified novel therapeutics molecules or candidates for such molecules. The author has recently been awarded the ISI-Thomson Scientific 'First Citation Laureateship' for work described in this thesis.

STATEMENT

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Mathew Alexander VADAS

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ACKNOWLEDGEMENTS

None of this work would have been possible but for the help and support of my family and colleagues. 'Thank you' seems inadequate to acknowledge the role of Jennifer Gamble, who is not only my wife and mother of our children, Camilla and Cecile, but also a colleague *sans pareil*. Angel Lopez has been a long-term collaborator, critic and friend, and more recently Frances Shannon, Peter Cockerill and Pu Xia have contributed extensively.

I also thank my parents for their unfailing support and belief in me and my son Alexander who is developing from a relative into a colleague.

Finally, I thank Anna Nitschke, who has not only helped assemble the thesis, but in her quiet and unassuming fashion, ensured that it is done.

LEUCOCYTE & ENDOTHELIAL CELL BIOLOGY:

THE MOLECULAR BASIS OF INFLAMMATION

INTRODUCTION

In submitting this body of work for the Doctor of Science degree at the University of Adelaide, I have highlighted the chief strand of my research, that is concerned with the molecular and cellular phenomena underlying the inflammatory process. This represents an internationally recognised contribution not only to Science but also to medicinal development as it has generated and continues to generate new intellectual property and therapeutic agents.

The Thesis summarises the work and provides a full Curriculum Vitae of the candidate before submitting reprints of key publications.

HISTORICAL BACKGROUND

Since the time of Waller (1847), a pupil of Magendie, the cellular events associated with inflammation were recognised. Then, some 150 years ago, Waller reported "The active migration of white blood corpuscles through the walls of capillary blood vessels". He chronicled, as the initial cellular event, "the arrest of leucocytes on the wall of blood vessels near an inflammatory stimulus". Once the leucocytes were arrested, they moved through the endothelial lining (transmigration) into the tissues where they engaged the inflammatory agent either by engulfing it or by elaborating toxic products that resulted in local damage.

Subsequently the relative importance to the inflammatory process of the "white blood corpuscle" and the "blood vessels" were hotly debated. Julius Cohnheim (1878), a pupil of Virchow, emphasised the role of blood vessels: "Process of migration (through the vessel wall) of white blood corpuscles is the essence of inflammation" ("Vorlesungen über Allgemeine Pathologie"). In contrast, Élie Metchinkoff (1884) was leucocyte centered in his "Phagocytic Theory of Immunity" ("Leçons sur la pathologie comparée de l'Inflammation").

Over the ensuing years, three steps in the inflammatory process became more formally described. These were:

1. Adhesion to endothelium
2. Transmigration through endothelium
3. Recognition of the irritant

However, this description failed to provide significant insights into the mechanisms that govern the process and as such the development of therapeutics remained largely empirical.

The work described in this thesis addresses each of these steps. The author claims not only to have added significantly to the understanding of the mechanisms involved but also to the development of novel reagents that interrupt these events and thus have particular therapeutic usefulness.

The work described in this thesis began approximately twenty years ago, at a time when the existence of growth factors or cytokines was just becoming accepted, but their molecular characters were not known. It was also the time when the cell biological tools of cell culture and surface phenotype analysis were robust enough to provide a sound basis from which the inflammatory process could be investigated.

CITATION LAUREATE

The work presented herein has been recently recognised by the award of a Citation Laureateship to the author in 2001 by ISI Thomson Scientific. The award was based on having a 'top 200' publication in one's discipline in 7 different years since 1981. The author has been cited more than 13000 times in that period. Of the 33 Citation Laureatships awarded, there were seven in the author's area of Immunology - and the author was one of two practicing physicians in the whole group to be thus recognised.

PRECIS OF AUTHOR'S CONTRIBUTION TO UNDERSTANDING THE INFLAMMATORY PROCESS

1. Adhesion to Endothelium

The mechanism of adhesion of leucocytes to endothelium has puzzled scientists over the century. Indeed there was continual debate whether the process was initiated by changes in the leucocyte becoming more 'sticky' or by changes in the endothelium that allowed leucocyte adhesion.

(a) *Cytokine Network*

In 1985, the author produced clear evidence showing that the endothelium was actively involved in the inflammatory process. The work arose from a hypothesis that locally elaborated growth factors or cytokines are responsible for inducing endothelial adhesiveness and described, in a seminal paper [now a citation classic, cited more than 1045 times], that the cytokine, tumour necrosis factor α (TNF), stimulated endothelial cells to become adhesive for neutrophils. This paper [1] documented, for the first time, the plasticity of endothelial cells as far as adhesiveness for leucocytes is concerned, and showed that the changes needed new protein synthesis. The drive for the development of clinically useful TNF antagonists such as Embrel and Remicade came at least partly from this observation. This observation also led to an intense program to discover the proteins that were responsible for such an event, resulting in the discovery (by others) of adhesion proteins such as E-selectin, VCAM-1 and P-selectin.

The author's second important contribution in this area was stimulated by the hypothesis that the simple presence or absence of a pro-inflammatory cytokine such as TNF is too simple a control system for an important pathological mechanism such as inflammation. In an important paper in Science [2] [and followed up by several in the Journal of Immunology [3, 4]], it was demonstrated that another cytokine, TGF-β, that is chronically present in the vessel wall, is able to inhibit TNF's pro-inflammatory effects preventing cellular adhesion and the expression of adhesion proteins. Those observations highlighted that a network of factors is involved in the setting of endothelial phenotype and that the relative quantity and timing of those factors governs the inflammatory response.

(b) *Endothelial - smooth muscle cell interactive unit*

Besides TGF-β, two other key mediators of endothelial phenotype were discovered. The first discovery related to the bigger picture of endothelial monolayer integrity and what is the purpose of the cellular junctions that ensure a continuous covering of vessel walls. Hitherto these junctions were thought to provide a mainly mechanical link and barrier to the passage of cells and solutes. However, in a paper in the Journal of Cell Biology [5], the author's group showed that endothelial cell junctions actually deliver an anti-inflammatory signal to endothelial cells and furthermore that the junctional molecule (PECAM-1) is the chief mediator of this process. This work is now being further expanded in experiments and discovered that the cytokine Angiopoietin-1 controls the integrity of these cell junctions [6]. As a further elaboration of this concept, the authors also demonstrated that smooth muscle cell-endothelial interactions were also important in sustaining a non-inflammatory state of endothelial cells [7].

These observations support the concept proposed by the author of an "endothelial - smooth muscle interactive cellular unit" in which these two cell types not only supply structural components of the artery well but communicate with one another through adhesion proteins (eg PECAM-1) or cytokines (eg Angiopoietin-1) to maintain functional integrity.

(c) *New Role for HDL*

The revolutionary work of Russell Ross defined atherosclerosis as an inflammatory phenomenon in which adhesion protein expression and cell migration played pivotal parts. Whilst the pro-atherogenic proteins (especially altered forms of LDL) have been shown to induce inflammatory-type reactions, chiefly in monocytes, the role of the chief anti atherosclerotic blood lipoprotein, high density lipoprotein (HDL) was thought to involve the stimulation of reverse cholesterol transport. However the author's laboratory showed that, like TGF-β, HDL has a potent anti-inflammatory effect, inhibiting the activity of agents such as TNF [8]. This observation occasioned considerable interest as it heralded a new paradigm in approach to atherosclerosis.

(d) *Sphingosine kinase: A mediator of activation and anti-apoptosis*

The preoccupation of the author with endothelial activation naturally led to investigations of the mechanisms involved inside the endothelial cells mediating activation associated phenotypic changes.

In a series of papers (and patents), my group showed that a novel enzyme, sphingosine kinase (SK), was at the fulcrum of signalling events [9] determining the inflammatory phenotype in endothelial cells. Furthermore, SK and its product sphingosine-1-phosphate (S1P), were also important in preventing apoptosis in endothelial cells .... and indeed play an important part in the transforming process in tumours initiated by some (eg ras) oncogenes [10, 11]. This work has now concentrated on discovering novel ways of inhibiting SK. An important step has been the recent description of G82D mutant SK, that is devoid of kinase activity and potently prevents the activation but not baseline function of SK [12]. The mutation, by abolishing the binding of ATP, created a dominant negative molecule that differentiated and defined baseline and stimulated functions of the enzyme.

Interestingly, both HDL [13] and TGF-β appeared to exert their anti-inflammatory effect by inhibiting SK, thus further confirming the key role of SK in endothelial physiology.

The body of work on SK has prompted a strong push aimed at discovering antagonists. The first of these, Phenoxodiol, has recently been shown to be an SK antagonist in the author's laboratory.

Thus over the last 15 years, the author has identified some of the chief regulators of the inflammatory phenotype of endothelial cells and discovered an essential component of the signalling apparatus within cells that mediates their effect, and has been instrumental in developing drugs or drug targets in this process.

2. Transmigration

Following the adhesion of leucocytes to endothelium, a process of transmigration (TM) takes place whereby leucocytes migrate through endothelial junctions into the tissue.

Interestingly, this process has chiefly been viewed as a chemoattractant mediated phenomenon, whether the chemoattractant is elaborated by a foreign micro-organism (eg f-met-leu-phe) or by cells in the form of chemokines (eg Interleukin-8) or mediators (eg Leukotriene B4). The observation that TNF (and other pro-inflammatory mediators) not only caused adhesion of leucocytes to endothelial cells but also stimulated a propulsion of the leucocytes through the endothelial junctions was initially explained by the secretion by endothelial cells of copious quantities of chemokines. The chief contribution that the author made to this area has been to show that there exists a qualitative difference between exogenous chemoattractant driven TM and one stimulated by treatment of endothelial cells with TNF. Experiments with desensitized neutrophils [14] and antibodies to endothelial antigens [15] have shown different leucocyte and endothelial junctional requirements for TNF mediated TM and ones driven by exogenous chemokines. This work is ongoing with the most significant observation showing that a novel class of endothelial active cytokine, angiopoietin-1, is able to 'tighten' endothelial junctions (as measured by permeability, PECAM-1 redistribution and catenin phosphorylation) to prevent TNF mediated TM totally, whilst leaving chemokine mediated ones intact [6].

3. Recognition of the Irritant

The final cellular step in inflammation - that of the recognition of the irritant, was actually the author's first contribution toward understanding the mechanisms of inflammation. The recognition that eosinophils have an unique capacity to kill parasites [16] and that this killing capacity was stimulated initially by eosinophil-active hemopoietic growth factors rich supernatants [17], subsequently IL-5 [18], GM-CSF [19] and IL-3 [20], was a fundamental demonstration that these growth factors were not only active on hemopoietic progenitors but also activated mature cells [21, 22]. The prototypic pro-inflammatory cytokine, TNF, was also found to have a similar effect [23]. The discovery that some adhesion proteins inhibit neutrophil activation [24, 25] suggested a mechanism of inflammation inhibition *in vivo*. The hypothesis has been recently confirmed by others in P-selectin knock-out mice. Several papers in this series have been cited more than 600 times.

This work expanded into work on structure function relationship of hemopoietic growth factors, GM-CSF and IL-3 and examination of the molecular mechanisms governing their transcriptional and post transcriptional regulation.

The structure-function work resulted in the discovery of the first hemopoietic cytokine antagonist - an antagonist of GM-CSF (a point mutation at residue 21 of glutamic acid to arginine) - a molecule covered by world-wide patents and the subject of an ongoing phase II clinical trial [26, 27]. In addition, a super-agonist of IL-3 was discovered [28], a molecule that in spite of having been granted international patents struggled to find a clinical application.

The study of transcriptional regulation of GM-CSF and IL-3 began with the discovery in 1987 [29] of the first nuclear factors binding to the GM-CSF promoter in a cell-specific and stimulus-specific manner and the cytokine-1 and -2 elements (CK-1, CK-2) therein involved [30]. There followed a detailed analysis of promoters and intergenic enhancers of GM-CSF and IL-3 culminating in a comprehensive insight into the mechanisms that govern cell-specific responses of these cytokines. The discovery of complex NFAT-AP1 sites in the GM-CSF enhancer was significant as it demonstrated synergism between these transcription factors as the basis of enhancer function [31, 32]. The subsequent demonstration of correct enhancer function in transgenic mice [33] underlined the relevance of transcription factor synergism during GM-CSF expression.

Most recently, the basis for T-cell restricted expression of IL-3 and non-T-cell restricted expression of GM-CSF was discovered to be due to their respective enhancers. Specifically the IL-3 enhancer, although significantly similar to that of GM-CSF, has complex NFAT-Oct sites. This enhancer then associates with T-cell specific co-factors which render the expression T cell specific and also mediate its interaction with the transcription machinery [34].

4. The Body of Work

All of the work described in this thesis arose from the author's interest in inflammation and his pioneering insights into the role that cytokines/growth factors play in this process. Over the span of some 20 years, there have been a series of collaborations, post-doctoral fellows and students involved in the work. Many of those have taken on aspects of the work and have begun independent careers within or without of the author's laboratory, in many instances continuing fruitful collaborations. Professor A Lopez, Associate Professor J Gamble, Associate Professor MF Shannon, Dr P Cockerill, Dr G Goodall and more recently, Dr P Xia, have been key contributors to the work described over the years.

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1987 Commonwealth AIDS Grants Advisory Committee

1988 Regional Grants Interviewing Committee, National Health and Medical Research Council

1989-91 Scholarships/Fellowships Subcommittee, Royal Adelaide Hospital; Research Review Committee, Adelaide Children's Hospital

1990-94 Grants Committee, National Health and Medical Research Council, Canberra

1990-95 Chairman, Regional Grants Interviewing Committee, National Health and Medical Research Council

1993- Australian Vascular Biology Society Councillor

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Honours:

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1988-89 Initiation of the concept of the **"Adelaide Cancer Research Centre"** (subsequently named the Hanson Centre for Cancer Research) establishment of linkages to ensure scientific success, gaining support for

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1989-1999 Inaugural Director of the **Hanson Centre for Cancer Research**

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1987 Establishment of a series of biennial scientific meetings, now called **"Hanson Symposia"**:
November 1998: *From Genes to Therapeutics*
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American Association of Allergy & Immunology, 1994 Cabo, 1996 New Orleans
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Patents Registered:

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US patent no. 5.591,427 granted 7 January 1997.
National phase in progress in Australia, Europe, Canada and Japan.
December 1998 (European patent no. 92921235.5) - being pursued in Austria (E179211), Belgium, Switzerland (92921235.5), Germany (692 28 997.6-08), Denmark, France, United Kingdom, Ireland, Italy (48708BE99), Luxembourg, Monaco, The Netherlands, Sweden, Greece and Spain.

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(Australian patent granted 73414/94; New Zealand granted 269766; US 08/591438, Singapore 9601109.3, and Canada 2168261 in National phase)

P3. Haemopoietic growth factor antagonists and uses therefor. (PCT/AU96/00382) 23 June 1995. VADAS MA, Lopez AF, Shannon M, Cheah K-C, Senn C, Bastiras S and Robins A. (Aust – granted, Patent No: 703052; USA – granted, Patent No: 5,939,063; Pending in Japan, App. No: 503469/97; Canada, App. No: 225517; NZ, App. No: 310366; Singapore, App No: 9705881-2; Europe, App. No: 96918517.2; Hong Kong, App. No: 99101674)

P4. Agonists of haemopoietic growth factors with Medvet Science Pty Ltd (PCT/AU96/00521) PN4812. 16 August 1995. D'Andrea R, Gonda TJ and VADAS MA. (Australian – granted, Patent No: 701107; UK – accepted 3/11/99, App. No: 2319034; Pending in US, App. No: 09/011617).

P5. An interleukin-5 antagonist (PCT/AU97/00322) 24 May 1996. Lopez AF, VADAS MA and Shannon MF. Pending in Japan, No: 541279/97; Singapore, No: 9805822-5; Canada, No: 2256368; NZ, No: 332946; USA, No: 09/180864; Europe, No: 97921538.1.

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P9. Novel therapeutic molecules and uses thereof - I. Provisional Patent (PQ0339/99) 13 May 1999. VADAS MA *et al*. App No PQ8408/00.

P10. Novel therapeutic molecules and uses thereof - II. Provisional Patent (Q1504/99) 8 July 1999. VADAS MA *et al*.
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P11. Novel therapeutic molecules and uses thereof - III. Application No PQ8699/00. September 2000.

P12. Novel therapeutic molecular variants and uses thereof.

P13. Novel therapeutic molecular variants and uses thereof - II

P14. Novel therapeutic molecular variants and uses thereof - III

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Stimulation of the adherence of neutrophils to umbilical vein endothelium by human recombinant tumor necrosis factor

(inflammation/tumor rejection/adherence proteins/blood vessel)

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ABSTRACT **Recombinant human tumor necrosis factor (TNF) was found to enhance the adherence of human peripheral blood neutrophils to human umbilical vein endothelial (HUVE) cell monolayers *in vitro*. The enhancement was due to the effects both on neutrophils and HUVE cells. The effect on neutrophils was maximally induced within 5 min and did not require protein or RNA synthesis. By contrast, maximal effects on HUVE cells took 4 hr to develop and required *de novo* protein and RNA synthesis; however, exposure of HUVE cells to TNF for as little as 5 min was sufficient to initiate changes leading to maximal adherence of neutrophils at 4 hr. Both the effect on neutrophils and that on HUVE cells were blocked by a monoclonal antibody against TNF. TNF also rapidly induced an increased surface expression of neutrophil antigens recognized by monoclonal antibodies directed against epitopes of a glycoprotein required for optimum adherence and for complement component C3bi receptor (CR3) function. Thus, the mechanism of action of TNF may involve the regulation of expression of cell surface molecules. Our observations show that TNF induces a process central to the development of all inflammatory reactions and that both blood neutrophils and endothelial cells are targets of TNF action. The regulation of inflammatory reactions by TNF or antagonists of TNF has wide-ranging clinical implications.**

The human cytokines tumor necrosis factor (TNF) and lymphotoxin have been identified by their tumoricidal and tumorostatic properties *in vivo* and *in vitro* (1–4). The cloning of these cytokines has allowed confirmation of their effects on malignant cells (5, 6) and the demonstration of their biological effects on the neutrophil, in which they enhanced phagocytic and antibody-dependent cytotoxic activities (7).

Since neutrophils play a central role in inflammatory reactions, which may be involved in tumor rejections, we hypothesized that TNF may alter one of the essential components of the development of these reactions: namely, adherence of neutrophils to endothelial cells. In this communication we demonstrate that TNF is a powerful stimulator of neutrophil adherence to human umbilical vein endothelium (HUVE) and that this stimulation is achieved by effects both on neutrophils and HUVE.

MATERIALS AND METHODS

Recombinant Human TNF. Lot number 3238-14, containing 2×10^7 units/ml was generously provided by Genentech, South San Francisco, CA. The molecule was produced in *Escherichia coli* (5) and purified (99.8%). The undiluted material contained 0.8 ng of endotoxin/ml, as determined by a *Limulus* amoebocyte lysate assay kit (M. A. Bioproducts, Walkersville, MD). Dilutions from stock material were made weekly into Hanks' balanced salts solution (HBSS, from GIBCO) containing 1 mM Ca^{2+}, 2 mM Mg^{2+}, and 0.5% bovine serum albumin. No detectable loss of activity was observed during a 7-day period of storage at 4°C at 2×10^4 units/ml.

Other Materials. Cycloheximide, actinomycin D, phorbol 12-myristate 13-acetate, and lipopolysaccharide (*Escherichia coli* 055:B5) were obtained from Sigma.

Endothelial Cells. HUVE cells were isolated by collagenase treatment of human umbilical cord veins and maintained in endotoxin-free RPMI 1640 medium (M. A. Bioproducts) with 10% fetal bovine serum (HyClone, Logan, UT), as described (8). Except where indicated, human endothelial cells were used in the first passage. Bovine aortic endothelial cells were isolated and maintained according to Schwartz (9).

Preparation and Radiolabeling of Neutrophils. Whole blood from normal healthy donors was drawn into syringes containing 0.2% ethylenediaminetetraacetic acid (EDTA). Purified neutrophils were isolated by Ficoll/Hypaque (Pharmacia) gradient centrifugation and dextran sedimentation with hypotonic lysis of contaminating erythrocytes (10).

Radiolabeled neutrophils were prepared according to the method of Gallin *et al.* (11). Purified neutrophils (average purity >98%) were suspended (24×10^6 per ml) in HBSS with 1 mM Ca^{2+}, 2 mM Mg^{2+}, and 0.1% gelatin and incubated with ^{51}Cr (24 μCi/ml, as sodium chromate, 200–500 Ci/g; New England Nuclear; 1 Ci = 37 GBq) at 37°C for 1 hr with periodic gentle agitation. After incubation, free ^{51}Cr was removed in two washes with HBSS. Labeled neutrophils were resuspended in RPMI 1640 medium (GIBCO) or in RPMI 1640 medium with 5% fetal bovine serum at a final concentration of 10^6 per ml. Neutrophils labeled in this manner had a radioactivity that was consistently greater than 90% cell-associated.

Neutrophil-Adherence Assay. For adherence assays, HUVE cells were plated in RPMI 1640 medium with 20% fetal bovine serum in 16-mm diameter wells (Costar, Cambridge, MA) at 10^5 cells per cm^2 and grown to confluence. Prior to assay, the medium was decanted and the HUVE monolayers were washed once with RPMI 1640 medium containing 5% fetal bovine serum. To each well, a total volume of 250 μl was added, containing ^{51}Cr-labeled neutrophils (5×10^5) and the test substance. The mixture was then gently agitated and the cells were incubated with the HUVE monolayers at 37°C with 5% CO_2. After a 30-min incubation, the supernatant medium and the nonadherent ^{51}Cr-labeled neutrophils were aspirated and each well was washed once with RPMI 1640 medium containing 5% fetal bovine serum. Aspirated nonadherent neutrophils, incubation medium, and the wash medium from each well were pooled in individual

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Abbreviations: HUVE, human umbilical vein endothelium; TNF, tumor necrosis factor; mAb, monoclonal antibody.

counting tubes for measurement of radioactivity in a gamma counter. The HUVE monolayers and the adherent ^{51}Cr-labeled neutrophils then were lysed for at least 2 hr with 1 M NH_4OH as described by Harlan *et al.* (10). The radioactivity in the NH_4OH lysate and wash from each well was measured in a gamma counter. Adherence was determined as the percent of the total ^{51}Cr cpm added: % adherence = (^{51}Cr cpm in NH_4OH lysate/total ^{51}Cr cpm added) × 100. Total ^{51}Cr cpm added was calculated for each well as the sum of ^{51}Cr cpm in supernatant medium, washes, and lysate. Total ^{51}Cr cpm varied between wells by <10%.

Monoclonal Antibodies. Mouse monoclonal anti-TNF [IgG1(κ)] was kindly provided by Genentech. It was purified from tissue culture medium to 100 μg/ml and had a neutralization titer of 269 units of TNF/μl in the mouse L929 cell cytolysis assay (5). mAbs 60.1, 60.3, and 60.5, kindly provided by P. Beatty (Fred Hutchison Cancer Center, Seattle, WA), are respectively IgG1(κ), IgG2a(κ), and IgG2a(κ) murine monoclonal antibodies. mAbs 60.1 and 60.3 recognize different epitopes on a cell-surface antigen complex present on peripheral blood neutrophils and mononuclear cells that is required for optimum adherence and C3bi receptor (CR3) function (10, 13, 14). The antigen recognized by mAb 60.3 is not present on HUVE cells on analysis by indirect immunofluorescence microscopy or radioimmunoassay (unpublished observations). mAb 60.5 identifies an HLA class I antigen present on all peripheral blood leukocytes and on cultured HUVE cells.

Measurement of Binding of Monoclonal Antibodies by Flow Cytometry. After appropriate incubations, the purified neutrophils were chilled, and aliquots (5×10^5 cells per well) were transferred to microtiter plates before the addition of saturating amounts of mAb and incubation for 30 min at 4°C. After this incubation the cells were washed at 4°C and fluorescein-conjugated goat anti-mouse IgG $F(ab')_2$ was added for a further 30-min incubation at 4°C before fixation with 1% formaldehyde solution containing 1.0% glucose. Fluorescence was measured on an Ortho Cytofluorograf 30150 fitted with a log amplifier. Ten thousand cells were counted in each experiment.

Statistical Methods. The variations are expressed as the standard error of the mean (SEM). *P* values were calculated by Student's *t* test.

RESULTS

TNF Stimulates Neutrophil Adherence to HUVE Cells. Fig. 1 demonstrates the dose-dependent stimulatory effect of TNF on the adherence of ^{51}Cr-labeled neutrophils to HUVE cells over a 30-min incubation period. The stimulation by TNF was observed at 1 unit/ml and adherence reached 50% with increasing TNF concentration. Heating TNF at 100°C for 5 min destroyed its activity. Phorbol 12-myristate 13-acetate, which is a potent stimulant of neutrophil adherence to HUVE (10), produced 82% adherence at 10 ng/ml under our experimental conditions.

To ensure that the effect of TNF was not due to bacterial lipopolysaccharide contamination, we performed two types of experiments (Table 1). First, polymyxin B (10 μg/ml) inhibited lipopolysaccharide-mediated stimulation but was without effect on TNF. Second, anti-TNF mAb totally inhibited TNF- but not lipopolysaccharide-mediated stimulation. The anti-TNF mAb or polymyxin B alone had no effect on adherence.

Preincubation of Neutrophils with TNF Stimulates Adherence to HUVE Cells. When neutrophils were preincubated with TNF for 5, 15, or 30 min and then washed prior to incubation with HUVE cells for 30 min, stimulation of adherence was similar in degree to that observed when TNF was added directly to the adherence assay mixture without preincubation (Table 2, Fig. 2). The dose–response curve was comparable when TNF was preincubated with the neutrophils for 30 min (Fig. 2) or present during the 30-min adherence assay (Fig. 1). Anti-TNF mAb completely blocked the stimulatory effect of TNF on adherence when added either at the beginning of a 30-min adherence assay containing TNF or at the beginning of a 30-min preincubation period with TNF. However, when anti-TNF mAb was added 5 min after the start of the preincubation period, inhibition was decreased, and a further decrease in inhibition was observed when neutrophils were incubated with TNF for 15 min prior to the addition of anti-TNF (Table 2).

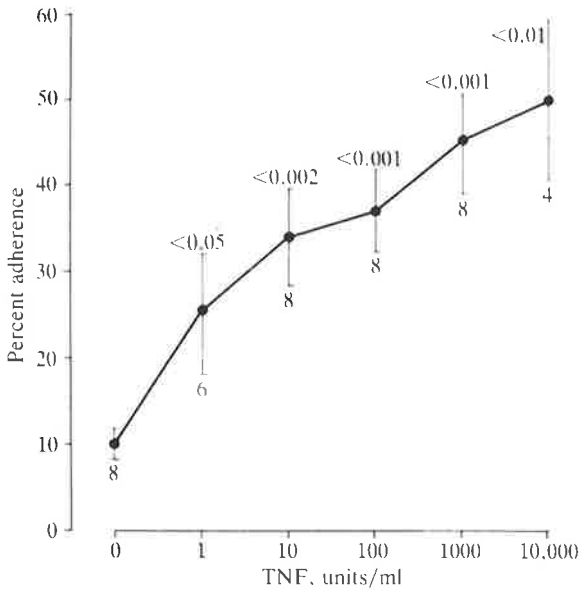

FIG. 1. Adherence of neutrophils to HUVE cells after a 30-min incubation with TNF. Means ± SEM are shown, with the number of experimental values indicated below, and the *P* value for the difference from zero indicated above, the bars.

Effect of Preincubation of HUVE Cells with TNF on Neutrophil Adherence. To test whether TNF also stimulated endothelial cells directly, we pretreated HUVE cells with graded amounts of TNF for 30 min and washed before the adherence of neutrophils was measured. There was a stimulation of adherence with as little as 100 units of TNF/ml (Fig. 3). This effect of TNF on HUVE cells was maximal at

Table 1. Effect of heat, anti-TNF mAb, or polymyxin B on TNF- and on lipopolysaccharide (LPS)-stimulated adherence

Supplement(s)*	% adherence†	*P* values‡		
		A	B	C
None	4.4 ± 0.3 (6)	—		
TNF	26.5 ± 1.7 (6)	<0.001		
Heated§ TNF	7.4 ± 2.3 (4)	NS	<0.001	
TNF + anti-TNF	7.7 ± 1.8 (6)	NS	<0.001	
TNF + polymyxin B	30.0 ± 3.8 (4)	<0.001	NS	
LPS	30.5 ± 5.2 (6)	<0.001		
LPS + anti-TNF	30.7 ± 6.3 (6)	<0.01		NS
LPS + polymyxin B	7.6 ± 2.3 (4)	NS		<0.01
anti-TNF	7.4 ± 2.3 (4)	NS		
Polymyxin B	4.6 ± 2.3 (4)	NS		

*Concentrations were as follows; TNF, 100 units/ml; anti-TNF, 2 μg/ml; polymyxin B, 10 μg/ml; LPS, 10 μg/ml.
†Mean ± SEM (of *n* experiments).
‡A, comparison with "None"; B, comparison with "TNF"; C, comparison with "LPS". NS, not significant ($P < 0.05$).
§Five minutes at 100°C.

Table 2. Effect of the duration of preincubation of neutrophils with TNF

Preincubation* with TNF, min	TNF added† to adherence assay	Time of anti-TNF addition‡, min	% adherence§
0	−		18.7 ± 0.5 (4)
5	−		51.7 ± 2.3 (4)
15	−		52.8 ± 3.4 (4)
30	−		57.5 ± 2.2 (4)
0	+		55.8 ± 1.6 (4)
0	+	0	17.3 ± 2.9 (3)
30	−	0	21.9 ± 2.3 (2)
30	−	5	33.5 ± 1.5 (2)
30	−	15	41.8 ± 1.2 (2)

*Neutrophils were preincubated with TNF (100 units/ml) for the periods indicated and washed before addition to HUVE cultures in a 30-min adherence assay.
†TNF (100 units/ml) was added directly to the 30-min adherence assay.
‡Anti-TNF mAb (2 μg/ml) was added before (0) or 5 or 15 min after TNF in the 30-min preincubation period or was added before TNF in the adherence assay.
§Mean ± SEM (of *n* determinations).

4 hr and then decreased with further preincubation (Fig. 4). With 4 hr of incubation a significant increase was seen with TNF at 0.1 unit/ml, 1/10th the dose needed when TNF was added directly to the adherence assay.

To establish the rapidity with which TNF initiates the processes in HUVE cells that lead, in 4 hr, to increased neutrophil adherence, free TNF was washed from HUVE cells various times after the beginning of a 4-hr incubation. Incubations as short as 5 min were sufficient to induce increased adherence manifest at 4 hr (Table 3). Anti-TNF mAb blocked this effect of TNF on HUVE cells (data not shown).

Although the first-passage HUVE cells used in these experiments were free of other detectable cell types, the possibility that TNF induced the above changes via a contaminating cell type was considered. For this reason, the effect of TNF on multiply passaged bovine endothelial cells was investigated. TNF induced similar changes in these cells. The adherence of neutrophils increased from 0.9 ± 0.1 to 9.6

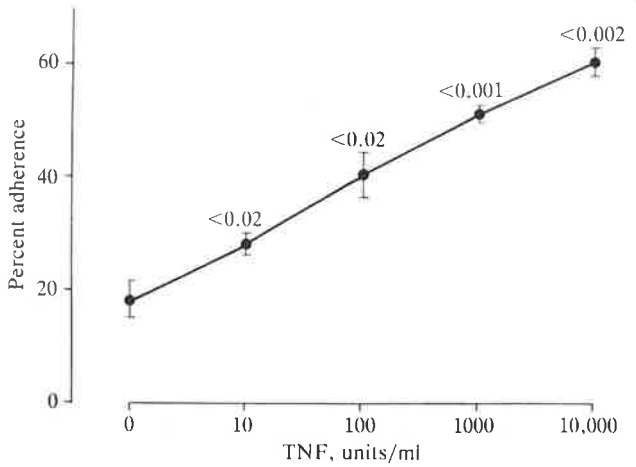

FIG. 2. Preincubation of neutrophils for 30 min with graded doses of TNF stimulates their capacity to adhere to HUVE cells during a subsequent 30-min incubation. Each point is the mean ± SEM of duplicate determinations. The *P* values for the differences from zero TNF are shown.

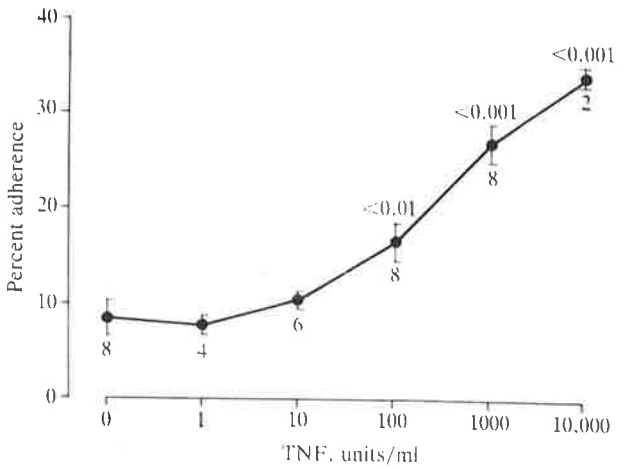

FIG. 3. Preincubation of HUVE cells for 30 min with graded doses of TNF stimulates neutrophil adherence during a subsequent 30-min incubation. Means ± SEM are shown, with the number of experimental values indicated below and the *P* value for the difference from zero TNF indicated above the bars where significant ($P < 0.05$).

± 0.3% ($P < 0.001$) following 4 hr of preincubation of TNF (100 units/ml) with the bovine endothelial cells.

Effects of Actinomycin D and Cycloheximide on TNF-Stimulated Adherence. To test whether RNA or protein synthesis was involved in TNF-mediated stimulation of adherence, neutrophils or HUVE cells were pretreated with actinomycin D or cycloheximide before the addition of TNF. Table 4 indicates that neither agent, when preincubated with neutrophils, inhibits adherence in the absence of TNF or following a 30-min incubation with TNF. By contrast, both agents inhibited or abolished the enhancement of adherence that resulted from TNF pretreatment of HUVE cells. To establish that actinomycin D or cycloheximide did not alter the capacity of HUVE cells to support adherence, it was shown that TNF- or phorbol myristate acetate-treated neutrophils adhered equally to untreated or to actinomycin D- or

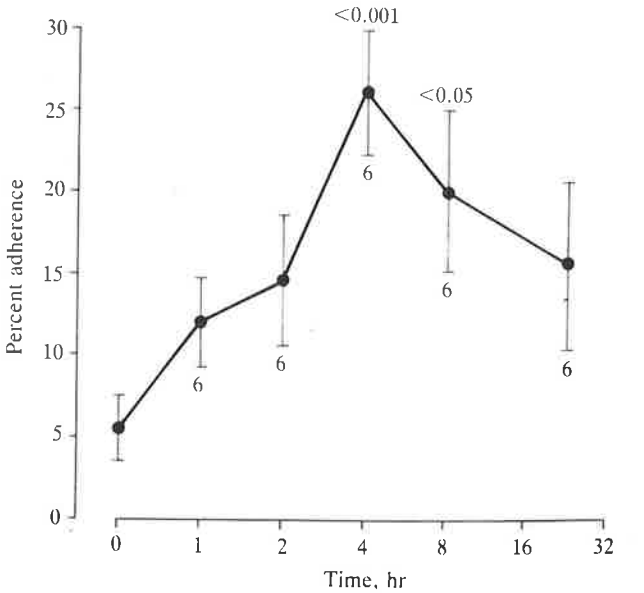

FIG. 4. Time course of the effect of incubation of HUVE cells with TNF (100 units/ml) on neutrophil adherence. Means ± SEM are shown with the number of experimental values indicated below, and the *P* value for the difference from zero time indicated above the bars where significant ($P < 0.05$).

Table 3. Incubation of HUVE cells with TNF stimulates adherence of neutrophils

Length of incubation with TNF	% adherence*	
	+TNF	−TNF
5 min	43.8 ± 4.1	12.4 ± 0.5
15 min	36.6 ± 10.6	12.7 ± 1.0
1 hr	41.8 ± 1.5	13.4 ± 2.6
4 hr	62.8 ± 1.5	10.2 ± 0.4

HUVE cells were incubated with TNF (100 units/ml) for the periods indicated, washed, and incubated further where needed for a total period of 4 hr. Neutrophils were then added and adherence was measured following a 30-min incubation. HUVE cells incubated in the absence of TNF were washed and further incubated as for the TNF-treated HUVE preparations.
*Mean ± SEM of 4 determinations.

cycloheximide-treated HUVE cells (data not shown). A constant, but unexplained, finding was the greater adherence of neutrophils to cycloheximide-exposed HUVE cells in the absence of TNF (Table 4).

Treatment of Neutrophils with mAb 60.1 or 60.3 Blocks the Adherence Reaction. mAbs 60.1 and 60.3 inhibit adherence of stimulated neutrophils to HUVE cells, and neutrophils from individuals from individuals lacking the antigens identified by these antibodies do not adhere to HUVE cells when stimulated (10). It was therefore of interest to determine whether TNF-stimulated adherence was also blocked by these monoclonal antibodies. As shown in Table 5, the addition of either of these mAbs to neutrophils, but not endothelium, blocked resting or TNF-stimulated adherence.

Incubation of Neutrophils with TNF Increases Expression of the Antigens Recognized by mAbs 60.1 and 60.3. The possibility that TNF stimulates neutrophil adherence by increasing the surface expression of the putative "adherence" proteins recognized by mAbs 60.1 and 60.3 was investigated by flow cytometry. Neutrophils were incubated with TNF for 30 min at 37°C before the addition at 4°C of the mAb, whose binding was measured with a fluorescein-conjugated second antibody. TNF at 100 units/ml increased the expression of the epitopes recognized by mAbs 60.1 and 60.3, respectively, 2-fold and 1.5-fold, as judged by mean or medium fluorescence, but did not increase the expression of class I HLA molecules recognized by mAb 60.5.

DISCUSSION

The adherence of blood cells to endothelium is a central reaction in the development of inflammatory reactions (15). The regulation of this interaction is therefore of importance not only in inflammation due to foreign microorganisms but also in situations where the inflammatory reaction is against self-components. Thus, the discovery of a cytokine that regulates this interaction by effects both on the adhering cell and on the endothelium is of potential interest.

TNF induces within 5 min (Table 2) a process in neutrophils that does not need RNA or protein synthesis (Table 4) and that leads to enhanced attachment to HUVE cells. This differs from the effect of TNF on HUVE cells, which needs 4 hr for maximal development and depends on RNA and protein synthesis (Table 4). An exposure of HUVE cells to TNF for as short a time as 5 min is sufficient to lead to the development of maximal effects at 4 hr. mAb against TNF blocks the induction of the changes in both neutrophils and HUVE cells which lead to increased adherence, but the mAb does not inhibit them after the initial exposure to TNF.

Our findings thus confirm the observations of Shalaby *et al.* (7), showing the neutrophil as another target cell for TNF. Our results show that HUVE cells are also affected by this cytokine. Since HUVE cells are not cloned, the possibility of an indirect effect via a cellular contaminant (perhaps mononuclear cells) exists, but this is unlikely since multiply passaged bovine endothelial cells, which represent pure populations, behave like HUVE cells in their response to TNF. Other than the requirement for *de novo* protein synthesis, the adhesion-promoting activity induced in HUVE cells by TNF is not yet characterized.

The mechanism of the effect of TNF on neutrophils is also not known, but the rapidity of the effect suggests the induction of surface expression of adhesion-promoting molecules. One such molecule is the receptor for complement component C3bi (CR3), since mAb against certain epitopes of

Table 5. mAbs 60.1 and 60.3 can block TNF stimulation of adherence of neutrophils to HUVE cells

Supplements	% adherence*	*P* values†	
		A	B
None	8.0 ± 0.9 (4)		
TNF	31.9 ± 7.3 (4)	<0.02	
60.3	5.7 ± 7.3 (4)	NS	
60.1	4.5 ± 0.8 (4)	<0.05	
9E8‡	7.9 ± 0.9 (2)	NS	
TNF + 60.3	3.6 ± 0.1 (4)	<0.01	<0.01
TNF + 60.1	8.0 ± 1.5 (4)	NS	<0.02
TNF + 9E8	42.2 ± 1.6 (2)	<0.001	NS

Neutrophils were incubated for 30 min with the indicated supplement(s) and then were assayed for adherence to HUVE cells.
*Mean ± SEM (of *n* experiments).
†A, comparison with control; B, comparison with TNF group.
‡9E8 is a mAb (10) of the same isotype as monoclonal antibody 60.3 but does not bind to neutrophils.

Table 4. Effects of cycloheximide and actinomycin D on TNF-induced adherence

Supplement(s)	Neutrophils			HUVE cells		
	% adherence	*P* values*		% adherence	*P* values*	
		A	B		A	B
None	13.5 ± 2.8			5.7 ± 1.3		
Actinomycin D (4 μg/ml)	12.2 ± 3.1	NS		5.8 ± 1.9	NS	
Cycloheximide (4 μg/ml)	12.3 ± 3.6	NS		16.2 ± 3.9	<0.02	
TNF (100 units/ml)	28.2 ± 5.3	<0.05		30.0 ± 4.9	<0.001	
TNF + actinomycin D	26.3 ± 4.2	<0.05	NS	7.8 ± 2.1	NS	<0.001
TNF + cycloheximide	36.6 ± 6.8	<0.01	NS	12.6 ± 2.9	<0.05	<0.01

The supplements were preincubated with neutrophils or HUVE cells for 30 min or 4 hr, respectively, and adherence was determined in a 30-min assay. Data are expressed as mean ± SEM of 4 determinations.
*A, comparison with control ("None"); B, comparison with TNF group.

this molecule block adhesion-dependent reactions. mAbs 60.1 and 60.3, previously shown to recognize epitopes of CR3 (13, 14), do block TNF-stimulated adhesion. TNF induces, in a time- and dose-dependent fashion, the expression on neutrophils of structures recognized by mAbs 60.1 and 60.3 but not control mAb 60.5 (which recognizes an HLA framework determinant). This observation shows that this cytokine, like phorbol myristate acetate (12), fMet-Leu-Phe (16), or the calcium ionophore A23187 (17), induces rapid modulation of an adherence protein on the neutrophil surface, possibly by translocation from specific granules (17), but does not unequivocally establish that this phenomenon is directly responsible for the enhanced adherence.

The role of TNF is unlikely to be restricted to the rejection of tumors (1), although this still remains one of the unique effects of this cytokine. Tumor rejection may, however, involve some of the phenomena described herein. TNF might induce changes in the local vasculature of tumors, leading to inflammatory reactions that may precede or enhance rejections. If this were the case, the local use of TNF for tumor therapy may be envisaged.

Note Added in Proof. Work by Bevilacqua *et al.* [Bevilacqua, M. P., Pober, J. S., Wheeler, M. E., Mendrick, D., Cotran, R. S. & Gimbrone, M. A., Jr. (1985) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **44,** 1494 (abstr. 6335)] has shown that interleukin 1 effects the adhesion of leukocytes to cultured human endothelial cells in a similar fashion to TNF. In contrast to TNF, however, interleukin 1 pretreatment of leukocytes or addition to the adhesion assay mixture does not alter endothelial–leukocyte adhesion.

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SCIENCE

Endothelial Adhesiveness for Blood Neutrophils Is Inhibited by Transforming Growth Factor–β

JENNIFER R. GAMBLE AND MATHEW A. VADAS

Endothelial Adhesiveness for Blood Neutrophils Is Inhibited by Transforming Growth Factor–β

JENNIFER R. GAMBLE AND MATHEW A. VADAS

Adhesion of blood cells to endothelial cells is an essential component of all inflammatory responses. The capacity of the endothelium to support adhesion of neutrophils is increased by cytokines such as tumor necrosis factor–α, interleukin-1, and endotoxin. Another cytokine, transforming growth factor–β (TGF-β), was a strong inhibitor of basalneutrophil adhesion and also decreased the adhesive response of endothelial cells to tumor necrosis factor–α (TNF-α). The ability of cells to respond to TGF-β was related to the duration of culture of endothelial cells after explantation from umbilical veins. TGF-β is likely to serve an anti-inflammatory role at sites of blood vessel injury undergoing active endothelial regeneration.

ATTACHMENT TO THE ENDOTHELIUM is essential in the movement of cells from the circulation into the site of an inflammatory reaction. The level of cell attachment is markedly increased by cytokines such as TNF and interleukin-1 (IL-1), which also increase the levels of some molecules on the endothelial surface involved in adhesive reactions (*1–3*).

Endothelial cells also elaborate an extracellular matrix composed of glycoproteins such as laminin (*4*), fibronectin (*5*), and collagen (*6*) involved in cell anchorage and migration. Recently, TGF-β was shown to increase the incorporation of fibronectin and collagen into the extracellular matrix of fibroblasts, epithelial, and endothelial cells (*7, 8*). Furthermore, TGF-β also inhibits endothelial cell proliferation in response to growth-promoting factors (*9*) and wounding (*10*), which suggests that TGF-β may be an important regulator of function at the endothelial surface. We therefore examined the effect of this molecule on adhesive interactions involving endothelial cells and neutrophils.

Primary cultures of human umbilical vein (HUVE) endothelial cells exposed to TGF-β for 24 hours showed a reduced capacity to support neutrophil binding (Fig. 1) that was dose-dependent but plateaued at maximum levels of inhibition with TGF-β (2 ng/ml). This dose response for inhibition of neutrophil attachment is consistent with reports for the effect of TGF-β on endothelial cell proliferation, locomotion, and angiogenesis (*8*). The response was blocked by a monospecific antibody against TGF-β (R&D Systems) and similar inhibition was demonstrated with recombinant (r) TGF-β (Genentech); the basal adherence of 0.149 ± 0.016 was reduced to 0.063 ± 0.002 by rTGF-β (2 ng/ml). This dose of TGF-β did not result in inhibition of endothelial cell proliferation; there was little or no change in the morphology or density of the endothelial monolayer as assessed by dye uptake and microscopy. Cell numbers after 24 hours of exposure to TGF-β (2 ng/ml) were $10.6 \pm 0.8 \times 10^3$ and without exposure to TGF-β were $9.6 \pm 0.8 \times 10^3$ (arithmetic mean ± SEM, $n = 5$) from an original 10^4 cells plated per well.

To test the effect of varying the time of addition of TGF-β on the inhibition of neutrophil attachment, endothelial cells were replated into microtiter wells, and treated for 3 to 24 hours with TGF-β (2 ng/ml) before assaying for neutrophil binding. Significant inhibition of adherence was seen after a 6-hour exposure; however, maximum inhibition was obtained when TGF-β was added at the initiation of the experiment and the endothelial cells were exposed for a full 24-hour period (Table 1). Again, no effect on endothelial cell growth was observed under these conditions.

TGF-β has been shown to decrease the number of high-affinity epidermal growth factor (EGF) receptors on a variety of endo-

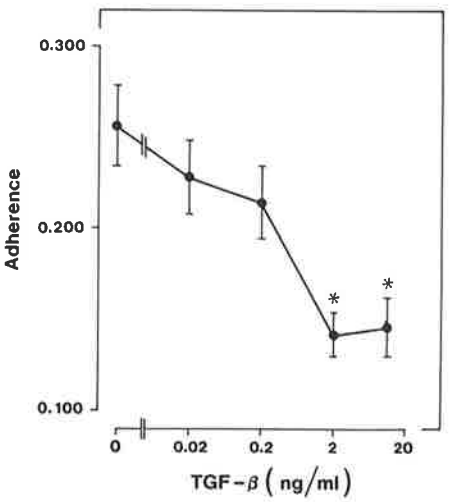

Fig. 1. The effect of TGF-β on adherence of neutrophils to endothelial monolayers. HUVEs obtained from collagenase treatment of umbilical cords were cultured for 3 to 7 days in 25 cm^2 flasks (Costar) in RPMI 1640 plus 20% fetal bovine serum (FBS) as described (*17*). Cells were then harvested by trypsinization (Flow) and plated into 6.41-mm^2 microtiter wells (NUNC) at 1.6×10^4 cells per well. TGF-β (97% pure isolate from human platelets, R&D Systems) was then added and after 24 hours the adherence of Ficoll-Hypaque–purified (*18*) venous blood neutrophils (mean purity 96%) was measured with the use of an assay based on the uptake of the viable stain Rose Bengal (*19*). Between 2 and 8×10^5 neutrophils in RPMI 1640 plus 5% FBS were added to wells of a 96-well flat-bottomed microtiter tray containing monolayers of HUVEs. After 30-min incubation at 37°C in 5% CO_2, the supernatant was removed and 100 μl of 0.25% Rose Bengal stain was added for 5 min at room temperature. Nonadherent cells were removed by two subsequent washes in medium; stain incorporated into the cells was released by the addition of 200 μl of ethanol:phosphate-buffered saline (1:1). After 45 min the wells were read in an enzyme-linked immunosorbent assay (ELISA) reader (Dynatech) at 570 nm. The level of adherence is given as the mean optical density reading at 570 nm (OD_{570}) of wells containing adhering cells and HUVEs minus the mean OD of wells containing HUVEs alone. On the average a tenfold change in OD_{570} represents a tenfold change in the numbers of cells adherent per square millimeter. Each point represents the arithmetic mean ± SEM of 9 to 15 determinations from four to five separate experiments. Points marked by an asterisk differ significantly from no added TGF-β ($P \leq 0.005$, two-tailed t test).

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thelial cell types and to prevent the EGF-mediated induction of several growth regulatory genes (*9*). This suggested that TGF-β might affect the adhesiveness of endothelial cells by altering the capacity of the cells to respond to other adhesion-promoting cytokines. HUVE monolayers exposed for 24 hours to TGF-β (2 ng/ml) were stimulated for 4 hours with the cytokine TNF-α, followed by assessment of neutrophil binding (Fig. 2). As we have previously shown (*1*) TNF-α stimulation of HUVEs increases neutrophil attachment in a dose-dependent fashion. Addition of TGF-β inhibited neutrophil binding to unstimulated HUVEs and also to TNF-α–stimulated HUVEs at all doses of TNF-α tested. Preincubation with TGF-β completely abolished the normal enhancement of adhesion caused by TNF-α.

Three types of TGF-β receptors have been found in bovine endothelial cells. The largest (280 kD, type III) receptor is only present on subconfluent cells, whereas the smaller receptors (85 kD and 72 kD, types I and II) are seen on cells from confluent cultures (*8*). Because the pattern of expression of these receptors varies with the stage of cell growth, we decided to test whether ligand binding to the TGF-β receptor on human endothelial cells may result in different functional effects when the cells were at different stages of cell growth. We therefore tested endothelial cells, grown for varying lengths of time, for their ability to respond to TGF-β as assessed by neutrophil attachment. HUVEs recently established in culture (3 to 6 days) showed responsiveness to TGF-β by a decreased level of neutrophil adherence. However, when subcultures were allowed to grow for an additional 3 to 8 days they no longer responded to TGF-β in this assay (Fig. 3). This change in TGF-β–responsive phenotype was not related to confluency of the HUVEs at the time of the adherence assay because the cells were always replated at the same densities. The phenomenon appeared to be associated with the age of the endothelial cells and may be related to the presence or absence of the high molecular weight receptors (*8*). Indeed, the triggering of the type III receptor in fibroblasts leads to the induction of the adhesion-promoting molecules fibronectin and collagen (*7, 11*) and to an increase in proteoglycan expression (*12*).

Our findings on adherence may be related to the expression on endothelial cells of a group of adhesion-promoting molecules such as ELAM-1 (endothelial-leukocyte adhesion molecule–1) (*13*) and ICAM-1 (intracellular adhesion molecule–1) (*14*), the expression of which are enhanced by TNF-α. TGF-β treatment of recently established and actively growing endothelial cells may inhibit adhesion by preventing the expression or up regulation of these molecules. Preliminary evidence suggests that TGF-β does down regulate the surface expression of ICAM-1 (*15*). The effect of TGF-β on adhesion thus appears to be highly selective, as (i) it promotes adhesion by elaborating extracellular matrix proteins such as fibronectin (*7*) and by increasing the expression of its receptor (*7*), but (ii) it prevents endothelial cells from exhibiting an adhesive phenotype for neutrophils and prevents the response to the proinflammatory cytokine, TNF-α.

In addition to enhancing endothelial cell adhesiveness, TNF also induces tissue factor and plasminogen activator inhibitor on endothelial cells (*16*) thus favoring a thrombotic state. It is possible that TGF-β also prevents expression of these structures. Regardless of the mechanism and range of the effect of TGF-β, our results demonstrate that the adhesive phenotype of human endothelial cells is capable of being inhibited. This phenomenon may be clinically relevant at sites of trauma, injury, or reperfusion

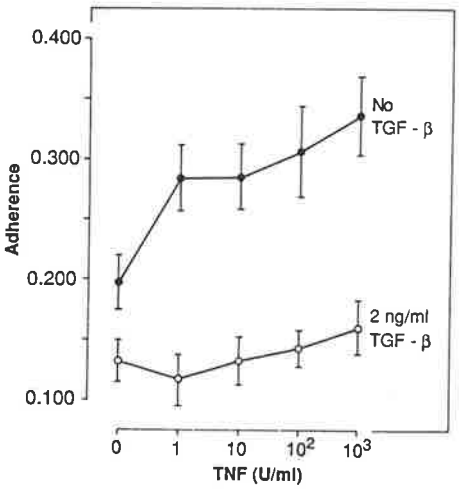

Fig. 2. TGF-β inhibits neutrophil attachment to unstimulated and TNF-α–stimulated endothelial cells. Endothelial cells cultured for 3 to 7 days were harvested by trypsinization and replated in 96-well microtiter trays with or without TGF-β (2 ng/ml). Various concentrations of recombinant human TNF-α (lot 3056-55, Genentech) were added for the final 4 hours of culture before being assayed for neutrophil adherence. The mean adherence ± SEM of 15 determinations from five separate experiments is given. All groups receiving TGF-β differed significantly from those not pretreated with TGF-β ($P \leq 0.03$). At all concentrations of TNF-α tested, adherence values for each group differed significantly from control value receiving no TNF-α ($P \leq 0.05$). However, all groups receiving TGF-β and TNF-α showed no significant increase in adherence when compared to groups receiving TGF-β alone.

Table 1. The effect of time of addition of TGF-β on polymorphonuclear cell (PMN) attachment to HUVEs. Endothelial cells harvested by trypsin from 4- to 6-day cultures and replated into microtiter wells at 1.5×10^4 cells per well were incubated with TGF-β (2 ng/ml) for various times and cultured for a total of 24 hours before assaying for neutrophil attachment. Time is the time (in hours) HUVEs were treated with TGF-β. Adherence is given as the OD_{570} of adhering neutrophils and HUVEs minus the OD_{570} of wells containing HUVEs alone. Inhibition is the percent inhibition of PMN binding. The *P* values for the difference between adherence in the presence of TGF-β and the control (no added TGF-β) are shown; NS, not significant. The mean (± SEM) of 7 to 12 determinations from three separate experiments is given.

Time (hour)	Adherence (OD_{570})	Inhibition (%)	*P* value
24	0.212 (± 0.010)	28	0.0002
18	0.237 (± 0.011)	19	0.003
6	0.253 (± 0.007)	14	0.007
3	0.259 (± 0.013)	12	NS
Control	0.294 (± 0.012)	—	

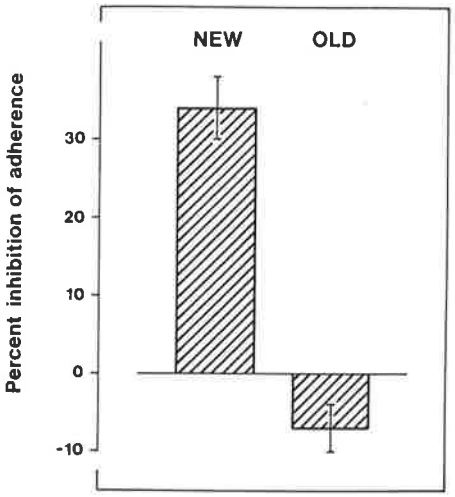

Fig. 3. Effect of age of endothelial cells on their response to TGF-β. After growth in culture for 3 to 6 days ("new" HUVEs) endothelial cells were harvested by trypsinization and replated at 1.5×10^4 cells per well into 96-well microtiter trays, with or without the addition of TGF-β (2 ng/ml) for 24 hours. The extent of neutrophil binding to the HUVEs was determined. A portion of these endothelial cells (0.2×10^6) were replated back into 25-cm^2 flasks for further growth in culture before being assayed at a later time. Three to 8 days (total time in culture 9 to 14 days, "old" HUVEs) later the cells were again harvested by trypsinization, replated into 96-well microtiter trays at 1.6×10^4 cells per well, and assayed for neutrophil attachment. The basal adherence to new HUVEs was 0.270 ± 0.030 and to old HUVEs was 0.256 ± 0.008 (arithmetic mean ± SEM, values not significantly different). The bars represent the mean percentage inhibition of adherence by TGF-β from four separate experiments, each performed in triplicate. The percentage inhibition of adherence after TGF-β treatment between new and old HUVEs differed significantly ($P = 0.0007$).

where vascular regeneration without excessive inflammation or thrombosis is essential.

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ENDOTHELIAL CELL ADHESIVENESS FOR HUMAN T LYMPHOCYTES IS INHIBITED BY TRANSFORMING GROWTH FACTOR-β[1]

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Recombinant human transforming growth factor-β (TGF-β) was found to inhibit the adhesive phenotype of human umbilical vein endothelial cells for human PBL, purified T lymphocytes, and PHA-activated lymphoblasts. TGF-β inhibited lymphocyte attachment to resting human umbilical vein endothelial cells and also to endothelial monolayers stimulated with the pro-inflammatory cytokines TNF-α and IL-1β. Our investigations also show that the ability of endothelial cells to respond to TGF-β by altering their adhesiveness is lost with prolonged culture of the cells. However, this loss is selective as TGF-β inhibits cell proliferation in both early and late passage endothelial cells. These results suggest that in vivo TGF-β may inhibit the adhesive phenotype of endothelial cells and also may limit the immunologic response occurring at the endothelial cell barrier.

The normal emigration of lymphocytes from the blood stream into the lymphatics occurs via binding to specialized endothelial cells in post-capillary venules of lymphoid tissues (1–4). However, lymphocyte adhesion and transendothelial cell migration also occurs at localized sites of inflammation, contributing to specific immunologic recognition, forms of chronic inflammation, and even to the atheromatous process.

The normal nonadhesiveness of EC[3] for lymphocytes is thought to be due to a lack of expression of adhesion molecules, which however can be induced by at least three classes of inflammatory cytokines: the TNF, IL-1s, and IFN-γ. In vitro, these cytokines increase adhesiveness of endothelium for lymphocytes and lymphoblasts and it is possible that in vivo they contribute to the specialized phenotype (such as that found in high endothelial venules) that regulate local lymphocyte traffic (5–8). The adhesion process involves several recognition systems that include members of the CD18 group of polypeptides and the integrin VLA-4 on lymphoid cells and their ligands, ICAM-1 and V-CAM, on the endothelium (9–12). In the case of IFN-γ-stimulated adherence, recognition of class II MHC antigens has also been shown to be important (13). Other less well characterized molecules (14, 15) together with ones as yet undefined may also play a part in this complex phenomenon of adhesion.

The possibility that non-adhesiveness is an active phenomenon (rather than the mere absence of pro-inflammatory stimuli) was raised by our previous observations that TGF-β inhibits endothelial adhesiveness for neutrophils (16). The acquisition of adhesiveness was therefore postulated to require two signals, the absence of TGF-β and the presence of a pro-inflammatory stimulus. We now extend these findings to T lymphocytes and lymphoblasts. Our findings point to the possibility that TGF-β may play an important role in vivo in preserving the nonadhesiveness of endothelial cells and to limit inflammation.

MATERIALS AND METHODS

Lymphocytes and lymphoblasts. Blood was obtained from either blood packs from the Red Cross, Adelaide, Australia or from fresh blood donated by laboratory staff. The mononuclear cells were prepared from density sedimentation of whole blood using Ficol-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ). The blood was centrifuged for 30 min at 400 × *g* and the mononuclear cells collected from the interface. The lymphocyte fraction was obtained by incubating the cells at 37°C on plastic dishes for 1 h before collecting the nonadherent cell population. Cells were stained with cell specific mAb and analyzed for immunofluorescence on a Coulter Epics V Coulter Electronics, Hialeah, FL). The lymphocytes used were found to be routinely 10% B lymphocytes, 80 to 85% T lymphocytes, and 2% monocytes. In some cases the lymphocytes were further purified by passage over nylon wool columns (Travenol, Sydney Australia). The cells obtained after such fractionation were routinely 95% T lymphocytes and less than 4% B lymphocytes as judged by immunofluorescence. Lymphoblasts were established by culture of mononuclear cells in 2 μg/ml PHA (HA16, Wellcome Reagents, Dartford, UK.) for 4 days. Tonsil conditioned medium (20%) (17) was added at day 3 of culture. Day 4 PHA blasts were judged to be approximately 80% T and 12% B lymphocytes.

Endothelial cells. These cells were obtained by collagenase treatment of human umbilical vein according to a modified version of Wall et al. (18). Cells were grown in gelatin coated Costar (Cambridge, MA) flasks in endotoxin free RPMI 1640 (Cytosystems, Sydney, Australia), 20% FCS (Flow, Sydney Australia), 20 mM HEPES at 37°C, 5% CO_2 with added sodium pyruvate, nonessential amino acids and fungizone (HUVE medium) and used between 2 and 14 days after establishment of cultures.

Adhesion assay. Endothelial cells were harvested by trypsin-EDTA treatment and plated at 1.5×10^4 cells/well onto gelatin-coated 96-well flat-bottomed microtiter trays and grown to confluence for 24 h. The measurement of adhesion is based on the uptake of the vital stain, rose Bengal, and details are given in (19). Briefly, the microtiter wells containing the HUVE monolayers were washed twice with RPMI 1640, 10 mM HEPES, 10% FCS before the addition of 2 to 5×10^4 lymphocytes/well in a total volume of 200 μl. The cells were incubated for 60 min at 37°C, 5% CO_2. After this, the supernatant was removed by suction and 100 μl of 0.25% rose Bengal in PBS added for 10 min at room temperature. Nonattached cells

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[3] Abbreviations used in this paper: EC, endothelial cells; HUVE, human umbilical vein endothelial cells; TGF, transforming growth factor; ECGS, endothelial cell growth supplement.

and excess stain were then removed by two washes in medium before the addition of 200 μl PBS:ethanol (1:1) solution. The OD reading at 570/630 nm (OD570nm) was taken after at least 60 min. The level of adhesion is given as the OD reading at 570 nm (OD570nm) of wells containing HUVE and attached lymphocytes minus OD570nm of wells containing HUVE only. For estimation of percent of cells adhering, an aliquot of the lymphocytes used in the adhesion assay were stained as a pellet for 10 min before four washes in medium to remove excess stain. The cells were resuspended in PBS and the concentration determined. Known numbers of cells (usually from 5×10^5 down to 0.625×10^5 cells) were plated out in 100 μl PBS into microtiter wells. A total of 100 μl of ethanol was added and the OD570nm determined after 60 min. From these values a calculation of the number of cells bound in the experimental system could be determined. Using this procedure we find that essentially a linear relationship exists between the OD570nm and the number of attached cells.

^{51}Cr labeling of HUVE and calculation of percent release. 2×10^6 endothelial cells were resuspended in 0.5 ml of HUVE medium, 100 μCi ^{51}Cr as sodium dichronate (Amersham, Buckinghamshire, UK) added and incubated for 20 min at 37°C. The cells were then washed three times in HUVE medium to remove unbound radioactivity. Cells were recounted before plating and then grown overnight. After the appropriate treatment with cytokines, the supernatant of each well was removed and saved and the cells harvested by trypsin-EDTA. Both the supernatant and cell samples for each well were counted in a Packard gamma-counter (Hewlett-Packard Co., Palo Alto, CA) and the percent ^{51}Cr release for each well was determined

$$\% \text{ release} = \text{cpm in supernatant}/(\text{cpm in supernatant} + \text{cpm in cell pellet}) \times 100$$

Proliferation assay. EC were plated at 5×10^3 cells/well into flat-bottomed microtiter trays either with or without ECGS (Collaborative Research, Bedford, MA 50 μg/ml) and heparin (Sigma, 50 μg/ml). ^3H-thymidine (1 μCi/well) was added on day 3 and the cells collected using an automated cell harvester 18 h later.

Cell surface Ag expression. The level of ICAM-1 and VCAM-1 expression on endothelial cells was assessed by flow cytometry. mAb to ICAM-1 (WEHI-CAM-1) (20) was kindly supplied by Dr. Andrew Boyd, Melbourne, Australia and mAb to VCAM-1 by Dr. M. Gallatin (Fred Hutchinson Cancer Research Centre, Seattle, WA). HUVE (at 1.3×10^5/well were plated into 24-well culture trays (growth area 2 cm^2/well) either with or without the addition of 2 ng/ml of TGF-β. To some groups TNF-α (10 U/ml) was added for 6 h before staining. Cells were stained for 30 min at 4°C with the appropriate mAb, washed, and fluoresceinated sheep anti-mouse Ig added for 30 min at 4°C before the cells were washed three times, harvested by trypsin, and resuspended in a formaldehyde fixative before analysis on a Coulter Epics V. A total of 10,000 cells was analyzed for each sample. Using this method of in situ fluorescence labeling, similar levels of Ag expression were seen as that obtained when the HUVE were harvested and stained in suspension (data not shown).

Cytokines. Human rTNF-α (lot N9017AX), specific activity of 3.8×10^7 U/mg and human rTGF-β-1 (lot 7466/59, 0.8 mg/ml) were kindly supplied by Genentech, South San Francisco, CA. Human rIL-1β (10^8 thymocyte mitogenesis units/mg) was kindly supplied by Immunex).

Statistics. Significance was determined by either the unpaired two-tailed *t*-test or by ANOVA test for analysis of variance.

RESULTS

Effect of TGF-β on resting and TNF-α-stimulated lymphocyte adherence to HUVE. HUVE were grown for 2 to 5 days after harvest from umbilical cords. The cells were then trypsinized and replated at 1.5×10^4 cells/well into gelatin-coated microtiter plates. TGF-β, 2 ng/ml, or control medium were added at the time of plating and the cells were incubated for 24 h. For the last 6 h of this incubation TNF-α was added to some wells. The monolayers were then washed twice, unfractionated lymphocytes added, and their adhesion measured 60 min later. As shown in Figure 1, the pooled results from 16 consecutive experiments, TGF-β inhibited the resting adherence of lymphocytes to endothelium and also limited the responsiveness of the endothelium to graded doses of TNF-α. Notably in the presence of TGF-β even the highest dose

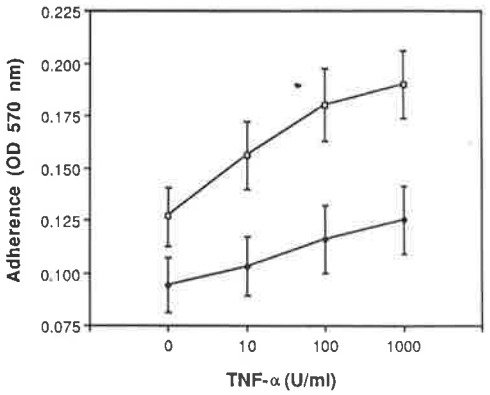

Figure 1. The effect of 2 ng/ml of TGF-β on resting and TNF-α-stimulated adherence of EC for blood lymphocytes. Monolayers of EC treated either with (●) or without (○) TGF-β were stimulated for 6 h with different concentrations of TNF-α before adherence of lymphocytes determined. Adherence is given as the OD at 570 nm (OD570nm). The pooled results of 16 experiments are shown. Each *point* in each experiment was performed in triplicate. Using ANOVA test for analysis of variance $p = 0.008$ between groups with and without TGF-β treatment.

TABLE I

Effect of TGF-β and TNF-α on endothelial cell viability[a]

Cytokine Addition		No. Cells ($\times 10^4$)[b]	OD570 nm	Percent ^{51}Cr Release
TGF-β	TNF-α			
Nil	Nil	1.1 (±0.1)	0.102 (±0.006)	42.4 (±2.5)
2 ng/ml	Nil	1.1 (±0.1)	0.108 (±0.008)	41.7 (±2.7)
Nil	10 U/ml	1.0 (±0.1)	0.100 (±0.009)	45.2 (±2.4)
2 ng/ml	10 U/ml	1.1 (±0.2)	0.102 (±0.009)	44.9 (±2.2)
Nil	1 U/ml	ND	0.098 (±0.009)	44.4 (±2.5)
2 ng/ml	1 U/ml	ND	0.104 (±0.009)	44.4 (±2.3)

[a] Endothelial cells (either ^{51}Cr labeled or not) were plated into microtiter wells either with or without the addition of 2 ng/ml TGF-β. The next day TNF-α was added to the appropriate wells for 6 h. Some wells were then extracted with trypsin and cell counts performed ([b]), other wells washed and stained with rose Bengal (OD570 nm) or the percent ^{51}Cr release determined for the third group. The results are the mean (±SEM) of three to four experiments with at least triplicate determinations performed in each group in each experiment.

of TNF-α did not stimulate adherence to a level higher than normal resting adherence. The confluency of endothelial cell monolayers was assessed microscopically in each experiment before the addition of lymphocytes. No change in the integrity of monolayer was observed. However, because both TGF-β and TNF-α have been reported to inhibit endothelial cell proliferation, further experiments were conducted to rule out the possibility of toxic effects by these two agents either individually or together. Endothelial cells were harvested and some cells plated into microtiter wells at 1.5×10^4 cells/well (with or without the addition of 2 ng/ml TGF-β). Another aliquot of HUVE was labeled with ^{51}Cr before plating. The next day TNF-α was added for 6 h to the appropriate groups. After this time, cell counts were performed on some of the unlabeled HUVE to estimate the number of viable cells whereas another group of cells were stained with rose Bengal. For the group of cells labeled with ^{51}Cr, the amount of ^{51}Cr release was determined. The results from three to four experiments performed are given in Table I. No significant differences in the amount of ^{51}Cr release, in cell viability, or amount of staining with rose Bengal over this 24-h time was observed between groups receiving either TGF-β or TGF-β and TNF-α to those groups untreated or treated only with TNF-α.

TGF-β inhibits T lymphocyte adhesion to endothe-

lium. To characterize the subtype of lymphocyte whose adherence was being inhibited by TGF-β, cells were further fractionated on leukopak leukocyte filters and populations of >95% T lymphocytes were obtained. As shown in Figure 2, pretreatment of endothelial cells with 2 ng/ml TGF-β, inhibited the basal attachment of T lymphocytes to endothelium as well as inhibiting the level of attachment to TNF-α-stimulated endothelium. Furthermore, the level of inhibition induced by TGF-β on the adhesion of unfractionated and purified T lymphocytes from the same blood donor and on the same EC population was similar (data not shown). This inhibition of adhesion occurred regardless of the contact time between T lymphocytes and the EC. When T lymphocytes were added to EC monolayers for 45 min TGF-β caused a 28 and 32% inhibition of the TNF-α-stimulated adherence (data not shown). If the same cells were incubated for 90 min on the EC, TGF-β caused a 28 and 26% inhibition of the TNF-α stimulated adherence (mean of two separate experiments).

Inasmuch as IL-1 has been shown to increase the adhesiveness of endothelial cells for lymphocytes (6) we tested whether TGF-β also inhibited the IL-1-mediated effect. As shown in Figure 3, TGF-β inhibited T lymphocyte adhesion to IL-1-stimulated EC.

Age of HUVE influences response to TGF-β. In the course of these experiments we noted that recently established endothelial cells became less adhesive after TGF-β treatment but that multiple passaged cells did not show this change in adhesion. We therefore examined the effect of age of endothelial cells on TGF-β responsiveness. After collagenase digestion to remove cells from the umbilical vein, HUVE were grown for 2 to 5 days ("young") in 25-cm^2 flasks and then harvested by trypsinization and either replated at 1.5×10^4 cells/well in gelatin-coated microtiter wells or replated back into 25-cm^2 flasks at approximately 0.5×10^6 cells/flask. The cells in flasks were then grown to confluence (total time in culture 10 to 14 days) and are referred to as "old" HUVES. At this time the cells were again harvested by trypsinization and plated at 1.5×10^4 cells/microtiter well. To assess the effect of TGF-β on adhesion of either young or old EC, TGF-β was added at the time of plating for a total of 24 h to half of the wells. Graded doses (1, 10, and 100 U/ml) of TNF-α were added to appropriate wells for the last 6 h of this incubation. The monolayers were washed twice and the adhesion of lymphocytes was measured. The results (Fig. 4) show that TGF-β inhibited the adhesion of lymphocytes to resting and TNF-α stimulated HUVE using young but not old endothelial cells. The results are shown using young and old HUVE derived from the same donor population but assayed 8 days apart and using T lymphocytes from the same donor on the 2 days. If young and old EC (derived from different donors) are tested for their response to TGF-β on the same day using T lymphocytes from a single donor, then inhibition of adhesion is seen in young but not old EC (data not

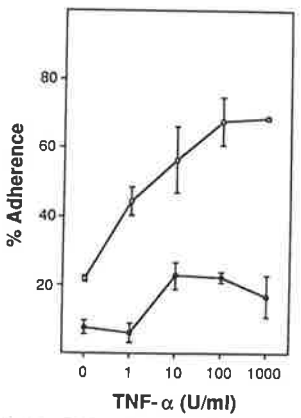

Figure 2. TGF-β inhibits T lymphocyte adherence to TNF-α stimulated endothelial cells. T lymphocytes were purified from blood as described in *Materials and Methods* and assessed for the level of adherence to monolayers of EC either untreated (○) or treated for 24 h with 2 ng/ml TGF-β (●). Monolayers were stimulated with different concentrations of TNF-α for 6 h before adherence was determined. The OD570nm has been converted to percentage adherance. The results of a single experiment are given that is representative of four different experiments. Each *point* represents the mean ± SEM of triplicate determinations. $p < 0.005$ comparing groups with and without TGF-β using ANOVA test for analysis of variance.

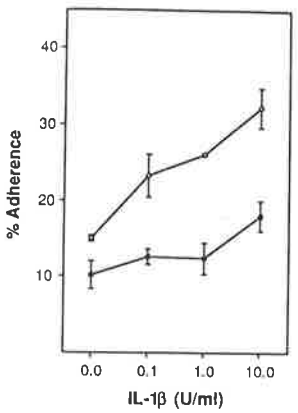

Figure 3. The effect of TGF-β pretreatment of EC on IL-1β-stimulated T lymphocyte adhesion. EC monolayers were either pretreated with 2 ng/ml TGF-β for 24 h (●) or untreated (○). IL-1β was added for 6 h before assaying for T lymphocyte adhesion. A representative experiment of three experiments performed is shown with the OD570nm converted to percentage adherence. Each point is the mean ± SEM of triplicate determinations. $p < 0.005$ comparing groups with and without TGF-β treatment using ANOVA test for analysis of variance.

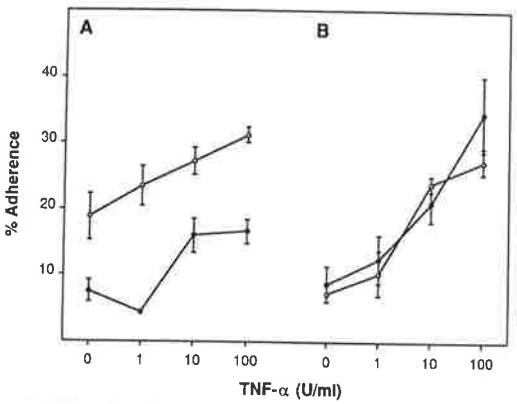

Figure 4. TGF-β inhibits T lymphocyte adherence only on young endothelial cells. Two days after harvesting from the umbilical cord, HUVE were trypsinized and replated into either microtiter wells (= young HUVES) for measurement of T lymphocyte adherence or plated back into flasks. Those in flasks were grown for a further 8 days before again being harvested and plated into microtiter trays (= old HUVES). At the time of plating of both young and old HUVES 2 ng/ml TGF-β was added to half the wells for 24 h. TNF-α was added to the appropriate wells 6 h before the adherence assay was performed. *A*, T lymphocyte adherence to young EC; *B*, T lymphocyte adherence to old EC. Each *point* represents the mean ± SEM of triplicate determinations in a representative of two such experiments. The OD570nm has been converted to percentage adherence. T lymphocytes were extracted from the same donor for adherence to both young and old EC. In *A*, $p < 0.05$ comparing groups with and without TGF-β treatment using ANOVA test for analysis of variance.

shown). In addition, the growth of old HUVES in ECGS and heparin did not confer on them TGF-β responsiveness (data not shown).

TGF-β has previously been shown to inhibit long term endothelial cell proliferation (21). To test whether old HUVE are able to respond to TGF-β the proliferation of cells over a 4-day period in the presence or absence of ECGS and heparin was measured. As shown in Table II, TGF-β inhibited proliferation of old as well as young cells.

Effect of various doses of TGF-β on resting T lymphocyte adherence. Young HUVE were incubated with a range of doses of TGF-β for 24 h before washing and measurement of T lymphocyte binding. As shown in Figure 5 maximum inhibition of both basal- (*A*) and TNF-α-stimulated lymphocyte adherence (*B*) was observed at about 2 ng/ml which is similar to the dose determined to have maximum inhibitory effects on neutrophil adhesion (16) and is similar to doses used to inhibit EC proliferation (21). Using these same doses of TGF-β, old HUVES did not alter their adhesiveness for T lymphocytes (data not shown).

Effect of TGF-β on lymphoblast binding to endothelial cells. Lymphoblasts have been shown previously (22) to be more adherent than resting lymphocytes to cultured endothelium. We therefore tested whether TGF-β is able to also inhibit lymphoblast attachment to endothelial cells in a manner similar to that observed for nonactivated lymphocytes. In each of three experiments treatment of the endothelial cells with 2 ng/ml TGF-β resulted in significant inhibition of attachment of day-4 PHA-activated lymphoblasts (Table III).

ICAM-1 and VCAM-1 expression on endothelium is not altered by TGF-β. One possibility for the mechanism whereby TGF-β inhibits lymphocyte adhesion is that TGF-β is able to regulate the level of expression of adhesion molecules. To test this possibility, young endothelial cells were stained for ICAM-1 and VCAM-1 expression and analyzed by immunofluorescence. Six-h TNF-α stimulation of the HUVE increased the expression of ICAM-1 and VCAM-1 (Fig. 6 *A* and *B*, respectively) but no alteration in this enhanced level was seen with pretreatment of the cells with TGF-β. TGF-β did not alter the basal expression of ICAM-1 (data not shown). In parallel experiments, TGF-β was shown to inhibit leukocyte adhesion

TABLE II

Inhibition of endothelial proliferation by TGF-β[a]

	No TGF-β	With TGF-β	p Value
Young HUVE			
−ECGF	8,431 (±526)	2,475 (±198)	0.0001
+ECGF	48,291 (±1728)	33,348 (±2696)	0.0002
Old HUVE			
−ECGF	2,296 (±420)	808 (±134)	0.05
+ECGF	22,958 (±1102)	14,372 (±900)	0.002

[a] Old HUVE (10 days in culture) and young HUVE (6 days in culture) were plated either with endothelial cell growth factor and heparin or without. A total of 2.0 ng/ml of TGF-β was added and the cells cultured for a total of 4 days, ^{3}H-thymidine (1 μCi/well) was added on day 3. The results are expressed as the mean ^{3}H-thymidine cpm (±SEM) of six determinations performed for each group. This is a representative of four similar experiments.

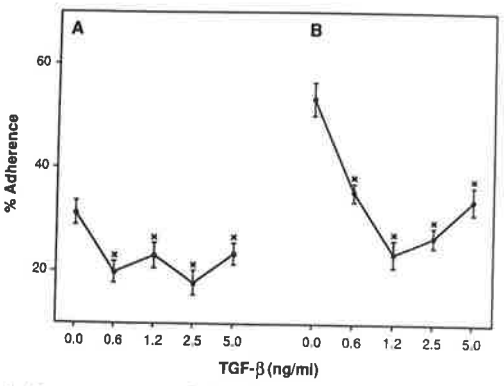

Figure 5. Dose response of TGF-β on T lymphocyte adhesion to unstimulated and TNF-α stimulated EC monolayers. EC were plated into microtiter wells with increasing doses of TGF-β. The next day some monolayers were left unstimulated and others stimulated for 6 h with 10U/ml TNF-α. At the end of this time, the monolayers were washed twice before T lymphocyte adhesion measured. The effect of TGF-β on T lymphocyte adhesion is shown to unstimulated (*A*) or TNF-α-stimulated (*B*) EC monolayers. The results show the mean ± SEM of three experiments with each point performed in triplicate in each experiment. x, p <0.05 by t-test compared with no TGF-β group.

TABLE III

Effect of TGF-β on lymphoblast adhesion[a]

TGF-β	Percent Adherence	p Value
Nil	50.2 ± 3.3	
2 ng/ml	34.4 ± 3.5	0.007

[a] HUVE were plated into microtiter wells either with or without the addition of 2 ng/ml TGF-β. Then 24 h later the monolayers were washed twice and 2×10^5 day 4 PHA-activated lymphoblasts added. Adherence was measured 30 min later as described in *Materials and Methods*. The results are the mean (±SEM) of three experiments with triplicate determinations performed in each group for each experiment.

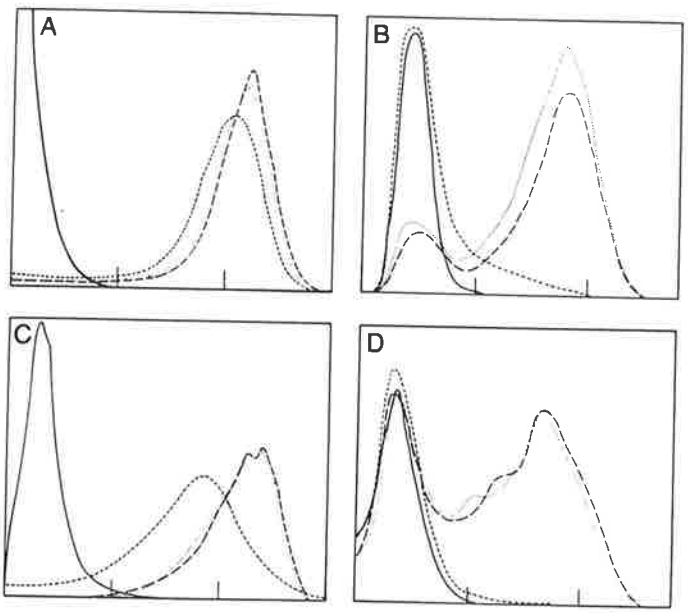

Figure 6. Regulation of expression of ICAM-1 and VCAM-1 on endothelial cells by TGF-β treatment. Young and old HUVES were plated with or without the addition of 2 ng/ml TGF-β for 24 h. During the final 6 h of this incubation 10U/ml of TNF-α was also added to the appropriate groups. The wells were then washed and stained with mAb recognising either ICAM-1 or VCAM-1 followed by a fluoresceinated sheep anti-mouse Ig. The cells were harvested, fixed, and analysed for fluorescence. The results of four separate experiments are given which are similar to those seen in at least two other experiments performed for each. In each of these experiments, the effect of TGF-β treatment on the adherence of T lymphocytes was also determined. *A*, Young HUVES; *C*, Old HUVES. (——) no antibody, (– – –) basal ICAM-1 expression, (—— —— ——) TNF-α induced ICAM-1, (· · ·) TGF-β pretreated TNF-α induced ICAM-1; *B*, Young HUVES; *D*, old HUVES. (——) no antibody, (– – –) basal VCAM-1 expression, (—— —— ——) TNF-α induced VCAM-1 expression, (· · ·) TGF-β-pretreated TNF-α-induced VCAM-1 expression.

to unstimulated and to TNF-α-stimulated HUVE (data not shown). The basal level of VCAM-1 expression varied between HUVE lines although most lines show no expression of VCAM-1. However, when some level of expression of VCAM-1 was seen, TGF-β was not able to alter this (data not shown). Further experiments were performed with old HUVE that showed similar immunofluorescence results (Fig. 6, *C* and *D*) but TGF-β did not influence lymphocyte adhesion to the old HUVE used in these studies (data not shown).

DISCUSSION

Our results show that TGF-β inhibits the attachment of lymphocytes and more specifically T lymphocyte to unstimulated and TNF-α- and IL-1β-stimulated EC in a manner similar to that which we described for neutrophils (16). In addition, the level of adhesion of activated lymphocytes (lymphoblasts) to endothelium is also inhibited by TGF-β. Importantly the extent of inhibition seen by pretreatment of endothelial monolayers with TGF-β either alone or in combination with TNF-α cannot be explained by toxicity to the cells (Table I).

EC play a key role in the development of inflammatory and immunologic responses. Adhesion is necessary for specific immunological recognition by T cells of antigen presented by EC in the context of MHC molecules (23). In addition, initial contact between EC and T cells is required for transmigration of T cells into the tissues. Immunologic mediators such as IFN-γ, TNF, and IL-1 have been shown to promote these functions in vitro (5, 6, 24, 25). Our findings suggest that another cytokine, TGF-β, is able to inhibit at least one component of these phenomena, namely adhesion.

The demonstration that EC lose responsiveness to TGF-β with regard to adhesion with age in culture may define an endothelial cell phenotype that is acquired for some days after endothelial cell injury, a process that may be mimicked in the harvesting of endothelial cells from umbilical cords. Clearly, the EC do not lose all responsiveness to TGF-β with extended growth in vitro as EC proliferation can be inhibited by TGF-β during all stages of cell growth (Table II). In addition, cross-linking studies show the same m.w. TGF-β-binding proteins on both old and young cells (data not shown). Our findings may therefore be analogous to the alteration in responsiveness to TGF-β upon differentiation in fetal (26) and myogenic (27) systems and suggest that the responsiveness of EC to exogenous hormones (such as TGF-β) is dependent on their state of differentiation.

Equally the TGF-β responsiveness described here may reflect the constant steady state situation in vivo and its loss be an artefact of culture conditions. This possibility is especially attractive as the nonadhesiveness of EC is a central feature of normal blood circulation and is supported by a recent report (28) that active TGF-β is produced when capillary endothelial cells and pericytes (or smooth muscle cells) are cocultured. It could thus be hypothesized that production of TGF-β serves in part to maintain the anti-adhesive state of EC.

Our observations raise questions not only about the regulation of TGF-β responsiveness but also how TGF-β works. The simplest hypothesis for the mode of action of TGF-β may be that the expression of structures on endothelium involved in adhesion (such as ICAM-1 and VCAM-1 for lymphocyte adhesion), is inhibited by TGF-β. However, on young HUVE where TGF-β inhibits lymphocyte attachment, no alteration in the basal level of surface expression of ICAM-1 or VCAM-1 nor in the level induced by TNF-α, was observed. Old HUVE also showed no response to TGF-β at the level of ICAM-1 or VCAM-1 expression. It should be noted that regulation of the level of expression of adhesion molecules is not the only mechanism by which the extent of adherence of leukocytes to endothelium may be altered. Changes in the conformational state of Mac-1 on neutrophils (29–32) and ELAM-1 (33) on endothelial cells has been implicated in regulatory adhesive phenomena, and raises the possibility that TGF-β may work through this mechanism. In addition, adhesion of lymphocytes to EC involves multiple receptor-ligand interactions (14). Of these we have analyzed the effect of TGF-β on ICAM-1 and VCAM-1 and the possibility remains that TGF-β has its chief effect on other structures. Regardless of the mechanism we suggest that TGF-β may have an important role in vivo to limit endothelial cell-lymphocyte and endothelial-neutrophil interactions.

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Transforming Growth Factor-β Inhibits E-Selectin Expression on Human Endothelial Cells[1]

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Abstract. Transforming growth factor-β (TGF-β), a pleiotropic cytokine that is elaborated in the active form upon co-culture of endothelial cells and pericytes or smooth muscle cells, has been shown to decrease the adhesiveness of endothelial cells for neutrophils, lymphocytes, and tumor cells. The mechanism whereby TGF-β inhibits the adhesiveness of human endothelial cells was investigated. TGF-β inhibited the basal E-selectin (formerly ELAM-1) expression by 55 ± 7% and TNF-stimulated expression by 57 ± 4%. Similar decreases of IL-1-stimulated expression were also seen. Peak inhibition was seen at TGF-β doses between 0.2 and 2 ng/ml. Both TGF-β_1 and -β_2 were functional. The effectiveness of TGF-β in inhibiting E-selectin expression was dependent on cell density and incubation time. TGF-β also inhibited E-selectin mRNA levels in endothelial cells. TGF-β had no effect on the expression of VCAM-1 and ICAM-1, but was additive with IL-4 in inhibiting the expression of E-selectin. The expression of E-selectin has been shown to mediate several aspects of the inflammatory response involving neutrophils and memory T lymphocytes. Perivascular TGF-β appears to act as an inhibitor of the expression of the endothelium-specific selectin, E-selectin, and therefore of inflammatory responses involving neutrophils and (a subset of) lymphocytes. *Journal of Immunology,* 1993, 150: 4494.

The adhesion of blood cells to endothelium is an essential process during the development of the inflammatory response. The treatment of EC[3] with TNF-α (1) or IL-1 (2) increases the adhesion of WBC due to the de novo or increased expression of adhesion molecules. In the case of neutrophils, the increased adhesion to IL-1- or TNF-treated HUVEC is due to the expression on HUVEC of the adhesion molecule, E-selectin, previously known as ELAM-1 (3–5). E-selectin is only expressed in EC and, in keeping with the function of all the three members of the selectin family, may also be responsible for the capture of neutrophils from the rapidly flowing axial blood stream to the marginated or rolling pool of cells (6). E-selectin is thought to provide the shear-resistant form of adhesion that is necessary before the integrin-mediated steps of adhesion and transmigration can begin to operate (7). E-selectin also stimulates the recognition by neutrophils of C-coated particles and may act as a chemotactic stimulus for neutrophils (8). In vivo E-selectin expression is seen at sites of inflammation mainly in the skin (9). It is also expressed in various tissues after inflammatory assaults and in the vasculature of lymphoid tumors (10, 11).

E-selectin also supports the adhesion of memory and skin homing T lymphocytes (12, 13), but not other types of lymphoid cells. Lymphocyte adhesion is supported by two other adhesion molecules, ICAM-1 and VCAM-1, the expression of which is also induced by TNF and IL-1 (14, 15). Whereas ICAM-1 is constitutively expressed, VCAM-1 is either absent or present in very low levels in resting EC (15). VCAM-1 expression, however, has also been observed in vivo at sites of chronic inflammation (16, 17),

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[3] Abbreviations used in this paper: EC, endothelial cells; HUVEC, human umbilical vein endothelial cells; TIE, TGF-β inhibitory element; nt, nucleotide; ECGS, endothelial cell growth supplement; TGF-β, transforming growth factor-β.

notably in the aorta at sites of atheroma formation (17), where presumably it mediates the influx of T lymphocytes and monocytes into these lesions. The regulation of VCAM-1 expression has been shown to differ from that of E-selectin. IL-4 induces VCAM-1 expression, whereas it inhibits TNF or IL-1 induction of E-selectin (14, 18).

TGF-β and related cytokines have been shown to be involved in processes of tissue formation and also to have immunosuppressive properties (19, 20). Active TGF-β is made by EC in vitro when they are co-cultured with pericytes or smooth muscle cells (21, 22). Inasmuch as these cells are in juxtaposition in vivo, it is suggested that the TGF-β found perivascularly is in the active form. Vascular TGF-β has been hypothesized to control the composition of the extracellular matrix deposited by EC, pericytes or smooth muscle cells, and fibroblasts. We have previously shown that treatment of HUVEC with TGF-β decreases basal adhesiveness to neutrophils and lymphocytes (23, 24). In addition, TGF-β inhibited neutrophil and lymphocyte adhesion stimulated by the cytokines TNF and IL-1β (23, 24) and supported the concept that perivascular TGF-β has an anti-inflammatory role. The inhibitory effects of TGF-β on adhesion were evident on recently explanted (or "young") HUVEC but were absent or much weaker on cells that had been in culture for more than approximately 2 wk ("old"). Subsequently, TGF-β has been shown to inhibit the adhesion of murine lymphocytes to Peyers patch endothelium and high endothelial venule cells (25) and of murine tumor cells to IL-1- or TNF-treated murine EC (26). These results suggested that TGF-β is an important regulator of endothelial function. We now show that TGF-β inhibits the expression of the endothelium-specific selectin, E-selectin, potentially providing an important mechanism for the prevention of inflammation.

Materials and Methods

EC

EC were extracted from umbilical veins by collagenase treatment according to a modified version of Wall et al. (27). Cells were grown in 25-cm^2 gelatin-coated Costar flasks (Cambridge, MA) in endotoxin-free M199 (Cytosystems, Sydney, Australia), 20% FCS (PA Biologicals, Sydney, Australia), 20 mM HEPES, with sodium pyruvate, nonessential amino acids and Fungizone. Young cells were used 2 to 5 days after establishment of culture. For additional growth, cells were harvested by trypsin-EDTA treatment (Flow, Australia) replated into 75-cm^2 gelatin-coated flasks in endotoxin-free M199, 20% FCS, 20 mM HEPES, sodium pyruvate, nonessential amino acids, and Fungizone with the addition of ECGS (Collaborative Research, Bedford, MA) and heparin (Sigma, St. Louis, MO), both at a final concentration of 50 μg/ml. Microvessel EC were prepared from neonatal foreskins according to the method of Marks et al. (28). Cells were frozen in liquid nitrogen at 1 to 2 $\times$ 10^6/vial at passages 2 to 6 and thawed as required. Medium for growth and maintenance of these cells was M199 with Earle's salts, 25 mM HEPES, 50% human serum, sodium bicarbonate, 2 mM glutanine, Fungizone, penicillin, streptomycin, 3.3 $\times$ 10^{-4} M cAMP, ECGS (50 μg/ml), and heparin (50 μg/ml).

Cell surface Ag expression

EC were plated onto gelatin-coated 24-well tissue culture trays (Nunc, Denmark) at 10^5 cells/well in 500 μl of HUVEC medium without ECGS or heparin. TGF-β was added at the time of plating. In some cases, TNF or IL-1β was added 19 to 20 h later for 4 to 5 h, giving a total incubation time of approximately 24 h. After this, medium was removed and the cells washed twice with fresh medium, and 200 μl of antibody directed to E-selectin, VCAM-1, or ICAM-1 were added. Cells were incubated for 30 min at 37°C and washed twice, and then sheep anti-IgG Fab_2-FITC-labeled antibody (Silenus, Victoria, Australia) added in a volume of 200 μl. The cells were incubated on ice for 30 min, washed three times in PBS, harvested by trypsin-EDTA treatment, pelleted, and resuspended in fixative (1% formaldehyde, 2% glucose, 5 mM sodium azide in PBS, pH 7.3). The fluorescence profiles were analyzed by flow cytometry using an EPICS Profile II; 10,000 cells/group were analyzed. The above method for fluorescence staining of attached cells was tested in parallel with cells detached before labeling. We found no difference in the level of expression of E-selectin, VCAM-1, or ICAM-1 with these two methods. Since fewer cells could be used to stain attached cells, this was chosen for all experiments described here.

ELISA assay for E-selectin expression

Freshly trypsinized EC were plated onto collagen-coated 96-well flat bottomed trays at 2 $\times$ 10^4 cells/well and incubated. TGF-β was added to wells at plating and TNF-α the next day 4 h before assay. For assay, medium was flicked off the plates and anti-E-selectin antibody incubated at 37°C for 1 h. Supernatants were flicked and plates washed once with RPMI + 10% FCS. The mouse Ig signal was amplified with rabbit anti-mouse Ig (Dako 2412) at 37°C for 30 min followed by one wash with assay medium and detected with goat anti-rabbit horseradish peroxidase (Dako P448) at 37°C for 30 min, followed by three washes. Positive signals were detected with *O*-phenylene diameric (Sigma P-1526) 1 mg/ml in 0.1 M citrate, pH 6.5, + 0.03% H_2O_2, at room temperature for 8 min and quenched with 1 M H_2SO_4, and OD read at 490 nm.

RNase protection assays

A 348-bp fragment of E-selectin cDNA (a gift from Dr. B Seed, Boston, MA) spanning nt 1–348 (5), was subcloned into the vector pGEM-1. The plasmid was linearized 74 bp 3′ of the cloning site and transcribed using SP6 RNA polymerase in the presence of [^{32}P]UTP as previously described (29) to generate a full length cRNA of 422 bases. A human β-actin cRNA probe of 330 bases containing 120 complementary nt to the human β-actin mRNA was used as an internal standard. The human β-actin cRNA probe used had approximately two times higher specific radioactivity than the E-selectin cRNA probe.

Total RNA from EC was isolated according to the method of Chomczynski and Sacchi (30); 5 μg of total RNA were hybridized to 20,000 cpm of each of the E-selectin and β-actin cRNA probes followed by digestion with 40 μg/ml RNase A essentially as described (31). The protected fragments of 348 bases (E-selectin) and 120 bases (β-actin) were resolved by electrophoresis on 6% polyacrylamide gels containing 8 M urea.

The amount of radioactivity in each band was visualized and quantitated using a Molecular Dynamics Phosphorimager. The extent of stimulation or inhibition of E-selectin mRNA by TNF or TGF-β was calculated after normalization using the β-actin mRNA as an internal control.

Antibodies

The antibody 1.2B6 (IgG1) (32), which reacts with E-selectin, was kindly provided by Dr. Dorian Haskard, Hammersmith Hospital, London, United Kingdom. The anti-VCAM-1 antibody was kindly supplied by Dr. Mike Gallatin and Dr. Boris Masinovsky, ICOS, Seattle, WA. The anti-TGF-β antibody (lot 8755.81) was supplied by Genentech, South San Francisco, CA; 1 mg of the antibody neutralizes 160 ng TGF-β_1, TGF-β_2, and TGF-β_3.

Cytokines

TNF-α (lot S9010AX; sp. act., 6.27×10^7 U/mg) and TGF-β (lots 8987–53 and G098AD) were kindly supplied by Genentech. Porcine TGF-β_1 and -β_2 were obtained from R&D Systems (British Biotechnology Ltd., Oxford, United Kingdom). IL-1β (10^8 thymocyte mitogenesis U/mg) was kindly supplied by Immunex, Seattle, WA. All reagents contained less than 3 Ehrlich U/mg of LPS as detected by limulus amoebocyte assay.

Statistics

Significance was determined by the unpaired two-tailed *t*-test.

Table I
Effect of TGF on basal and stimulated E-selectin expression

TGF-β (ng/ml)	E-Selectin			
	0	0.2	*p* value	% Inhibition
Basal (n = 11)	1.7 ± 0.6[a]	0.9 ± 0.4	0.008[b]	55 ± 7[c]
TNF stimulated (n = 16)[d]	20.0 ± 4.3	7.1 ± 1.6	0.002	57 ± 4

[a] Mean fluorescence expressed as mean channel number ± SEM of 10,000 cells.
[b] *p* value of difference between 0 and 0.2 ng/ml TGF-β.
[c] Inhibition was calculated for each experiment and mean then calculated.
[d] These 16 experiments include all 11 used for "basal" studies. The results were not significantly different if the additional five experiments for which there were no corresponding basal studies were excluded.

Results

Effect of TGF-β on basal expression of E-selectin

EC derived from umbilical veins have a low expression of E-selectin for about the first week in culture, perhaps due to cytokines secreted by passenger leukocytes (J. R. Gamble and M. A. Vadas, unpublished observations). We noted that this expression declines with passage in culture as the number of contaminating cells also declines. In young EC as measured by flow cytometry, 23 ± 8% (mean ± SEM n = 6) of the cells were positive for E-selectin, with a mean intensity of positive cells of 5.7 ± 0.2 channels. In old EC, only 2 ± 1% (n = 4) of cells were positive, with a mean intensity of positive cells of 2.8 ± 0.7 channels. TGF-β was added to young HUVEC for 24 h, and the expression of E-selectin was determined by flow cytometry. TGF-β inhibited basal mean E-selectin expression by 55 ± 7% (mean ± SEM of 11 separate experiments) (Table I). Figure 1*A* gives an example of the flow cytometry profile of young HUVEC cultured in the presence or absence of 0.2 ng/ml TGF-β. The inhibition was maximal with 0.2 ng/ml and was less at higher and lower doses (Fig. 2*A*). The bell shaped dose-response curve was a consistent observation, but the most effective dose of TGF-β varied between 0.2 and 2.0 ng/ml, depending on the batch of TGF-β used. A bell-shaped dose-response curve to TGF-β is also seen with neutrophil or lymphocyte adhesion (23, 24) in the inhibition of smooth muscle cell proliferation (33), and in the TGF-β_1-induced neutrophil chemotaxis (34).

Effect of TGF-β on TNF-stimulated expression of E-selectin

TNF stimulates the expression of E-selectin on EC, with peak expression being seen 4 to 6 h after the addition of TNF (4, 5). In young cells, after 1 U/ml TNF, 92 ± 9% (n = 6) of cells became positive with a mean intensity of 17.7 ± 2.4 channels. In old cells, only 49 ± 4% of cells became positive with a mean intensity of the positive population of 5 ± 1 channels. TGF-β added 20 h before TNF inhibited this effect of TNF on young cells, and as with

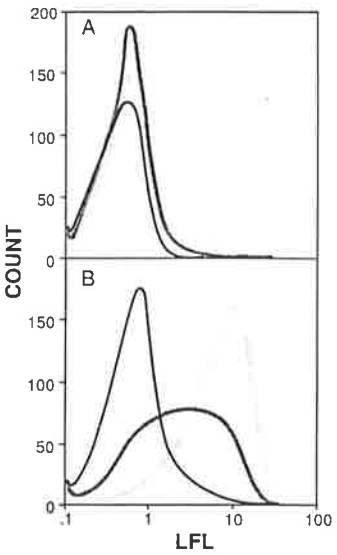

FIGURE 1. Flow cytometry profiles of the expression of E-selectin (*light lines*) in young (3 days in culture) HUVEC that were not (*A*) or were (*B*) treated for 4 h with 1 U/ml TNF. The effect of 0.2 ng/ml TGF-β added 24 h before assay is also shown (*dark lines*). *Black lines* show fluorescence with control antibody. *Vertical axis* shows cell numbers in each channel and *horizontal axis*, the intensity of fluorescence on a 3-decade scale.

basal E-selectin expression, 0.2 ng/ml TGF-β gave maximal inhibition (Fig. 2*B*). Inhibition was seen at various doses of TNF (Fig. 3), however the largest inhibitions were observed at submaximal doses of TNF. In a series of 16 experiments, 0.2 ng/ml TGF-β inhibited 1 U/ml TNF-stimulated E-selectin expression by a mean of 57 ± 4% (Table I). The flow cytometry profile (Fig. 1*B*) of a typical experiment shows the shift in E-selectin expression after TNF treatment and its inhibition by TGF-β. In all experiments, flow cytometry profiles showed a single bell-shaped curve of E-selectin expression after TNF induction. Therefore, in all other experiments, fluorescence levels are reported as the mean channel fluorescence. Similar levels of increased E-selectin expression with TNF treatment and decreased E-selectin expression after TGF-β were observed in an ELISA in which no trypsinization of the cells was requaired (data not shown). As previously shown for adhesion (24), TGF-β was effective in inhibiting E-selectin expression when added 24 h, 15 h, but not 6 h or 3 h before assay (data not shown). The effect of TGF-β was not evident if added 48 h before assay (Table II, group II compared to group III), however, this was most likely due to either exhaustion of the amount or effectiveness of TGF-β present or conversion to an inactive (35) form, since re-addition of TGF-β after 24 h of culture resulted in equivalent levels of inhibition of E-selectin as that seen with only 24-h incubation (Table II, groups IV and V). TGF-β inhibited IL-1-induced E-selectin expression in a similar fashion to that of TNF (data not shown). A mAb against TGF-β prevented the inhibition of E-selectin expression (Table III), and recombinant human and purified porcine TGF-β_1 were both active, as was purified porcine TGF-β_2 (Table IV). The ability of TGF-β to inhibit TNF-induced E-selectin expression was dependent on the density of the cells. Cells plated at high density (Table V) or allowed to grow to high density before the addition of TGF-β (data not shown) were less responsive than cells plated at lower cell numbers. For three experiments, the mean inhibition for cells plated at 1×10^5/well was 64.1 ± 3.5 and for cells at 2×10^5/well was 20.1 ± 4.5 ($p = 0.009$). The effect of TGF-β was also seen in old EC, however the decrease observed was of a lesser magnitude, was not observed in all EC lines tested and, if seen, was observed only at a single concentration of 0.2 ng/ml of TGF-β (Table VI). For 13 experiments with old cells, 0.2 ng/ml of TGF-β-inhibited TNF-α stimulated E-selectin expression by 22.6 ± 6.3%. Cell lines of capillary endothelium derived from human foreskin were also tested for E-selectin regulation by TGF-β. Of four lines analyzed, two showed no change in E-selectin and two showed a decrease (by 98 and 62%), with 0.2 ng/ml of TGF-β in TNF-induced E-selectin expression.

TGF-β inhibits E-selectin mRNA accumulation

E-selectin mRNA was measured by RNase protection in young or old EC that were or were not pretreated with 0.2 ng/ml TGF-β (Fig. 4). The higher basal and stimulated expression of E-selectin mRNA in young rather than old EC is evident, as is the inhibitory effect of TGF-β. Using a phosphorimager to quantitate the amount of radioactivity, the mean E-selectin mRNA levels in three experiments (calculated as a ratio of E-selectin to actin) was obtained (Table VII). A 7.1 ± 1.5 (mean ± SEM, $n = 3$, $p = 0.008$)-fold higher basal expression of E-selectin mRNA was observed in young EC compared to old EC. TNF (1 U/ml) stimulated expression by 6.7 ± 0.9-fold in young and 17.4 ± 0.8-fold in old cells. Notably the level of E-selectin mRNA and surface expression after any dose of TNF was always higher in young than old EC (data not shown). However, inasmuch as young EC also always had higher basal expression, correspondingly the increase was about twofold less in young than old HUVEC. TGF-β inhibited basal E-selectin mRNA in young cells in every experiment and by a mean of 36 ± 10% ($p = 0.03$, $n = 3$), but not significantly ($p = 0.3$) in old ($n = 6$) cells. TNF-induced E-selectin mRNA was inhibited by TGF-β in each experiment with young EC (mean inhibition, 23 ± 6%, $n = 3$, $p = 0.01$) but was more variable in old cells (mean inhibition, 25 ± 9%, $n = 6$, $p = 0.06$).

Effect of TGF-β on VCAM-1 expression

We have previously shown that TGF-β did not alter the expression of ICAM-1 or VCAM-1 on HUVEC (24). We

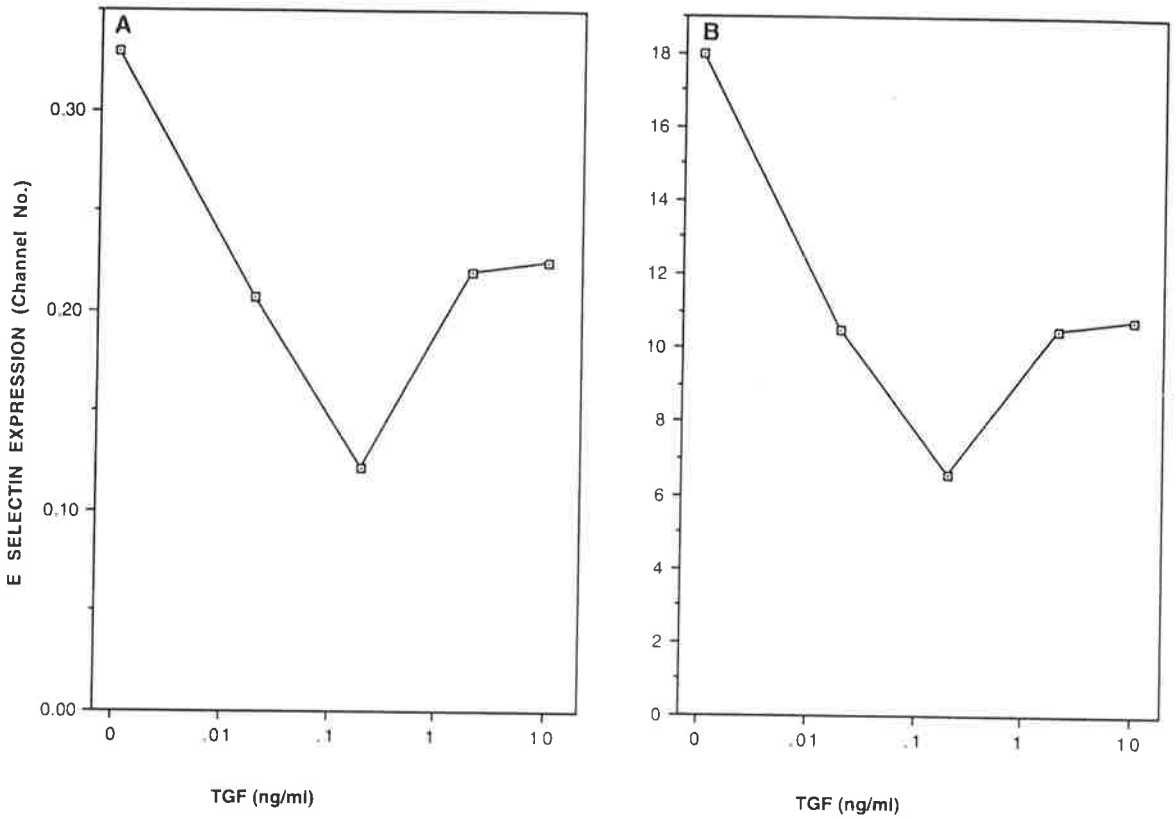

FIGURE 2. Effect of a range of doses of TGF-β on mean E-selectin expression by young EC. *A*, Basal expression; *B*, expression stimulated by 1 U/ml TNF. Ten other experiments gave similar results, although on occasion peak inhibition was seen with 2 ng/ml TGF-β.

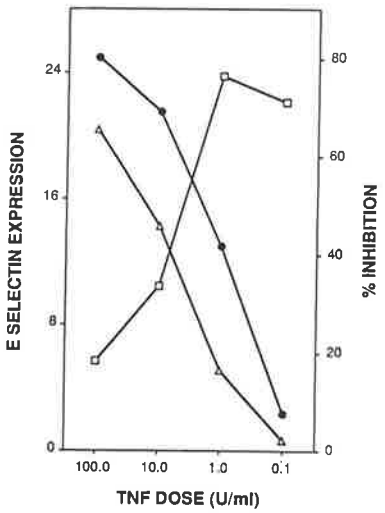

FIGURE 3. Effect of TGF-β on E-selectin expression stimulated by various doses of TNF. Expression (mean channel number) in the absence of TGF-β (●) or with 0.2 ng/ml TGF-β (△) is shown. The calculated percentage inhibition (□) is also shown. The experiment was repeated with similar results.

now confirm the findings on VCAM-1, in which case TGF-β did not significantly decrease basal expression (without TGF-β mean expression was 6.10 ± 2.19, and with TGF-β 5.89 ± 1.73, $n = 5$) nor TNF-induced expression (without TGF-β mean expression was 16.70 ± 5.27 and with TGF-β 15.67 ± 5.06, $n = 8$) in young EC. In the latter instance, we did however note a significant decrease in two of the eight lines examined.

Table II
Effect of time of addition of TGF-β on E-selectin expression[a]

Group	Time of Addition of TGF-β[b]	TNF[c] (U/ml)	E-Selectin Expression after: 24-h culture	48-h culture
I	Nil	0	1.05[d]	0.67
II	Nil	1	9.73	18.80
III	At plating	1	3.33 (66)[e]	17.82 (5.2)
IV	24 h after plating	1		7.04 (63)
V	At plating and 24 h after plating	1		8.39 (55)

[a] Data are representative of three similar experiments.
[b] TGF-β used at 0.2 ng/ml.
[c] TNF added 4 h before assay.
[d] Mean fluorescence expressed as mean channel number of 10,000 cells.
[e] Numbers in parentheses, percentage inhibition compared to no TGF-β.

TGF-β and IL-4 show additive effects at decreasing E-selectin expression

It was previously shown (14) that IL-4 added 20 h before TNF inhibits the induction of E-selectin. We now confirm these findings and in addition observe a decrease in basal

Table III
Inhibition of TGF-β by anti-TGF-β antibodies

TGF-β[a] (ng/ml)	Antibody[b] (μg/ml)	TNF[c] U/ml)	E-Selectin Experiment 1	E-Selectin Experiment 2
0	0	0	0.4[d]	1.6
0	0	1	7.6	11.0
0.2	0	1	4.4	8.3
0.2	56	1	9.1	12.1

[a] TGF-β was added 24 h before assay.
[b] Anti-TGF-β was added to TGF-β for 30 min at room temperature before the mixture was added to HUVEC.
[c] TNF was added 4 h before assay.
[d] Mean fluorescence expressed as mean channel number of 10,000 cells.

Table IV
Comparison of the effect of TGF-β_1 and β_2 on TNF-stimulated E-selectin expression

TGF-β Type	TGF-β ng/ml	TNF (U/ml)	E-Selectin Experiment 1	E-Selectin Experiment II
—	0	0	1.2[a]	0.7
—	0	1	11.9	6.8
rhβ_1[b]	2	1	4.9	2.3
rhβ_1[b]	0.2	1	7.1	4.0
pβ_1[c]	2	1	5.7	2.8
pβ_1[c]	0.2	1	5.8	3.7
pβ_2[c]	2	1	6.2	3.9
pβ_2[c]	0.2	1	8.4	5.3

[a] Mean fluorescence expressed as mean channel number of 10,000 cells.
[b] rhβ_1, Recombinant human TGF-β_1. For this batch of human rTGF-β, maximum inhibition was generally seen at 2.0 ng/ml.
[c] pβ_1 or pβ_2, Purified porcine TGF-β_1 or -β_2

Table V
Effect of cell density on EC responsiveness to TGF-β[a]

No. EC Plated/Well	TGF-β (ng/ml)	TNF-α[b] (U/ml)	E-Selectin Expression after 24-h Exposure to TGF-β
1×10^5[c]	0	0	0.53[d]
	0	1	26.68
	0.2	1	9.85 (63)[e]
2×10^5[c]	0	0	0.22
	0	1	17.05
	0.2	1	12.36 (28)

[a] Data are representative of three similar experiments.
[b] TNF-α added 4 h before assay.
[c] EC plated at 1×10^5/well formed a monolayer but did not assume the cobblestone morphology after 24 h of culture. When plated at 2×10^5 cells/well, EC showed a cobblestone confluent morphology after 24 h of culture.
[d] Mean fluorescence expressed as the mean channel number of 10,000 cells.
[e] Numbers in parentheses, percent inhibition compared to no TGF-β.

E-selectin expression in the presence of IL-4. We also note that the magnitude of the decrease of E-selectin expression is greater with IL-4 alone than with TGF-β alone, but show that at maximal doses of IL-4, TGF-β has an additional suppressive effect on E-selectin expression (Table VIII).

Discussion

The expression of adhesion proteins by EC is essential in the development of inflammatory responses. We now show

Table VI
Effect of TGF-β on E-selectin expression in young and old HUVEC[a]

HUVEC	TNF (U/ml)	0 TGF-β	0.2 ng/ml TGF-β	% Inhibition[b]
Young	0	0.2[c]	0.07	70
Young	1	5.2	2.4	53
Old	0	0	0.04	0
Old	1	0.8	0.6	22[d]

[a] Data are representative of 10 to 13 similar experiments.
[b] The calculation for percent inhibition included the second decimal point where necessary.
[c] Mean fluorescence expressed as mean channel number of 10,000 cells.
[d] This inhibition was only observed at 0.2 ng/ml, whereas in young HUVEC, inhibition was seen with doses between 0.02 and 10 ng/ml (see Fig. 2).

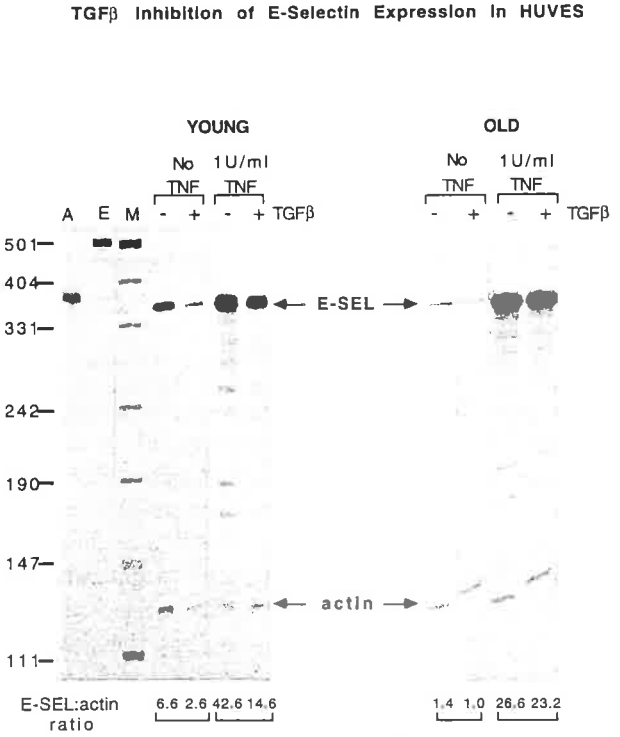

FIGURE 4. RNase protection assay of E-selectin and actin mRNA expression in young EC. The basal expression of E-selectin, its induction by 1 U/ml TNF, and the inhibitory effect of 0.2 ng/ml TGF-β are demonstrated; 5 μg of total RNA from EC were hybridized to ^{32}P-labeled E-selectin and β-actin cRNA probes followed by RNase A digestion, PAGE, and autoradiography. The autoradiogram shows full length,protected fragments by E-selectin and β-actin mRNA (indicated by *arrowheads*) as well as three minor fragments between m.w. markers of 147 bases and 190 bases. These three minor bands are probably the result of polymorphism within the E-selectin mRNA. *Lane A*, Full length actin cRNA probe; *Lane E*, full length E-selectin cRNA probe; *Lane M*, m.w. markers. The E-selectin:actin ratio was obtained by quantitating the E-selectin and action bands using a phosphorimager.

that TGF-β inhibits the expression of E-selectin on venous (HUVEC) EC. The inhibition is seen both with basal expression of E-selectin, seen in recently explanted or young HUVEC, and of TNF- and IL-1-stimulated expression in

Table VII
Inhibition of E-selectin mRNA by TGF-β in young and old HUVEC

TGF-β (ng/ml)	TNF (U/ml)	E-Selectin:Actin Ratio	
		Young	Old
0	0	7.4[a]	1.1
0	1	50.0	19.7
0.2	0	4.5	0.7
0.2	1	38.0	13.1

[a] The amount of radioactivity was quantitated using a phosphorimager. Arithmetic mean of three experiments.

Table VIII
Additive effect of TGF-β and IL-4 on E-selectin expression[a]

TGF-β (ng/ml)	IL-4 (ng/ml)	TNF (U/ml)	E-Selectin	
			Experiment I[b]	Experiment II[b]
—	—	—	6.7[c]	4.0
0.2	—	—	4.2	1.9
—	10	—	2.4	1.3
0.2	10	—	1.8	1.0
—	—	1	13.1	11.4
0.2	—	1	8.5	4.5
—	10	1	5.7	8.8
0.2	10	1	4.3	2.5

[a] Data are representative of four similar experiments.
[b] Young HUVEC were used for these experiments.
[c] Mean fluorescence expressed as mean channel number of 10,000 cells.

both young and old EC and in two of four capillary EC lines tested. The extent of inhibition was approximately the same as the inhibition of adhesion that we had observed previously (23, 24). The presence of immunoreactive TGF-β around blood vessels in vivo (36) and the production of active TGF-β by a co-culture in vitro of the cellular components of blood vessels, EC and pericytes (21), or smooth muscle cells (22) suggests that this is an important mechanism that tonically inhibits E-selectin expression and therefore the types of inflammation in which E-selectin is involved.

TGF-β inhibits steady state E-selectin mRNA, suggesting that TGF-β may exert its effect at the transcriptional level or at the level of mRNA stability. The necessity for TGF-β to be present at least 10 h before the addition of TNF or IL-1 argues for synthesis of a factor that prevents transcription. This is in accordance with the observations that cyclophosphamide abolishes the inhibitory effect of TGF-β on adhesion in a murine system (26). Bereta et al. (26) also showed that okadaic acid, an inhibitor of phosphatases, reverses the effect of TGF-β on adhesion in a murine system, suggesting that dephosphorylation of a protein is a step in this pathway.

The transcription rates of several genes are inhibited by TGF-β. Based on sequence homology in the promoters of these genes, Kerr et al. (37) have suggested a putative TIE with the consensus sequence, GNNTTGGtGa (Table IX).

Table IX
TGF-β inhibitory element-like sequences in the E-selectin promoter

nt[a]	TIE-Like Sequence in E-Selectin Promoter
Consensus	GNNTTGGtGa[b]
−74 to −65	GATGTGGACA
−98 to −89	CCATTGGGGA
−160 to −151	GAGTTTCTGA
−194 to −185	GGCATGGACA
−460 to −451	GAATTGGCAG
−800 to −791	GAGATGGCGT
−824 to −833	GGCATGGTGG[c]

[a] The nt numbering used is according to the method of Whelan et al. (38).
[b] n = any nt; capital letters = invariant nt; lower case letters = preferred nt.
[c] This sequence lies on the opposite strand.

They have also demonstrated the presence of a TGF-β-inducible nuclear protein complex that specifically binds to the TIE in the transin/stromelysin promoter. We have compared the E-selectin promoter sequence (38) (European Molecular Biology Laboratories database) from nt −900 to the start site of transcription (+1) with the TIE consensus sequence. Seven TIE-like elements are present in the E-selectin promoter (Table VIII). Whether any of these sequences is sufficient for binding of the nuclear protein complex that interacts with the TIE remains to be determined. Interestingly, two of these sequences (nt −74 to −65 and nt −98 to −89) overlap with the CAAT-box and the NF-κB consensus sequence suggested to be responsible for the TNF-induced transcription of E-selectin (39) and, hence, TGF-β may inhibit E-selectin transcription by inducing a nuclear protein complex that competes with the CAAT-binding protein and/or NF-κB for their respective binding sites.

The other striking observation in this paper is the constitutive expression of E-selectin as seen in young EC (Tables IV and V). This could be due to TNF or IL-1 made by passenger leukocytes. However, it has been well described that TNF, IL-1, and endotoxin induce E-selectin expression that peaks at 4 to 6 h and largely disappears by 24 h (40). The expression of E-selectin in young EC is not transient but maintained for about 1 wk, suggesting that a factor other than TNF, IL-1, or LPS is involved. It should be noted that the expression of E-selectin in skin EC is present in chronic skin diseases, such as atopic dermatitis (10). One possibility is that IFN-γ, which by itself has no effect on E-selectin expression, but, in conjunction with TNF can maintain or stabilize E-selectin expression (41), is responsible. However, IFN-γ only increases the percent of EC showing positivity and has no effect on the degree of positivity of the cells (41). In the case of young EC, both the percent positivity and the amount of E-selectin-positive cells are increased compared to old EC. Furthermore, these young EC are capable of expressing more E-selectin mRNA than old EC regardless of the dose of TNF used, suggesting that a

factor other than IFN-γ is responsible. Preliminary experiments from our laboratory have shown that supernatant from young EC can stabilize and increase the expression of E-selectin on multi-passaged EC (J. R. Gamble and M. A. Vadas, unpublished observations). Regardless of the nature of this factor, we note that TGF-β inhibits or eliminates basal E-selectin expression and mRNA in young, but not in old, EC. The reason for the loss of responsiveness to TGF-β with regard to regulation of adhesion and E-selectin expression is not known at present. One possibility is that old EC may actually be secreting active TGF-β. However, supernatant taken from confluent old EC does not result in inhibition of TNF-induced E-selectin expression in young cells (data not shown).

The effect of TGF-β on E-selectin expression is dependent on cell density. E-selectin expression is significantly inhibited by TGF-β in EC that are semiconfluent. Cells that are either plated down at high cell density or that are allowed to grow to high density before the addition of TGF-β respond poorly. This density dependence of the activity of TGF-β has been reported for fibroblasts (42, 43), smooth muscle cells (44), and EC (35). The mechanism for the variation in response to TGF-β is not known but may depend on the extracellular matrix deposited by the cells, the proliferative potential of EC, or the expression of TGF-β binding proteins.

Although TGF-β has been shown to alter the expression of many proteins, notably those of the extracellular matrix and of enzymes responsible for degrading these (19, 20), this is the first description of the effect of TGF-β on selectins. The effect of TGF-β on adhesion proteins is selective, inasmuch as there is no significant change in the expression of the Ig-like adhesion protein, ICAM-1 or VCAM-1 (23). The lack of effect on VCAM-1 is notable, inasmuch as it is another adhesion molecule with an absent or low basal expression in which expression is induced by TNF or IL-1.

The selectivity of TGF-β for E-selectin expression vs ICAM-1 and VCAM-1 may be important given the different roles for these EC adhesion molecules. E-selectin binds neutrophils, eosinophils, monocytes, and a subset of T lymphocytes, and is believed to play an essential role in the capture of leukocytes from the circulation. ICAM-1 and VCAM-1 molecules bind a similar spectrum of cells (although VCAM-1 does not bind neutrophils (18)) and interact with the β_2 and $\alpha_4\beta_1$ integrins, respectively, allowing the adhesion of cells at low shear forces and their transmigration from blood to tissue. Indeed we have noted that TGF-β does not inhibit the capacity of HUVEC to allow cellular transmigration (W. B. Smith, J. R. Gamble, and M. A. Vadas, unpublished observations). Thus, it is envisaged that in areas where blood stagnates or shear forces are low, the inhibitory effect of TGF-β will not be significant, and that the main site of action of TGF-β is likely to be in capillaries and postcapillary venules. The dominant role of E-selectin in skin (9, 10) and in the adhesion of skin homing and memory T lymphocytes (12, 13) suggests that the powerful in vivo anti-inflammatory and immunosuppressive action of TGF-β (45) is mediated in part by inhibiting the localization of T cells to the site of antigenic challenge and hence, of the orchestration of a specific immune response.

IL-4 is similar to TGF-β in that it inhibits the expression of E-selectin, however, it differs from TGF-β in being a strong inducer of the expression of VCAM-1 (14). Indeed, IL-4 and TGF-β are to some extent additive in inhibiting E-selectin expression (Table VIII), suggesting that their combined presence could eliminate the contribution of E-selectin to inflammatory responses. The failure to find E-selectin in certain tissues such as kidney (M. A. Vadas and D. Gillis, unpublished observations) may be due to the combined effect of these cytokines.

TGF-β is a cytokine that is made by blood vessel cells, the expression of which increases after arterial injury (36). The therapeutic administration of TGF-β has been shown to diminish the size of experimental myocardial infarction (46), suggesting an important role for perivascular TGF-β. The importance of TGF-β in the vasculature is further substantiated by the results with TGF-β_1-deficient mice in which multifocal inflammatory disease with gross cellular infiltration is seen (47). Our results showing that TGF-β inhibits the expression of E-selectin on EC suggest that TGF-β may be an important molecule in the control of EC function.

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Novel Cytokine-independent Induction of Endothelial Adhesion Molecules Regulated by Platelet/Endothelial Cell Adhesion Molecule (CD31)

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Abstract. Tumor necrosis factor–α, interleukin-1, and endotoxin stimulate the expression of vascular endothelial cell (EC) adhesion molecules. Here we describe a novel pathway of adhesion molecule induction that is independent of exogenous factors, but which is dependent on integrin signaling and cell–cell interactions. Cells plated onto gelatin, fibronectin, collagen or fibrinogen, or anti-integrin antibodies, expressed increased amounts of E-selectin, vascular cell adhesion molecule–1, and intercellular adhesion molecule–1. In contrast, ECs failed to express E-selectin when plated on poly-L-lysine or when plated on fibrinogen in the presence of attachment-inhibiting, cyclic Arg-Gly-Asp peptides. The duration and magnitude of adhesion molecule expression was dependent on EC density. Induction of E-selectin on ECs plated at confluent density was transient and returned to basal levels by 15 h after plating when only 7 ± 2% (n = 5) of cells were positive. In contrast, cells plated at low density displayed a 17-fold greater expression of E-selectin than did high density ECs with 57 ± 4% (n = 5) positive for E-selectin expression 15 h after plating, and significant expression still evident 72 h after plating. The confluency-dependent inhibition of expression of E-selectin was at least partly mediated through the cell junctional protein, platelet/endothelial cell adhesion molecule–1 (PECAM-1). Antibodies against PECAM-1, but not against VE-cadherin, increased E-selectin expression on confluent ECs. Co–culture of subconfluent ECs with PECAM-1– coated beads or with L cells transfected with full-length PECAM-1 or with a cytoplasmic truncation PECAM-1 mutant, inhibited E-selectin expression. In contrast, untransfected L cells or L cells transfected with an adhesion-defective domain 2 deletion PECAM-1 mutant failed to regulate E-selectin expression. In an in vitro model of wounding the wound front displayed an increase in the number of E-selectin–expressing cells, and also an increase in the intensity of expression of E-selectin positive cells compared to the nonwounded monolayer. Thus we propose that the EC junction, and in particular, the junctional molecule PECAM-1, is a powerful regulator of endothelial adhesiveness.

THE endothelial lining of the vascular system normally displays a nonactivated, nonadhesive phenotype. Stimulation with agents such as tumor necrosis factor-α (TNF-α)[1], interleukin-1 (IL-1), or lipopolysaccharide (LPS) are known to induce the expression of proteins on the endothelial surface that mediate coagulation (Bevilacqua et al., 1986), leukocyte adhesion (Bevilacqua et al., 1985; Gamble et al., 1985; Pober et al., 1986*b*; Doherty et al., 1989), and leukocyte transendothelial migration (Furie et al., 1989; Moser et al., 1989). The endothelial antigens that are important for the adhesion of leukocytes are members of the selectin family, E- and P-selectin, and the immunoglobulin gene superfamily, vascular cell adhesion molecule–1 (VCAM-1) and intercellular adhesion molecule–1 (ICAM-1) (Carlos and Harlan, 1994; Litwin et al., 1995).

The induction of E-selectin expression on endothelial cells (ECs) in vitro after cytokine stimulation is transient and independent of the continued presence of the stimulant (Pober et al., 1986*a*). Previous studies have shown that

M. Vadas and J. Gamble contributed equally to this paper.

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1. *Abbreviations used in this paper*: CSF, colony stimulating factor; EC, endothelial cell; HUVEC, human umbilical vein endothelial cells; ICAM-1, intercellular adhesion molecule–1; IL-1 and IL-1ra, interleukin-1 and IL-1 receptor agonist; LPS, lipopolysaccharide; MFI, mean fluorescence intensity; PECAM, platelet/endothelial cell adhesion molecule; TNF-α, tumor necrosis factor-α; VCAM-1, vascular cell adhesion molecule–1.

E-selectin mRNA and protein levels peak between 2 and 4 h, respectively, after treatment with an agonist, returning to near basal levels by 24 h (Bevilacqua et al., 1989; Read et al., 1994). VCAM-1 (Osborn et al., 1989) and ICAM-1 (Pober et al., 1986*b*) are maximal 6 and 12 h, respectively, after stimulation.

In contrast to the transiency of E-selectin and VCAM expression demonstrated by the in vitro data, these antigens have been detected on venular endothelium in chronic inflammatory lesions, such as the synovium in rheumatoid arthritis (Koch et al., 1991), and the skin in psoriasis (Petzelbauer et al., 1994). E-selectin expression is also detected on angiogenic vessels in human hemangiomas, a noninflammatory angiogenic disease (Kraling et al., 1996). Moreover, the architecture and anatomic localization of capillary loops influence the pattern of endothelial expression of E-selectin and VCAM-1, independently of the availability of cytokines (Petzelbauer et al., 1994). Thus it is likely that alternate control mechanisms exist to allow prolonged, locality-based expression of adhesion molecules on the endothelium. At least one of these alternate mechanisms may be flow, since increased shear stress has been shown to selectively modulate adhesion molecule expression, upregulating ICAM-1 but not E-selectin or VCAM-1 (Nagel et al., 1994).

Since sites of inflammation are often associated with morphological changes including cell retraction of the endothelium (Schumacher, 1973), we hypothesized that cell contacts may be important in the regulation of endothelial phenotype. We describe here the central role of the junctional protein, platelet/endothelial cell adhesion molecule–1 (PECAM-1), through the formation of cell–cell interactions, in the maintenance of the functional integrity of the endothelial monolayer. Furthermore, we demonstrate a novel pathway for the induction of adhesion molecules on endothelial cells that is independent of exogenous addition of cytokines, but is related to integrin- and cell shape–associated signaling events.

Materials and Methods

Cell Culture

ECs were extracted from human umbilical veins by collagenase treatment, according to a modified method of Wall et al. (1978). Cells were grown in 25-cm^2, gelatin-coated Costar flasks (Costar Corp., Cambridge, MA), maintained with endotoxin-free medium 199 (Cytosystems, Sydney, Australia), 20% FCS (Commonwealth Serum Laboratories, Melbourne, Australia), 20 mM Hepes, 2 mM glutamine, 1 mM sodium pyruvate, 1% nonessential amino acids (Cytosystems), 0.225% sodium bicarbonate and antibiotics in the absence of exogenously added heparin or basic fibroblast growth factor, at 37°C in a humidified, 5% CO_2 (in air) atmosphere. After 2–5 d of culture, the cells were harvested by trypsin-EDTA treatment (Cytosystems) and replated at specified cell densities on 1.9-cm^2, gelatin- and fibronectin-coated multiwell dishes (Nunc, Roskilde, Denmark) for flow cytometry experiments, or on fibronectin-coated glass or Permanox® chamber slides (Labtek, Nunc, Roskilde, Denmark) for immunofluorescence and adhesion assays. The preparation of PECAM-1–transfected L cells has been reported previously (DeLisser et al., 1993). The cells were maintained in RPMI 1640 (GIBCO BRL, Gaithersburg, MD) 10% FCS with 0.5 mg/ml G418.

Antibodies

Mouse mAbs directed against E-selectin (49-1B11), VCAM-1 (51-10C9), PECAM-1 (51-9H6, 51-6F6, 55-3D2), VE-cadherin (55-7H1), integrin β_1 (61-2C4), colony-stimulating factor (CSF), common β_c chain (3D7, supplied by Q. Sun, Hanson Centre for Cancer Research [HCCR], Adelaide, Australia), and Keyhole Limpet Hemacyanin (23-1F11) were raised at the HCCR. The anti-PECAM-1 mAbs were functional in an assay of neutrophil transendothelial migration and anti-VE-cadherin antibody was functional in an assay of EC aggregation. For coating of wells with antibody, plates were coated with rabbit anti-mouse Ig (50 μg/ml) for 18 h, blocked with 1% BSA, and then anti-integrin or anti-CSFβ_c chain–purified antibodies added for 1 h at 37°C. Wells were then washed and further blocked before addition of cells.

Flow Cytometry

Flow cytometric analysis of in situ endothelial monolayers was performed as previously described (Gamble et al., 1993). EC monolayers were blocked in 5% sheep serum, and then were stained with primary antibody for 30 min at 37°C, washed twice with RPMI 1640 containing 2.5% FCS, and then stained with FITC-conjugated, anti-mouse Ig (Fab2, DAF; Silenus Laboratories, Hawthorn, Australia) for 30 min at room temperature. Cells were washed twice, removed by trypsin-EDTA treatment, and then fixed in 1% formaldehyde, 0.02% azide, and 0.02% glucose. In experiments involving endothelial pretreatment with mAbs, E-selectin was detected using a single layer, FITC-conjugated, anti–E-selectin mAb (49-1B11).

A minimum of 1,000 events per test was analyzed using an EPICS Profile II (Coulter Immunology, Hialeah, FL). Results of individual EC lines are expressed either as a plot of frequency versus log fluorescence, or as the mean fluorescence channel number, subtracting the accompanying value for the negative control Ig. When results from multiple EC lines have been pooled, the mean fluorescence intensity (MFI) represents *n* cell lines.

In cocultures of ECs and L cells prepared for flow cytometric analysis, the cells were stained with anti–VE-cadherin, detected with phycoerythrin (PE)-conjugated anti-mouse F(ab')2 (DDAPE; Silenus Laboratories, Hawthorn, Australia) and simultaneously stained with goat anti–E-selectin detected with FITC–conjugated, anti-goat antibody (Silenus Laboratories). L cells were negative for VE-cadherin, EC were 100% positive for VE-cadherin and were selected for analysis of E-selectin (FITC staining) using a second fluorescence detector. The flow cytometer was calibrated using single PE- or FITC-stained cells.

Immunofluorescence Confocal Microscopy

Confocal microscopy was performed on ECs cultured on fibronectin-coated glass or Permanox® chamber slides. A staining three layer method was used in wounding assays and involved initial fixation in ice-cold methanol for 5 min and then acetone 1 min and washing in M199-containing 2.5% FCS. Cells were stained with saturating amounts of mAb for 30 min at 25°C. After two washes, cells were incubated with biotin-conjugated, affinity-purified anti-mouse Ig (Vector Labs, Burlingame, CA), washed twice, and incubated with avidin-FITC (Dako Corp., Carpinteria, CA). Slides were mounted using 2% propylgallate in glycerol as an anti-fade agent. Images were captured with a laser scanning confocal microscope (MRC600; Bio Rad Laboratories, Hercules, CA). Comparison images were subjected to equivalent amounts of contrast enhancement.

Neutrophil–Endothelial Adhesion

Peripheral blood from normal volunteers was sedimented on dextran, followed by density-gradient centrifugation on Lymphoprep (Nycomed, Oslo, Norway) at 450 *g*. Contaminating erythrocytes were then lysed by hypotonic 0.2% sodium chloride. Cells were resuspended in RPMI-1640 with 2.5% FCS and yielded a purity of >98%. 5×10^5 neutrophils were added in 125 μl medium to human umbilical vein endothelial cells (HUVECs), which had been plated 16 h earlier onto fibronectin-coated chamber slides at cobblestone and subconfluent densities. After 25 min at 37°C in a humidified 5% CO_2 in air atmosphere, the wells were washed three times removing unattached neutrophils. The slides were fixed in 0.5% glutaraldehyde, examined by confocal microscopy, and the number of neutrophils attached per EC counted. At least 160 ECs were assessed.

Preparation of Protein-coupled Beads

Tosyl-activated paramagnetic beads (Dynabeads M-450; DYNAL A.S.,

Oslo, Norway) were coated with purified platelet PECAM-1 as previously described (Plopper and Ingber, 1993). Essentially 99% of beads were coated with PECAM-1 as assessed by flow cytometry using polyclonal anti-PECAM-1 antibody staining.

Endotoxin Assay

A quantitative, photometric assay (Coatest; Kabi Diagnostica, Stockholm, Sweden) based upon activation of a proenzyme in limulus amoebocyte lysate was used, which detected endotoxin at 0.1–1.2 EU/ml.

Cytokines and Cytokine Antagonists

TNF-α (lot S9010AX; sp act 6.27×10^7 U/mg), TGF-β (lot 8987-53), and a monoclonal anti-TNF were gifts from Genentech, Inc. (South San Francisco, CA) IL-1β (10^8 thymocyte mitogenesis U/mg) was kindly supplied by Immunex (Seattle, WA). IL-1ra was a gift from Synergen (Boulder, CO). All cytokines contained <3 U/ml of LPS.

Reagents and Peptides

Polymyxin B sulfate (Sigma Chemical Co., St. Louis, MO) was used at 10 μg/ml. When added at plating, it effectively abolished induction of E-selectin by LPS on ECs. Soluble PECAM-1 protein was purified from platelet and was used at 0.01–100 μg/ml. Cyclic RGD and RAD peptides (EMD66203, 67679, 69601) were kindly supplied by A. Jonczyk from Merck KGaA (Darmstadt, Germany). These peptides were identical to those used by Brooks et al. (1994) in inhibiting $\alpha_v\beta_3$-dependent angiogenesis.

Enumeration of EC Contacts

Multiple photomicrographs of low power, phase contrast fields (see Fig. 1) were obtained of ECs plated at 0.25 and 10^5 cells per cm^2. The number of cell contacts made with adjoining ECs were counted for 10 ECs per field.

Wounding EC Monolayers

ECs plated at cobblestone density on fibronectin-coated chamber slides were wounded by scraping with the tip of a 1,000-μl pipette. The wells were washed three times with medium. All wounds consisted of a clearly demarcated cross in the center of a monolayer and healed as an advancing front of elongated, flattened cells. At specified times after wounding, cells were stained for immunofluorescence confocal microscopy as described above. Phase contrast microscopy with ×100 magnification was used to select fields at wound fronts and on areas of cellular monolayer, at least two fields were removed from a wound. Selected fields were then examined with the laser confocal microscope at ×200 magnification and the images stored. Images were retrieved in SETCOL format (COMOS 7.0; Bio Rad Laboratories, Hercules, CA), which displays fluorescence intensity on a color scale (green being minimal, red maximal), and were then examined for the number of ECs positive and negative for E-selectin per ×200 field. An EC was determined to be positive if any part of its surface, >5 mm in diam was red. At least 1,700 ECs were counted at wounds or monolayers per well and positivity was expressed as a percentage of all cells counted. The proportion of ECs expressing E-selectin in the top 50% range of expression intensity was assessed by analysis of pixel intensity in areas of monolayer and wound front and calculated using the histogram format.

Statistics

The statistical significance of results was assessed using the two-tailed Student's *t* test with either paired or unpaired groups of data as indicated. The frequency histograms of neutrophil adherence to ECs were compared by the Kolmogorov-Smirnov test and the effects of PECAM-1–coated beads on E-selectin expression by the analysis of variance (ANOVA) test.

Results

Confluency-dependent Expression of Endothelial Adhesion Molecules

HUVECs were seeded onto gelatin-coated tissue culture plates at varying numbers (Fig. 1). 15 h after plating, the EC surface expression of E-selectin was measured by flow cytometry. Confluent, cobblestone cultures as seen in Fig. 1 *a*, showed negligible E-selectin expression (Fig. 2 *a*). This is in agreement with published work demonstrating a lack of E-selectin on unstimulated ECs (Pober et al., 1986*b*; Bevilacqua et al., 1989). However, subconfluent ECs (Fig. 1 *b*) displayed substantial expression of E-selectin (Fig. 2 *a*). A comparison between subconfluent and confluent density cells using five separate EC lines showed a 17-fold greater induction in subconfluent ECs, (6.4 ± 1.0 vs. 0.37 ± 0.19 mean fluorescence intensity units ± SEM, $P = 0.0003$, unpaired *t* test). In these five experiments, 57 ± 4% of ECs at subconfluent density were positive for E-selectin, as opposed to 7 ± 2% of cells in a cobblestone monolayer. The confluency-dependent expression of E-selectin was evident over a range of EC densities (Fig. 2 *b*) and was seen on HUVECs extracted from their original monolayer culture by treatment with trypsin-EDTA or EDTA alone (data not shown).

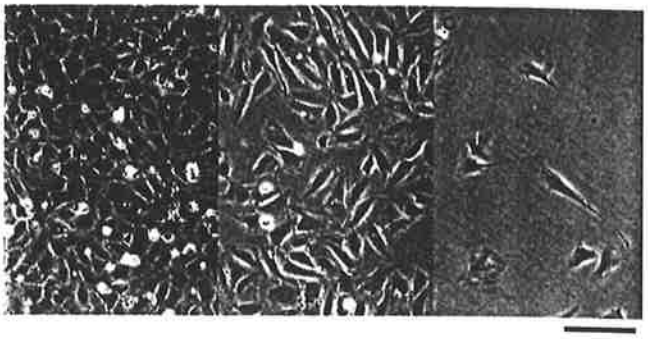

Figure 1. Phase contrast photomicrographs of EC monolayers 15 h after seeding at confluent 1.0 (*a*), subconfluent 0.25 (*b*), and sparse 0.05×10^5 cells per cm^2 densities (*c*). Bar, 40 μm.

Time Course of E-selectin Expression

The expression of E-selectin was measured on ECs at varying times after plating. Fig. 3 shows that cells plated at high and low densities both displayed significant levels of E-selectin early after plating that is within 4 to 12 h. However, expression of E-selectin on high density ECs was fourfold less at its maximum 8 h after plating, was transient and returned to basal levels by 24 h. In contrast, E-selectin expression on ECs plated at low density peaked at 12 h after plating and was still evident at 32 h. ECs at very sparse densities, such that single cells were maintained over the course of the experiment, displayed a persistent and significant expression of E-selectin even 72 h after plating (data not shown). There was considerable variation in the absolute levels of E-selectin between different EC preparations, the reason for which is not known. However, the fold induction calculated between high and low density cells was relatively consistent (21.1 ± 3.6%, $n = 30$) when measured 16–20 h after plating.

VCAM-1 and ICAM-1 (as well as E-selectin) demonstrated EC density–dependent expression, but the expression of PECAM-1 (or CD31), a non-inducible adhesion protein was not altered (Fig. 4). Increased expression of VCAM-1, ICAM-1, and E-selectin was independent of cell size, as identical forward scatter gates of high and low

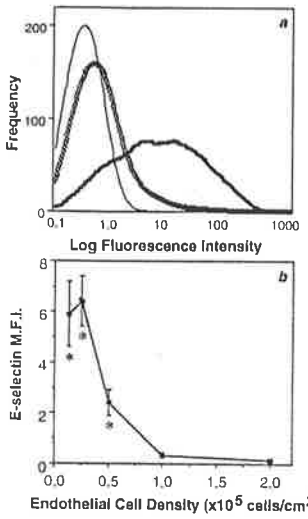

Figure 2. E-selectin expression varies with EC density. (*a*) Flow cytometry profile of a representative EC line stained for E-selectin 15 h after plating. Cells were plated at cobblestone (◇◇◇, 10^5 cells per cm^2) or subconfluent densities (*thick line*; 0.25×10^5 cells per cm^2). (*thin line*) Nonbinding control immunoglobulin, which gave a similar profile for confluent or subconfluent cells. (*b*) ECs were cultured for 15 h after plating at cell densities ranging from sparse to confluent ($0.125–2.0 \times 10^5$ cells per cm^2). The MFI ± SEM of E-selectin expression is shown for five EC lines, except for values at densities 0.5 and 2.0×10^5 cells per cm^2, which are triplicates. Asterisks indicate values significantly different ($P < 0.003$) from confluent density ECs (10^5 cells per cm^2) by unpaired *t* test.

density cells were always used in the FACS® analysis. Furthermore, the change in adhesion molecule expression was observed, whether the cells were stained in situ before detachment, or after extraction from matrix (data not shown).

E-selectin Expression by Subconfluent ECs Is Independent of Cytokines and Supports Neutrophil Adhesion

The time course of expression seen in Fig. 2 suggested two phases in the regulation of E-selectin expression: an induction phase, and a maintenance phase. Induction of the expression of adhesion molecules by subconfluent ECs occurred in the absence of exogenous cytokines. As shown in Fig. 5 *a*, IL-1 receptor antagonist (IL-1ra) or blocking anti–TNF-α antibody potently and specifically inhibited IL-1 or TNF-α–mediated induction of E-selectin, respectively, but these agents were ineffective on the induction of E-selectin by subconfluent ECs (Fig. 5 *b*). Furthermore, conditioned medium taken from subconfluent ECs or from cells multiply wounded such that the majority of cells were undergoing migration, did not induce E-selectin expression on confluent density ECs (Table I). Thus a role

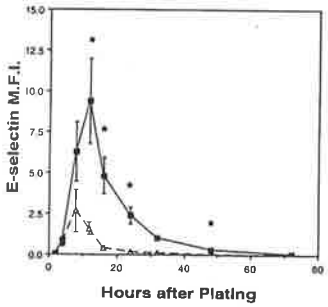

Figure 3. Time course of E-selectin expression after EC plating. ECs were plated at confluent (△, 10^5 cells per cm^2) and subconfluent (■, 0.25×10^5 cells per cm^2) densities. The expression of E-selectin was assayed by flow cytometry at specified times after plating. The MFI (± SEM) of three to five cell lines is shown but values at 2, 32, and 72 h after plating are singlicates. Asterisks denote values significantly different between the two cell densities ($P < 0.03$) by paired *t* test.

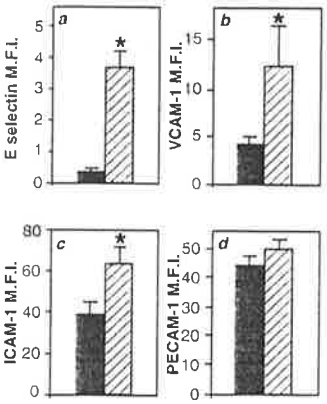

Figure 4. Expression of E-selectin, VCAM-1, and ICAM-1 are confluency dependent. ECs plated at subconfluent density (*stripes*, 0.25×10^5 cells per cm^2) and confluent density (*solid*, 10^5 cells per cm^2) were stained 20 h later for *a*, E-selectin, *b*, VCAM-1, *c*, ICAM-1, or *d*, PECAM-1. Expression is shown as the MFI of 23, 11, 7, and 7 EC lines, respectively. Error bars represent the SEM. Asterisks denote values significantly different from cells plated at cobblestone density ($P < 0.03$) by paired *t* test.

for endogenous endothelial cytokine production, or the release of some other stimulant from the EC, appeared unlikely.

Further, the induction of E-selectin is not related to cell proliferation. Rapamycin, an inhibitor of the G_1-S transition (Kato et al., 1994) showed no effects on E-selectin induction in subconfluent ECs (data not shown) although TGF-β, another inhibitor of cell proliferation (Heimark et al., 1986), which we have shown previously to inhibit cytokine-induced E-selectin expression (Gamble et al., 1993) did inhibit the cytokine-independent induction of E-selectin (data not shown). Moreover, E-selectin induction was independent of cell cycling since no difference in the level of E-selectin expression was seen in cells in G_0/G_1 with respect to $S/G_2/M$ at either cell density, both up to 20 h and even at 72 h after EC plating (data not shown).

Low doses of TNF-α were able to increase E-selectin expression on both low and high density ECs (Fig. 6 *a*) suggesting that the cytokine and non-cytokine pathways of induction are at least additive. Although the level of E-selectin expression induced by the cytokine-independent mechanism is less than that induced by TNF or IL-1 (Figs. 5 and 6), it is however, functionally relevant. Assessment of the number of neutrophils adherent per EC showed that there

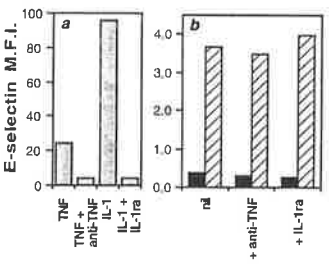

Figure 5. The induction of E-selectin on subconfluent ECs is not mediated through TNF-α or IL-1. (*a*) ECs plated at subconfluent density (0.25×10^5 cells per cm^2) were stimulated with 10 U/ml TNF-α (*TNF*) or 1 ng/ml IL-1β (*IL-1*) in the presence of anti–TNF-α (*TNF + anti-TNF*) (1:1,000), or IL-1ra (*IL-1 + IL-1ra*) (100 ng/ml), respectively. Inhibitors and agonists were added immediately after EC plating and E-selectin expression was assessed 12 h later. (*b*) ECs were plated at subconfluent density (*stripes*, 0.25×10^5 cells per cm^2) and confluent density (*solid*, 10^5 cells per cm^2). Anti–TNF-α or IL-1ra were added at plating. E-selectin expression was assessed 12 h later. Results shown are of one representative experiment of four performed.

Table I. E-selectin Expression Is Not Stimulated by Supernatant from Migrating Endothelial Cells

Addition	MFI
Unwounded SN	0.01 ± 0.01
6 h SN	0.04 ± 0.04
24 h SN	0.02 ± 0.02
TNF (10 U/ml)	20.5 ± 5.9

A confluent EC monolayer was multiply wounded such that all cells in the well were induced to migrate. The cells were washed twice to remove debris and replaced with fresh medium. Supernatants (*SNs*) were collected at 6 and 24 h thereafter and added to other wells containing confluent EC. TNF (10 U/ml) was added to some of these wells as a control, and E-selectin expression measured 4 h later. The results are given as the MFI ± SEM of two experiments. Neither the 6 nor the 24 SN gave significant increases in E-selectin expression compared to SN from unwounded cells.

was an increase in the number of neutrophils attached per EC in cells plated at low versus high density EC (Fig. 6 *b*). In three experiments, the percentage of EC supporting the adhesion of two or more neutrophils was 24.3% ± 14% for low density EC and 1.4% ± 1.0% for high density EC (mean ± SEM; $P = 0.02$).

E-selectin Induction Is Dependent on Integrin Engagement

ECs plated on collagen 1, gelatin, fibronectin, fibrinogen, and laminin in BSA-containing media resulted in an up-regulation of E-selectin when measured 6 h after plating (Fig. 7 *a*). The level of induction on these matrices in the absence of FCS in the media was never as high as in the presence of FCS. E-selectin expression was not induced on ECs plated on poly-L-lysine (Fig. 7 *a*) although they responded to TNF stimulation, suggesting that the poly-L-lysine was not toxic (data not shown). The induction was seen whether cells were plated at subconfluent density (Fig. 7) or at high density (data not shown). Cells plated on anti-β_1 integrin-coated surfaces, attached, spread, and induced E-selectin. In contrast, cells plated on an antibody to the common β chain (β_c) of GM-CSF, IL-3, and IL-5 receptor, which is also expressed on EC (Korpelainen et al., 1993), attached but failed to spread and also did not result in E-selectin induction (data not shown). Cells plated on fibrinogen in the presence of cyclic RGD peptide also failed to induce E-selectin (Fig. 7 *b*) although the peptide had no effect on E-selectin expression on cells plated on collagen (data not shown). These results suggest integrin engagement and possibly cell shape changes also are important in E-selectin induction.

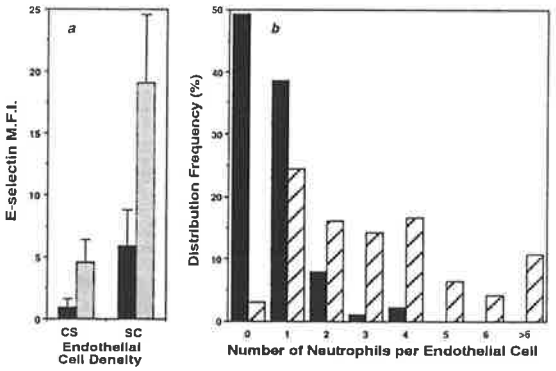

Figure 6. The non–cytokine-mediated pathway of E-selectin expression is additive with TNF-induced E-selectin expression and supports neutrophil adherence. (*a*) E-selectin expression on ECs plated at cobblestone density (10^5 cells per cm^2) or subconfluent densities (0.25×10^5 cells per cm^2) 15 h after plating (*black*). In some groups 1 U/ml of TNF-α was added 4 h before analysis (*gray*). Results shown are the mean ± SEM of four EC lines. Differences between non–cytokine-induced expression and expression after TNF stimulation, were significant by paired *t* test ($P = 0.04$, confluent density ECs and $P = 0.02$ subconfluent density ECs). (*B*) 15 h after plating EC at either confluent (*solid*, 10^5 cells per cm^2) or subconfluent densities (*stripes*, 0.25×10^5 cells per cm^2) neutrophils were added. 30 min later, attached neutrophils were removed by gentle washing and the number of neutrophils adherent per EC were counted using microscopy. A plot of the distribution frequency is shown. 176 ECs at cobblestone density and 168 ECs at subconfluent density were counted in the experiment shown, which is representative of at least three experiments. The two groups were statistically different ($P = 0.01$) by the Kolmogorov-Smirnov test.

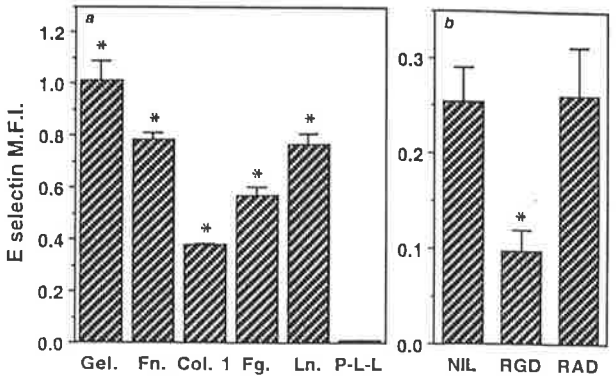

Figure 7. Integrin engagement is critical for E-selectin induction. (*a*) ECs were plated onto multiwell dishes coated with 0.2% gelatin (*Gel.*) or 50 μg/ml of fibrinogen (*Fg.*), collagen 1 (*Col. 1*), fibronectin (*Fn.*), laminin (*Ln.*), or poly-L-lysine (*P-L-L*) for 2 h. The cells were plated at a density of 0.25×10^5 cells per cm^2 in 1% BSA without added FCS. Cells were stained with anti–E-selectin or control antibody 6 h later and results given as the mean ± SEM of triplicate determinations from one experiment, which is representative of at least three similar experiments. The background level of staining of cells on each matrix, using a nonbinding antibody has been subtracted. Asterisks denote values significantly different from control group, which was stained with nonbinding antibody ($P < 0.002$). (*b*) EC at subconfluent density (0.25×10^5 cells per cm^2) were plated onto fibrinogen-coated wells in the presence of cyclic RGD (Arg-Gly-Asp) or RAD (Arg-Ala-Asp) peptides (final concentration 10 μM) in 1% BSA-containing media. E-selectin expression was measured 6 h after plating and is given as the mean ± SEM of a representative experiment where each group was performed in triplicate. Asterisks denote values significantly different from control peptide ($P < 0.01$).

PECAM-1 Regulates the Maintenance of Endothelial Adhesion Molecules

Although E-selectin is induced on all cells, only subconfluent EC maintain this expression. The chief difference between these density phenotypes lies in the number of cell–cell contacts and their establishment rate. ECs plated at confluent density (10^5 cells per cm^2) were rapidly sur-

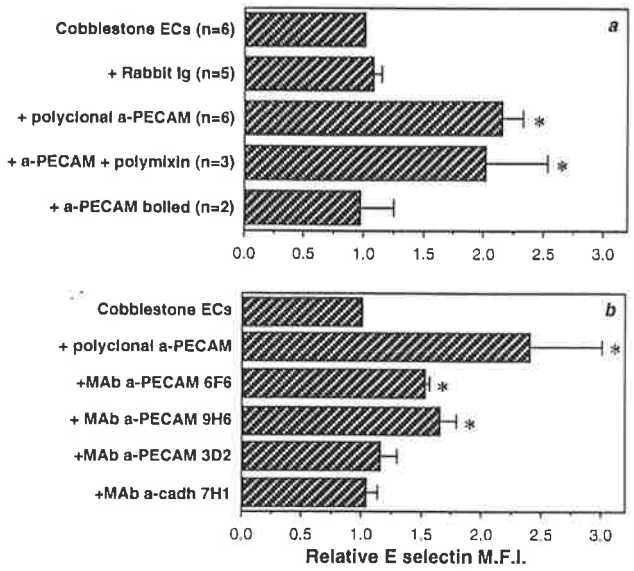

Figure 8. Anti–PECAM-1 antibody upregulates E-selectin on ECs plated at cobblestone density. (*a*) ECs at cobblestone density (10^5 cells per cm^2) were treated with Ig-purified rabbit polyclonal antibody to PECAM-1 (5 μg/ml) or a control nonimmune rabbit Ig at the time of plating. E-selectin expression was assessed by flow cytometry 12 h later. The MFI (± SEM) of two to six experiments is given where untreated confluent density ECs have been normalized to 1.0. Subconfluent ECs showed a 5.1 ± 1.4-fold increase in E-selectin expression relative to confluent EC. Asterisks denote a significant difference ($P < 0.05$) compared with control rabbit Ig by unpaired *t* test. (*b*) ECs at confluent density were treated with 5 μg/ml Ig-purified mAbs to PECAM-1 or VE-cadherin at the time of plating or polyclonal anti–PECAM-1 antibody. E-selectin expression was measured 12 h later. The MFI (± SEM) of three experiments is given where untreated confluent density ECs have been normalized to 1.0. Subconfluent ECs showed a 4.0 ± 0.7-fold relative increase in E-selectin expression relative to confluent EC. Asterisks denote a significant difference ($P < 0.05$) compared with cobblestone EC group.

rounded by other cells. Within 1 h they had established 5 ± 1 contacts with adjoining cells (mean ± SEM; $n = 3$). This number remained constant over the following 24 h. By contrast, subconfluent ECs (0.25×10^5 cells per cm^2) had an average of only 1 ± 1 contact ($n = 3$) at 1 h after plating and continued to form new associations such that 24 h later they had 4 ± 1 contacts ($n = 6$).

Two molecules known to be concentrated in cell–cell contacts and implicated in establishment of some of the junctional properties of endothelial cell monolayers are PECAM-1 (Albelda et al., 1991; DeLisser et al., 1994) and VE-cadherin (Lampugnani et al., 1992; Ayalon et al., 1994). To determine whether PECAM-1 was involved in the cytokine-independent regulation of E-selectin, three independent methods were used. Firstly, confluent density EC monolayers were exposed to functional antibodies directed to PECAM-1 or VE-cadherin. Monoclonal anti–VE-cadherin antibody (antibody 7H1) had no effect. Polyclonal anti–PECAM-1 resulted in a twofold increase in E-selectin expression (Fig. 8, *a* and *b*). Addition of both anti–PECAM-1 and anti–VE-cadherin antibodies produced no further increase than with anti–PECAM-1 antibody alone (data not shown). Although two mAb directed

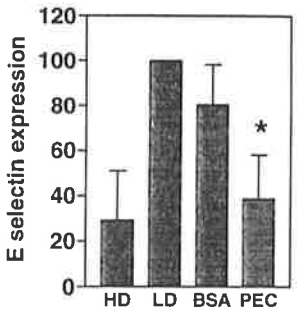

Figure 9. Adhesion through PECAM-1–coated beads downregulates E-selectin. ECs at subconfluent density (0.25×10^5 cells per cm^2) were plated in the absence or presence of beads (20 beads per cell) coated either with purified PECAM-1 or the blocking agent (BSA) alone. Cells at high density (10^5 cells per cm^2) were also plated. E-selectin expression was measured 18 h after plating. The data represents three experiments where each group was performed in triplicate and is presented relative to the E-selectin expression on subconfluent ECs, which is shown as 100%. (The mean MFI at 100% was 2.01 ± 0.09.) Asterisk indicates where PECAM-1–coated beads significantly decreased E-selectin ($P < 0.001$ by ANOVA) compared to BSA-coated beads.

to domain one of PECAM-1 consistently and significantly enhanced E-selectin expression their activity was always less than that seen with the polyclonal anti–PECAM-1 antibody (Fig. 8 *b*) suggesting the involvement of multiple domains. 55-3D2, an mAb directed to domain two-thirds of PECAM-1 was without function in these assays although it inhibits neutrophil transendothelial cell migration (Yan et al., 1995). The anti–PECAM-1 antibody effect was dose dependent (maximal efficacy at 5 μg/ml) and not due to contaminating endotoxin as polymyxin B did not abrogate the enhancement, the antibodies did not

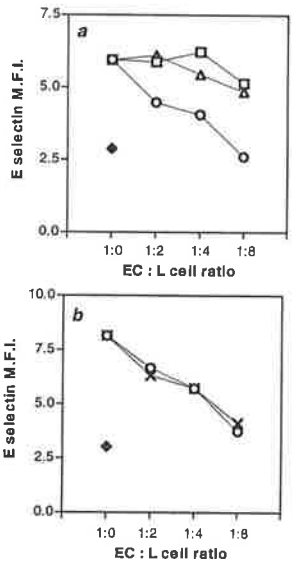

Figure 10. PECAM-1–transfected L cells regulate E-selectin expression. (*a*) EC at subconfluent density (0.2×10^5 cells per cm^2) were plated in the presence or absence of various concentrations of PECAM-1–transfected L cells (○, full-length PECAM-1; □, domain 2 deletion mutant; △, untransfected L cells). EC were also plated at high density (◆). A ratio of 1 EC to 8 L cells would be equivalent in cell number to a high density culture of EC. 18 h later, cells were harvested and stained with goat anti–E-selectin polyclonal antibody and detected using an anti–goat labeled polyclonal antibody together with a mouse anti–VE-cadherin antibody and detected with an anti–mouse, FITC-conjugated antibody. Cells were analyzed by two color analysis, and only the fluorescein positive (i.e., EC) were analyzed for E-selectin expression. The results are given as the MFI of one representative experiment of at least four performed where a similar trend was seen in each. (*b*) Subconfluent EC were plated as in *a* but either the full-length, PECAM-1 L cell transfectants (○), or cytoplasmic tail deletion mutant (X). Analysis was as for *a* and is given as the MFI of a representative experiment of at least four performed.

have detectable endotoxin and boiling the antibody abolished its potency (Fig. 8 *a*). VCAM-1 was also upregulated on cobblestone ECs by polyclonal anti–PECAM-1 antibody by 1.7 ± 0.19-fold (mean ± SEM, $n = 3$, $P = 0.03$, paired t test).

The second approach used platelet purified PECAM-1 immobilized on beads. E-selectin expression on EC plated at low density in the presence of PECAM-1–coated beads was inhibited when measured 18 h after plating (inhibition was 61 ± 20%; $n = 3$). BSA-coated beads had no effect (Fig. 9). Interestingly, neither purified, soluble PECAM-1, nor its immobilization on plastic was able to regulate E-selectin expression (data not shown) suggesting that valency, concentration, or microenvironment problems may be operating.

The third approach used PECAM-1 transfectants. EC were plated at subconfluent density in the presence of L cells expressing full-length PECAM-1, PECAM-1 lacking domain 2, or lacking the cytoplasmic tail. Both the full length and the truncation of the cytoplasmic tail inhibited E-selectin expression, whereas the mutant lacking domain 2 had no effect (Fig. 10 *a*). Untransfected L cells (Fig. 10 *b*) or L cells expressing the L1 adhesion glycoprotein (a member of the Ig superfamily expressed by neural cells and lymphocytes (Hubbe et al., 1993) failed to regulate E-selectin expression.

E-selectin Is Upregulated at Endothelial Wound Edges Whereas the Cell Junctional Molecule PECAM-1 Is Diminished

An in vitro wound assay was established as an in vivo correlate of EC migration (Schimmenti et al., 1992; Taylor and Alexander, 1993). ECs were plated and allowed to come to confluence. At variable times thereafter wounds were made and the cells stained 11 and 27 h later for E-selectin and PECAM-1. As seen in Fig. 11 *b*, the cells at the wound front displayed a spread, motile, morphology, and had advanced beyond the wound edge. These migrating cells had less PECAM-1 staining at the cell-to-cell borders. Significantly more ECs at the wound front and immediately behind the front expressed E-selectin in comparison

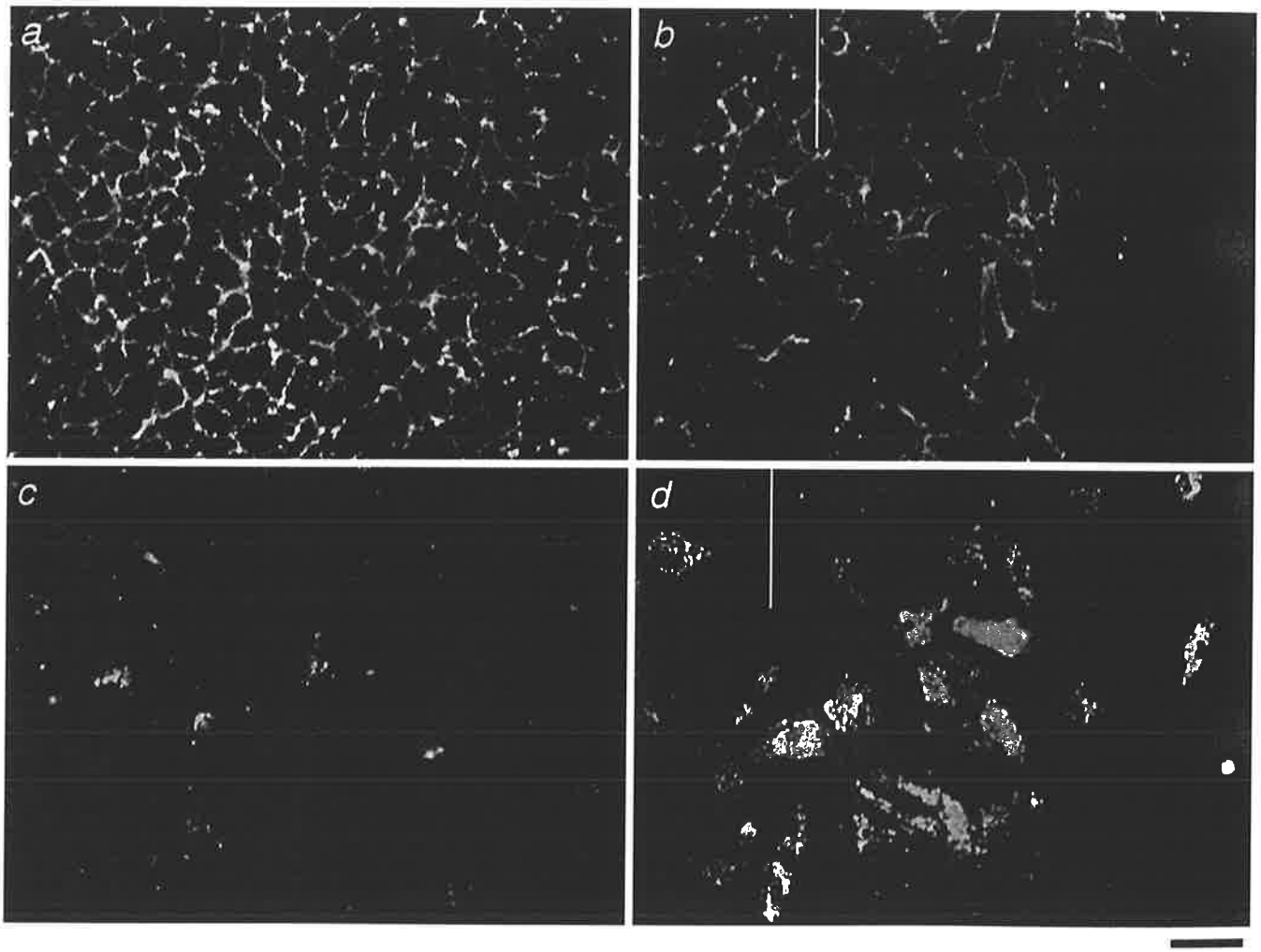

Figure 11. Immunofluorescence confocal microscopy of wounded ECs 40 h after plating and 11 h after wounding. *a* and *c* are cobblestone monolayers distant from the wound front. *b* and *d* are ECs at wound edges. *a* and *b* are stained for PECAM-1, whereas *c* and *d* are stained for E-selectin. *b* and *d* are not the same area but are of representative areas along the migrating front. The wound front is marked with a vertical bar. Bar, 40 μm.

Table II. E-selectin Expression by ECs at Wound Edges

Time after wounding	E-selectin–positive ECs		Mean increase in number of E-selectin–positive ECs ($n = 3$)
	%		
	Monolayer	*Wound front*	
11 h	4.1	13.5	2.2 ± 0.5
27 h	4.3	10.4	2.4 ± 0.5

Confluent EC monolayers were wounded by scraping with a pipette tip at either 13 or 29 h after plating. The cells were stained for E-selectin 27 or 11 h thereafter, respectively (i.e., 40 h after plating). An average of 2,600 cells was counted in the confluent monolayer, and 1,700 at the wound edge for each experiment, and the E-selectin–positive cells was calculated as described in Materials and Methods. The percent of positive cells at either the wound front or in the monolayer in a single experiment ($n = 1$) is shown. Also shown is the mean increase in the number of positive cells at the wound front compared to the monolayer for three experiments where all experiments showed the same trend.

to the nonwounded areas (Fig. 11, *c* and *d*), and this was substantiated by direct counts of the number of E-selectin expressing cells (Table II). The proportion of ECs expressing E-selectin in the top 50% range of intensity of expression was 1.7 ± 0.7% in the cell monolayer compared to 16 ± 5.0% at the wound front (mean ± SEM of three separate experiments at 16 h after wounding; $P = 0.05$).

Discussion

In this article, we have demonstrated a novel mechanism for the induction and expression of adhesion molecules on ECs. The induction is initiated through an integrin-dependent process and is associated with cell spreading. The intensity and duration of expression of the adhesion molecules is inversely proportional to cell density, and is mediated at least in part through the junctional protein, PECAM-1. This phenomenon does not involve the known cytokines and differs from the cytokine-dependent pathways in a number of ways: (*a*) it is dependent on integrin engagement and cell shape; (*b*) it is regulated by cell junctions; and (*c*) it can result in prolonged cell surface expression of adhesion molecules (Table III).

Since the phenomena described in this paper represent a fundamentally new mechanism underlying important pathological processes, we suggest nomenclature where type I induction refers to the classic cytokine-dependent process, and type II induction refers to the new cytokine-independent pathway. Although the level of E-selectin induced in type II conditions is less than that seen for maximal doses of cytokine-induced E-selectin, it is functional as evidenced by increased neutrophil attachment, and is at least additive with low doses of TNF. Such situations of low cytokine concentrations may be relevant during early phases of inflammatory lesions, suggesting the potential importance of both these induction processes in determining endothelial phenotype.

Type II induction of E-selectin described herein is independent of cell size, cell proliferation, and cell cycle, but is initially integrin mediated and seen in cells plated at both high and low density. Cells plated onto ligands that facilitated integrin-mediated attachment induce E-selectin expression (Fig. 7). However if the initial attachment is blocked, as with the addition of cyclic RGD peptides to cells on fibrinogen, E-selectin induction is inhibited. Binding of RGD to cells per se is insufficient to inhibit E-selectin induction since RGD peptides have no effect on cells plated onto collagen.

Table III. Mechanisms Controlling Expression of Endothelial Adhesion Molecules

	Type I	Type II
Induction	Cytokine, endotoxin	Integrin, cell shape
Peak	4–6 h	8–12 h
Duration	Transient	Prolonged
Regulation	Receptor mediated	Cell junctions

The intensity and duration of induction of type II responses was related to the density of cell plating. Cells plated at confluent density expressed less E-selectin when measured 8–48 h after plating, compared to cells plated at subconfluent density (Fig. 3). Morphologically, cells plated at high density form rapid cell–cell interactions in contrast to low density cells. Thus, junctional control of EC adhesion molecule expression was considered. Two molecules have been described that are known to be important in endothelial junctional integrity (Albelda et al., 1991; Lampugnani et al., 1992; Ayalon et al., 1994; DeLisser et al., 1994): PECAM-1, a transmembrane glycoprotein belonging to the Ig gene superfamily containing six extracellular, Ig-like domains; and VE-cadherin, an endothelium-specific member of the cadherin family of cell junctional molecules. The involvement of PECAM-1 in the control of E-selectin expression was shown by three independent methods. Firstly, anti–PECAM-1 but not anti–VE-cadherin antibodies induced E-selectin expression on confluent ECs (Fig. 8). Although the increases achieved with the antibodies were not to the level seen on low density cells, they were consistent. This may suggest that molecules other than PECAM-1 could exert secondary events that influence the level of expression of E-selectin. Secondly, PECAM-coated beads, when added at plating, inhibited the level of E-selectin expression on low density cells compared to BSA-coated beads (Fig. 9). Thirdly, transfectants expressing full-length PECAM-1, or those expressing the extracellular domains but lacking the cytoplasmic domain, inhibited E-selectin expression on subconfluent ECs (Fig. 10). In contrast, L cells expressing the domain 2 deletion mutant failed to effect E-selectin expression, suggesting a critical involvement of domain 2 in this regulation. These results are in agreement with other studies showing the importance of domain 2 in cell aggregation (Sun et al., 1996). Together with our antibody studies showing that antibodies to domain 1 increased the level of E-selectin expression on high density cells, our results suggest that both domains 1 and 2 of PECAM-1 are critical in regulation of E-selectin expression.

Cell–cell interactions are known to regulate a number of events, including cell proliferation (Gradl et al., 1995), release of bFGF from astrocytes (Murphy et al., 1988), responsiveness of fibroblasts to TGF-β (Paulsson et al., 1988), and the regulation of intracellular pH (Galkina et al., 1995). The mechanisms that underlie such regulation however have not been elucidated, although autocrine release of growth inhibitory factors (Antonelli-Orlidge et al., 1989; Gradl et al., 1995) and gap junction changes (Chen et

al., 1995) have been proposed. As described here, a cell surface junctional protein, PECAM-1, plays a central role in the density-dependent regulation of endothelial E-selectin expression. The possibility of a signaling role for PECAM-1 has been raised previously in a number of different systems. These include activation of integrins on T cells (Tanaka et al., 1992), natural killer cells (Berman et al., 1996), monocytes and neutrophils (Berman and Muller, 1995), and on $CD34^+$ hematopoietic progenitor cells (Leavesley et al., 1994), inhibition of EC proliferation (Fawcett et al., 1995), and platelet aggregation (Newman et al., 1992). The association of the tyrosine phosphatase-SHP2 with aggregated platelets (demonstrated recently by Jackson et al. [1997]) has put credence to a signaling pathway associated with PECAM-1 itself. Of interest to our study here is the recent observation by Lu et al. (1996) that integrin engagement and cell spreading results in PECAM-1 dephosphorylation in EC. Thus, the likely cross-talk between integrin and PECAM-1 is further strengthened.

Our in vitro model of cell wounding displays similar features to that described in the density-dependent regulation of adhesion molecules. Firstly, the induction of E-selectin expression is restricted to the migrating front and is associated with a change in cell shape and PECAM-1 redistribution away from the cell junction. Secondly, the E-selectin expression on these migrating cells is maintained at least up to 30 h after the wound signal. Thus we would suggest that this model may reflect identical signaling pathways as those operating in our plating experiments, and may therefore be appropriate as a model for in vivo endothelial regulation at wound sites.

Pathological tissue inflammation is characterized by increased and chronic expression of endothelial adhesion molecules (Koch et al., 1991; Petzelbauer et al., 1994; Kraling et al., 1996), changes in endothelial morphology and angiogenesis (Fitzgerald et al., 1991). These morphological changes include the formation of high endothelial venules (Freemont et al., 1983) and cell retraction with associated intercellular gaps (Nagel et al., 1994). In balloon angioplasty, expression of adhesion molecules at the wound front associated with morphological changes has been observed in contrast to the lack of expression in the area behind the wound edge (Tanaka et al., 1993). More recently, a role for E-selectin in angiogenesis has been postulated (Nguyen et al., 1993; Koch et al., 1995; Kraling et al., 1996). The results reported here, demonstrate: (*a*) a cytokine-independent mechanism of adhesion molecule expression, and (*b*) that cell–cell interactions influence the duration and magnitude of this expression, may explain some of these in vivo observations. Furthermore, the observation that restoration of PECAM-1 interactions can downregulate adhesion molecule expression on ECs offers the promise that manipulation of EC junctional molecules may permit the development of novel therapeutics.

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CIRCULATION RESEARCH 87:603-607, 2000 **MS# 1413/R1**

ANGIOPOIETIN-1 IS AN ANTI-PERMEABILITY AND ANTI-INFLAMMATORY AGENT *IN VITRO* AND TARGETS CELL JUNCTIONS

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ABSTRACT

Inflammation is a basic pathological mechanism that underlies many diseases. An important component of the inflammatory response is the passage of plasma components and leukocytes from the blood vessel into the tissues. The endothelial monolayer lining blood vessels reacts to stimuli such as thrombin or vascular endothelial growth factor (VEGF) by changes in cell-cell junctions, an increase in permeability and the leakage of plasma components into tissues. Other stimuli, such as tumor necrosis factor α (TNF), are responsible for stimulating the transmigration of leukocytes. Here we show that angiopoietin-1 (Ang1), a cytokine essential in fetal angiogenesis, not only supports the localisation of proteins such as PECAM-1 into junctions between endothelial cells and decreases the phosphorylation of PECAM-1 and VE cadherin, but also strengthens these junctions as evidenced by a decrease in basal permeability, and inhibition of permeability responses to thrombin and VEGF. Furthermore angiopoietin-1 inhibits TNF stimulated leukocyte transmigration. Angiopoietin-1 may thus have a major role in maintaining the integrity of endothelial monolayers.

Key words: endothelium, inflammation, permeability, angiogenesis, cell junctions

INTRODUCTION

Angiopoietin-1 is a recently identified ligand of the endothelial-specific tyrosine kinase receptor Tie-2 [1]. It is involved in the angiogenic phase of embryonic vascular development with major defects in the interaction of endothelial cells with the surrounding mesenchymal cells and extracellular matrix evident in Ang1 knock out mice [2]. As a result the vessels are poorly formed with a lack of branching and remodelling, ectatic and leaky. Tie-2 knock out mice have a similar phenotype with additional venous malformation brought about by a disruption of the endothelial-smooth muscle cell interactions [3]. Whilst these observations have shown that, in development, Ang1 is critical for the stabilisation of the interaction of endothelial cells with their surrounding matrix, little is known of how the integrity of mature vessels, and in particular that of the endothelial monolayer is maintained. Two recent publications have suggested that Ang1 is involved in mature blood vessel control. Firstly, mice transgenic for Ang1 have leakage-resistant blood vessels [4] and secondly Ang1, delivered by an adenovirus expression system, inhibited the tissue edema induced by VEGF and mustard oil, two powerful stimulators of vascular leak [5].

Junctions between endothelial cells give the structural basis for the regulation of the passage of plasma proteins or leukocytes into the tissues [6]. Two junctional structures involved in this regulation include the tight junctions [7] and the adherence junctions which are altered by agents that induce EC permeability or which mediate leukocyte transmigration [8-13]. Junctional proteins also serve to signal neighbouring endothelial cells to maintain their quiescent and anti-inflammatory phenotype. In particular as endothelial junctions are established PECAM-1 rapidly moves to these junctions [14], suppresses the expression of adhesion molecules such as E-selectin [15] and prevents endothelial cell apoptosis [16].

We show here that Ang1 inhibits endothelial cell permeability *in vitro* and suggest that a likely mechanism is through the regulation of the junctional complexes, PECAM-1 and VE cadherin.

MATERIALS & METHODS

Cells. Human umbilical vein endothelial cells were grown and used as described [15]. For VEGF induced permeability, human umbilical artery endothelial cells [17] were used since they more consistently responded to $VEGF_{165}$.

Reagents. Ang1 used is as described [18]. Anti VE cadherin and PECAM-1 antibodies have been described [15].

PECAM-1 localisation. Cells were fixed and stained for PECAM-1 as described [15]. Images were captured using a BIORAD Laser Scanning Confocal Microscope MR600. Images under comparison were subjected to equivalent amounts of contrast enhancement. At least 20 fields were analyzed for PECAM localisation in each group. These were consecutive fields within the one well and contained approximately 100-400 cells in total.

E-selectin expression was analysed as described [15].

Endothelial permeability assay. The assay was performed essentially as described [19].

Neutrophil transendothelial cell migration assay was performed and quantified by an MTT colorimetric assay [20, 21].

Immunoprecipitation. HUVEC monolayers, confluent for at least 24 hours were treated with control or Ang1. The lysis, extraction and analysis were performed as described [9].

RESULTS

Angiopoietin 1 Regulates PECAM-1 Localisation to Cell Junctions

Endothelial cells (EC) plated at low density initially attach to the matrix, flatten and establish contacts with neighbouring cells. Pretreatment of cells with Ang1 did not change the number of cells that adhered to the matrix nor the initial single cell morphology (not shown), however, it resulted in an enhanced PECAM-1 localisation to the junctions (Fig 1a, 1b) suggesting these junctions were forming more rapidly. At 45 minutes after cell plating, Ang1 did not affect the number of cell aggregates, the number of cells in each aggregate or the expression of cell-surface PECAM-1 or VE cadherin (Online Table 1). However the number of cell contacts that showed PECAM-1 localisation increased from 55±5% to 75±5% (n=2 experiments) between control and Ang1 treatment respectively and the percentage of cells with the most intense junctional staining of PECAM-1 increased from 1% to 27% (Online Figure 1). The changes were specific for Ang1 since the other angiogenic factors, VEGF and bFGF, had no such effects (not shown). This data is suggestive that Ang1 may affect cell junctions and therefore we extended our analysis to assays that involve junctional regulation.

Angiopoietin 1 Inhibits E-selectin Expression

PECAM-1 has been proposed as an important mechano-sensing molecule in EC [22, 23] mediating inhibition of proliferation [24], migration [25], apoptosis [16] and expression of adhesion molecules [15]. As such, the consequence of the enhanced PECAM-1-PECAM-1 engagement mediated by Ang1 should be to mature the cells into a quiescent, non-proliferative, non-inflammatory phenotype. E-selectin expression is involved in inflammation [15], proliferation [26] and angiogenesis [27] and thus its presence can be considered a marker of an activated endothelium. Cells plated at low density express low but significant levels of E-selectin. The induction under this situation is cytokine independent but dependent on integrin attachment and the loss of PECAM-PECAM interaction [15]. Treatment with Ang1 inhibited the E-selectin expression (Fig 2a, 2b) in a dose dependent manner (Online Figure 2). Ang1 had no effect on TNF induced E-selectin expression (Online Table 2, groups a-d).

Angiopoietin 1 Inhibits EC Permeability

Ang1 treatment of EC monolayers inhibited their basal permeability (Fig 3a) in a dose dependent manner. More strikingly, Ang1 inhibited the permeability induced by two classic EC permeability inducing agents, thrombin and VEGF (Fig 3b, 3c) by 70 and 100% respectively. The phosphatidylinositol 3 kinase (PI3K) pathway, although implicated in Ang1 mediated EC survival [28, 29] does not appear to be involved in the regulation of permeability. The PI3K specific inhibitor LY294002 had no effect on Ang1 induced inhibition of permeability (Online Figure 3) although it reversed, in parallel experiments, the protective effect of Ang1 on EC survival (not shown). This result suggests an alternate signalling pathway to PI3 kinase is utilized by the Tie2 receptor to mediate changes in cell junctions.

The transmigration of leukocytes induced by cytokines such as TNF is also regulated by endothelial junctions [30-33]. Ang1 pretreatment of EC abolished TNF induced transmigration (Fig 3d). The Ang1 effect was not mediated through changes in TNF signalling since maximum inhibition of transmigration was seen when Ang1 was added at the end of the stimulation period, 15 minutes prior to PMN addition. Under these conditions, Ang1 had no effect on the level of TNF induced E-selectin expression (Online Table 2, groups e and f). Furthermore, Ang1 treatment of the neutrophils did not alter their capacity to transmigrate (not shown).

The inhibitory effect of Ang1 on E-selectin expression, permeability and transmigration was mediated through the Tie2 receptor since the Tie2Fc soluble protein abolished these responses (Online Figures 4a, b, c).

Angiopoietin 1 Alters VE Cadherin and PECAM-1 Phosphorylation

PECAM-1, VE Cadherin and its associated signalling molecules the catenins have been implicated in the regulation of EC junctions. Changes in phosphorylation of PECAM-1 and association of the catenins with VE cadherin are seen during histamine, thrombin and VEGF induced permeability and during PMN transmigration [8-12]. Ang1 treatment for 10 minutes induced a decrease in the basal phosphorylation of VE cadherin which returned to normal levels by 30 minutes (Fig 4a). In two experiments performed, the decrease was 33% and 45% normalised to VE cadherin content. Although no significant change in phosphorylation of β catenin was evident, an increase in the amount of β catenin associated with VE cadherin was observed. Ang1 also induced a significant decrease in basal PECAM-1 phosphorylation (Fig 4b). In two experiments performed, the decrease was 48% and 51% normalized to PECAM-1 content. The changes in phosphorylation of PECAM-1 and VE cadherin and increase in the association of β catenin with VE cadherin are consistent with an increase in cell-cell interaction [6, 8, 9, 34].

DISCUSSION

Enhanced interaction of endothelial cells with their supporting structures has been the chief explanation for the angiogenic effects of Ang1 both in development and *in vitro* [2, 18]. The results presented here suggest the additional mechanism of stabilisation of cell-cell interactions may also be operative. Indeed this multi-level effect of Ang1 compounds the anti-proliferative and anti-apoptotic effects of cell-cell and cell-matrix interactions [16, 35-40].

Our *in vitro* findings highlight a potentially critical role for Ang1 in maintaining the integrity of endothelial monolayers in mature animals. To our knowledge this is the first agent that, by an action on endothelial cells, prevents the acute leakiness of blood vessels that is involved in the generation of swelling or edema seen in inflammatory or allergic reactions. Moreover the degree of inhibition of between 70-100% suggests this is a potentially potent and physiological mechanism. The effects on inhibition of permeability

and transmigration as well as on the prevention of cytokine-independent expression of adhesion proteins are consistent with its effect on cellular junctions, and this is supported by the alteration in two important molecules involved in EC integrity, namely PECAM-1 and VE cadherin. However, other mechanisms of action cannot be ruled out. Nevertheless, these findings suggest a role for Ang1 from embryogenesis to adulthood and open the possibility of its therapeutic use in inflammatory diseases.

ACKNOWLEDGMENTS

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FIGURE LEGENDS

Figure 1 Localisation of PECAM-1 following treatment with Ang1.
EC were incubated with control (a) or 0.1 μg/ml Ang (b) for 10 minutes and then allowed to attach to fibronectin coated slides. 60 minutes later the cells were stained for PECAM-1 expression.

Figure 2 Ang1 down-regulates E-selectin expression.
EC were treated with 0.1 μg/ml Ang1 (ANG), Vehicle (VEH) or left untreated (NIL) for 10 minutes, then plated onto fibronectin coated wells and cultured for 16 hours. Cells were then stained for E-selectin.
a. Typical FACS profile from one individual experiment showing E-selectin expression. CTRL shows cells stained with an irrelevant antibody.
b. The mean fluorescence intensity for E-selectin from six experiments. Results are expressed as the mean ± SEM. * $p < 0.006$ compared to Vehicle (unpaired t-test).

Figure 3. Ang1 inhibits EC permeability and TNF induced transmigration of neutrophils. The EC monolayer was treated for 30 minutes with Ang1 (Ang) or vehicle (Veh) prior to assay.
Figure 3a. Ang1 inhibits in a dose dependent manner the basal permeability of EC in the absence of exogenous stimulation. The mean ±SEM of 3-5 experiments is given where each group was performed in duplicate or triplicate. *$p < 0.05$ compared to no treatment.
Figure 3b. Ang1 inhibits the thrombin stimulated vascular permeability. Cells were stimulated in the upper well with (2U/ml) thrombin (T) for 15 minutes after 0.1 μg/ml Ang1 treatment. Results are given as the mean ±SEM, n=2 experiments where each group was performed in duplicate.
*$p < 0.02$, compared to Vehicle + Thrombin (paired t-test).
Figure 3c. Ang1 inhibits the VEGF stimulated vascular permeability. This was performed as for Figure 3b except that stimulation was with 50 ng/ml VEGF (V) for 30 minutes. n=3 experiments where each group was performed in singlecate or duplicate.
* $p < 0.05$ compared to Veh + VEGF (unpaired t-test).
Figure 3d. Ang1 inhibits transmigration. The EC monolayer was treated for 4 hours with TNF-α and then for 30 minutes with 0.1 μg/ml Ang1 (Ang) or Vehicle (Veh). The mean (±SEM) as a percentage of cells that transmigrated is given for four experiments where each group was performed in duplicate or triplicate. * $p < 0.002$ compared to Veh + TNF (unpaired t-test).

Figure 4. Ang1 decreases basal VE cadherin and PECAM-1 phosphorylation.
a. Cells were treated with vehicle control (C) or Ang1 (0.1 μg/ml) for 10 (A10) or 30 (A30) minutes. Lysates were immunoprecipitated with anti-VE cadherin coated beads. Equivalent samples of immuno-complexes were blotted with an anti phospho-tyrosine antibody and the phosphorylation of VE cadherin and associated β catenin are shown (upper two bands). The gels were stripped and reprobed for VE cadherin and β catenin (lower two bands respectively).
b. Cells were treated with Ang1 or vehicle control for 30 minutes, immunoprecipitated with anti PECAM-1 coated beads, blotted for anti phospho tyrosine (upper band), stripped and reprobed for PECAM-1 (lower band).

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MS#1413/R1

ANGIOPOIETIN-1 IS AN ANTI-PERMEABILITY AND ANTI-INFLAMMATORY AGENT *IN VITRO* AND TARGETS CELL JUNCTIONS

(ON-LINE MATERIAL)

Materials and Methods

Permeability assay

Endothelial cells ($8x10^4$ cells/well) were cultured on 0.4μ pore size Transwells (Costar, Corning Inc, USA) for three days to achieve a tight confluent monolayer. Medium was changed into culture media containing 2% FCS (Commonwealth Serum Laboratories, Melbourne, Australia) for two hours prior to assay. Horse radish peroxidase was added to the top well (5 μg/ml). Permeability (ng/ml) is given as the amount of HRP that passed into the lower chamber over a specific time period (usually 10-20 minutes) as assessed by an ELISA and compared to a standard curve.

Neutrophil Transendothelial Cell Migration Assay

$5x10^4$ HUVEC per well were cultured on 3μ pore size Transwells for three days. Wells were treated with TNF (0.2 ng/ml) for four hours. Wells were washed to remove the TNF and Ang1 added to both chambers for 30 minutes prior to the addition of neutrophils ($5x10^5$ cells/well). The number of cells that migrated across the monolayer into the lower chamber was assessed by an MTT colorimetric assay and compared to a standard curve in order to calculate % TM.

Immunoprecipitations

Endothelial cells in full medium were treated with control or Ang1 (0.1 μg/ml) for 10 or 30 minutes. Five minutes prior to the end of the stimulation sodium pervanadate was added, 100μmol/l for cells destined for VE cadherin precipitation and 30μmol/l for cells for PECAM-1 immunoprecipitation. Cells were washed once in ice cold PBS and solubilized on ice for 15 minutes in lysis buffer (137 mM NaCI, 10 mM Tris-HCI, pH7.4, 1% NP-40, 10% glycerol containing 10 μg/ml aprotinin, 0.1 mM phenylmethylsulphonyl fluoride, 1 μM leupeptin, 2 nM $CaCl_2$, 0.5 μM DTT, 2.5 μM benzamide, 1 μM β-glycerophosphate, 300 μM sodium pervanadate). Cells were scraped, the lysates centrifuged at 13,000 rpm for 10 minutes at 4°C. Lysates were precleared with CNBr activated Sepharose 4B control coupled beads and then immunoprecipitations performed with anti VE cadherin or anti PECAM-1 coupled beads. Immunoprecipitates from approximately $5x10^6$ EC were separated by SDS-PAGE, transferred onto nitrocellulose membranes and immunoblotted and detected using HRP conjugated secondary antibodies (Immunotech, Marseilles, France) and enhanced chemiluminescence (Amersham).

LEGENDS

Online Figure 1

The intensity of junctional staining was measured by a blinded observer on an arbitrary scale from least intense (+) to most intense (+++) in cells treated with Ang1 (black bars) or vehicle control (open bars) in two experiments counting a total of 450 cells. The vertical bar shows 1 SD and Ang1 and vehicle differ by $p< 0.001$ using a chi square method comparing + and +++.

Online Figure 2

The Ang1 induced inhibition of E-selectin expression is dose dependent.
EC were treated with decreasing concentrations of Ang1, the cells plated and E-selectin expression assessed 16 hours later. The results are given as the mean fluorescence intensity (MFI) of one experiment which is similar to three others performed. The numbers above the columns refer to the percentage of cells positive for E-selectin.

Online Figure 3

The PI3K Inhibitor LY294002 does not reverse the Ang1 mediated inhibition of basal permeability. The EC monolayer was treated for 15 minutes with 10μM of LY294002 (LY, Santa Cruz Inc USA) then Ang1 (0.1 μg/ml) for 30 minutes. The permeability (ng/ml HRP) is expressed as mean ± SEM of a pool of two experiments where each group in each experiment was performed in duplicate or triplicate. * $p< 0.001$ compared to LY + Veh (unpaired t – test). This dose of LY294002 was shown, in parallel experiments, to reverse the protective effect of Ang1 on EC survival.

Online Figure 4

The Tie2 receptor mediates the inhibitory action of Ang1 on E-selectin expression, permeability and neutrophil transmigration. Ang1 (0.1 μg/ml) was incubated with a 10 fold excess Tie2Fc soluble chimera protein prior to addition to the cells.

a) E-selectin expression. Cells were treated as indicated, plated for 16 hours and E-selectin expression measured. The mean fluorescence intensity (MFI) is given for one experiment which is representative of two performed. The values above the columns represent the number of E-selectin positive cells.

b) Thrombin induced permeability. Cells were treated with Ang1 for 30 minutes followed by thrombin for 15 minutes and then assayed for the passage of HRP through the monolayers. Groups are 1, NIL, no treatment; 2, thrombin (1μ/ml); 3, Tie2Fc + thrombin; 4, vehicle + thrombin; 5, Ang1 + thrombin; 6, Tie2Fc + Ang1 + thrombin. Results are expressed as the mean ±SEM of triplicate determinations from one experiment representative of two performed *$p<0.03$ compared to Ang1 + Tie2Fc + thrombin (group 6 compared to group 5, paired t-test).

c) Neutrophil transmigration. Endothelial cell monolayers were incubated for 4 hours with TNF, then Ang1 for 30 minutes prior to PMN addition. Groups are 1, NIL no treatment; 2, TNF; 3, Tie 2Fc + TNF; 4, Veh + TNF; 5, Ang1 + TNF; 6, Ang1 + Tie 2Fc +TNF. Results are given as the mean ± SEM of two experiments where each group in each experiment was performed in duplicate or triplicate *$p<0.01$ compared to Ang1 + Tie2Fc + TNF (group 6 compared to group 5, paired t-test).

Online Table 1

Ang1 Treatment Does Not Alter Expression of PECAM-1 or VE Cadherin

Treatment	Expression (MFI) of
	PECAM-1
NIL	39.6 ± 0.3
Ang1	38.4 ± 1.6
Veh	37.3 ± 0.8
	VE Cadherin
NIL	17.7 ± 0.3
Ang1	17.9 ± 0.8
Veh	18.9 ± 0.4

Confluent monolayers of EC were stimulated with 0.1 μg/ml Ang1 or Vehicle control for 30 minutes and then stained for PECAM-1 or VE Cadherin expression. The mean fluorescence Intensity (MFI) ± SEM (n=2) from one experiment is given. Similar results were obtained with suspension treatment of cells with Ang1.

Online Table 2

Ang1 has no effect on TNF mediated E-selectin Expression

Group	Treatment	Mean Fluorescence Intensity
a.	Nil	0.56 ± 0.02
b.	TNF	8.33 ± 0.35
c.	Ang1 + TNF	7.78 ± 0.2
d.	Veh + TNF	8.93 ± 0.05
e.	TNF + Ang1	8.39 ± 0.01
f.	TNF +Veh	8.40 ± 0.01

Confluent monolayers of EC were stimulated with 0.2 ng/ml TNFα for 4 hours. Groups c. and d. received 0.1 μg/ml Ang1 or Vehicle control 30 minutes prior to TNF, groups e. and f. received Ang1 or Vehicle 3.5 hours after TNF stimulation (ie 30 minutes prior to assay). Results (Mean ± SEM) from one experiment performed in duplicate is given which is similar to the results seen in three other experiments.

Figure1

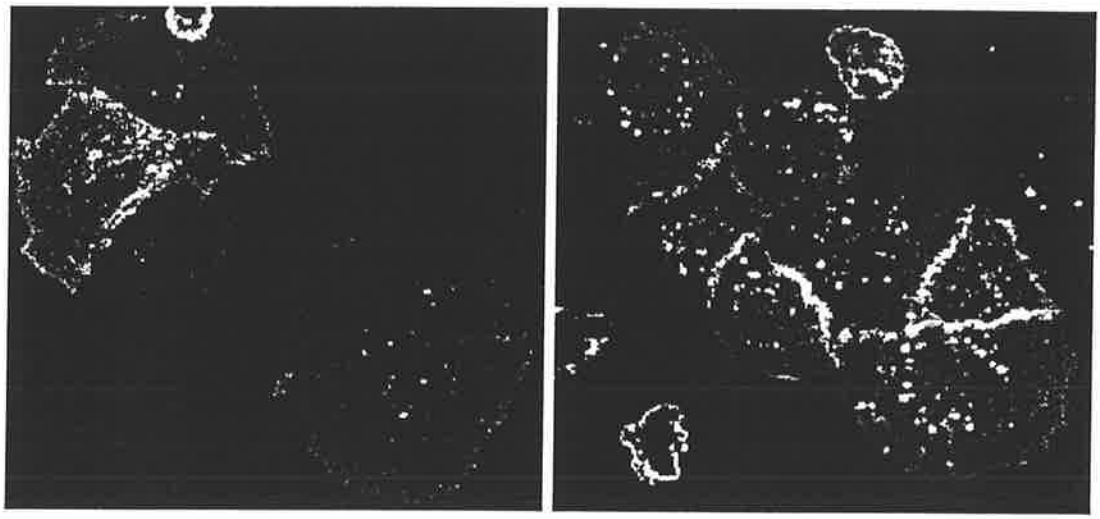


Figure2

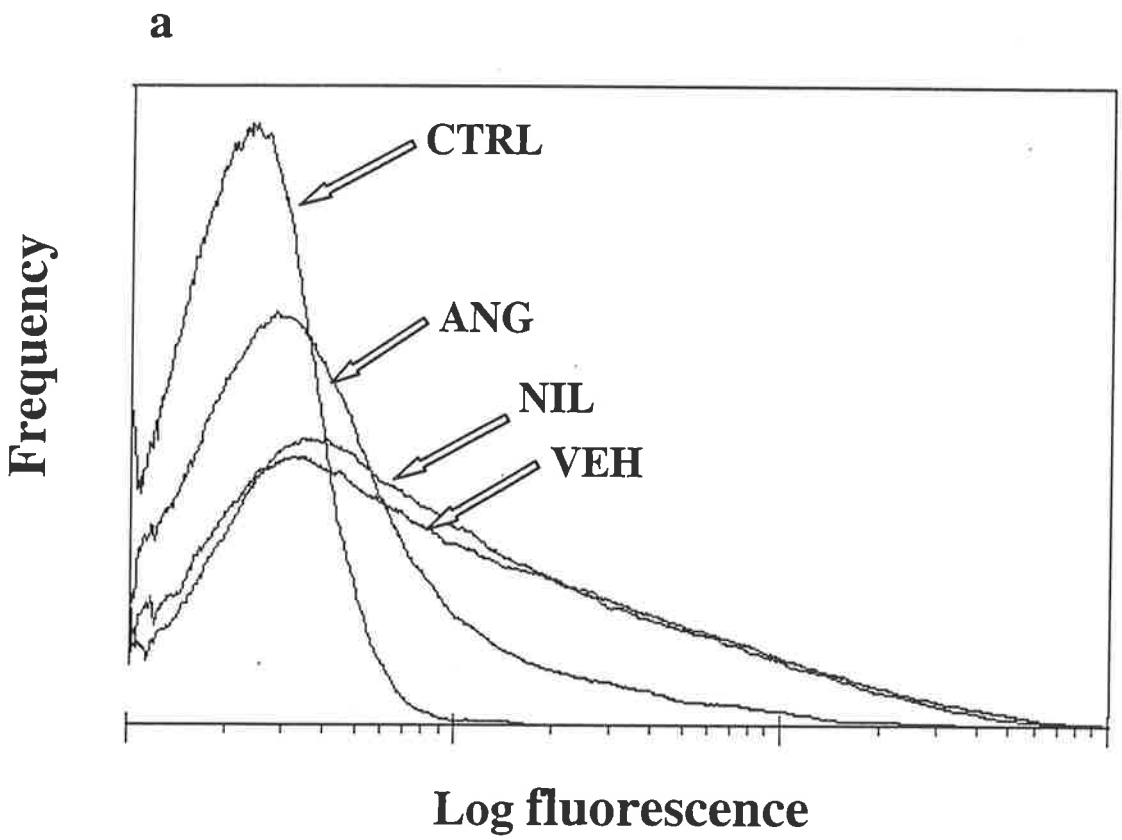


Figure2

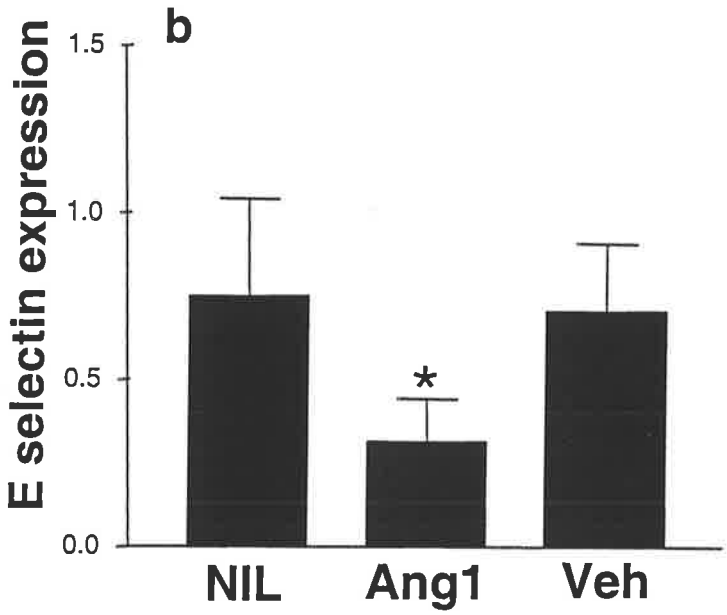
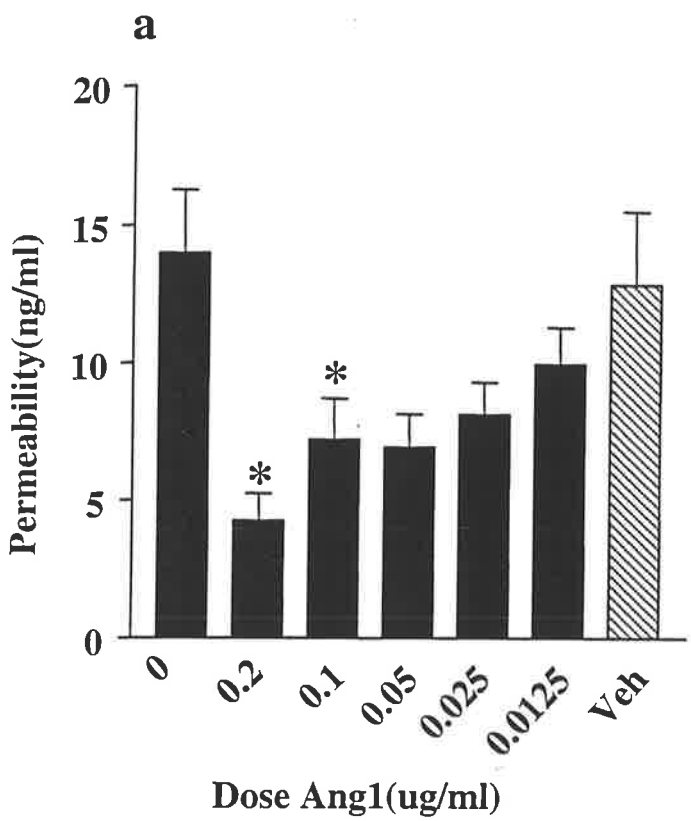


Figure3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Permeability(ng/ml)
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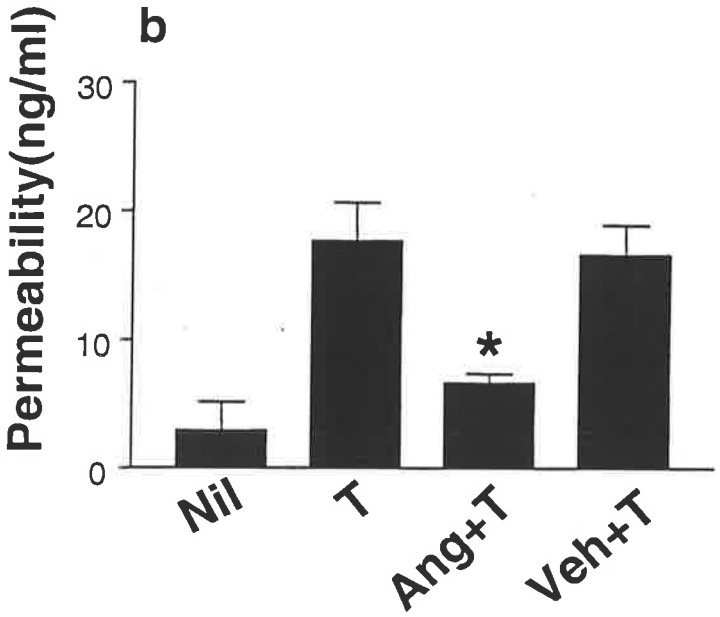
Dose Ang1(ug/ml)

Figure3



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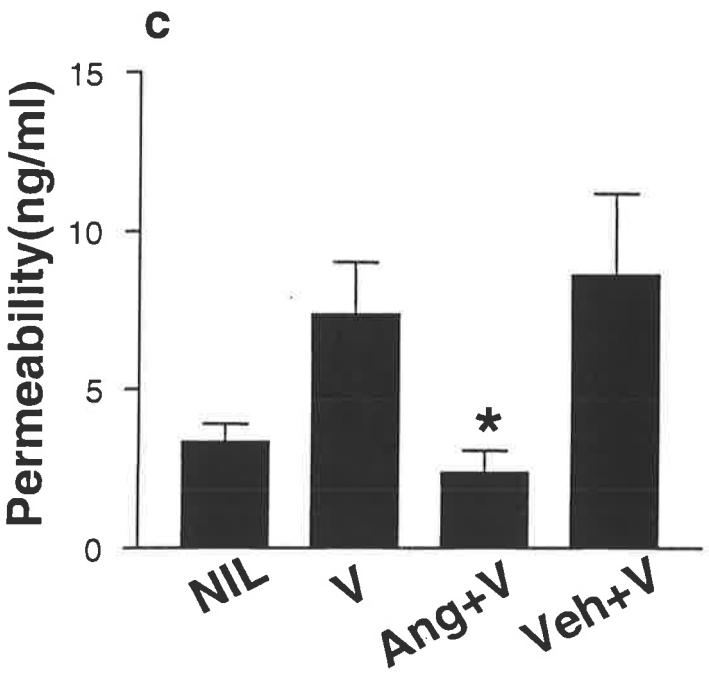

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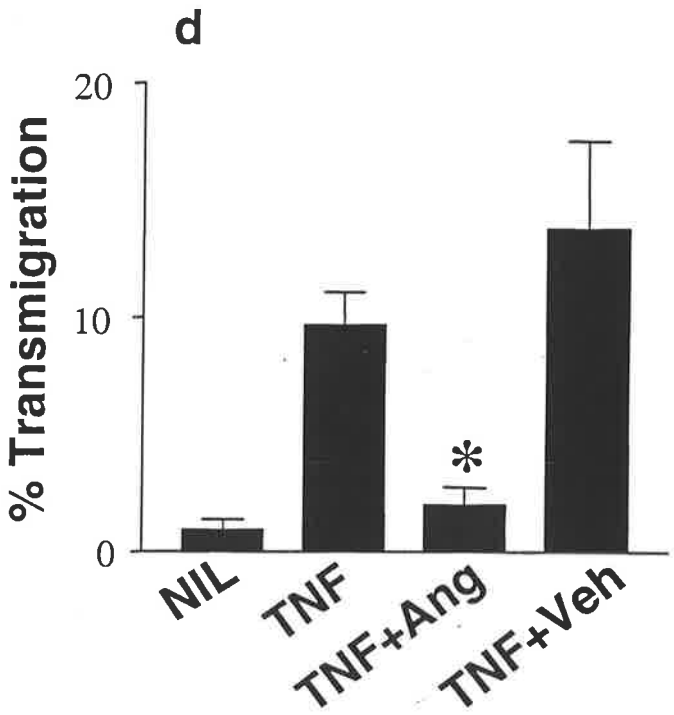
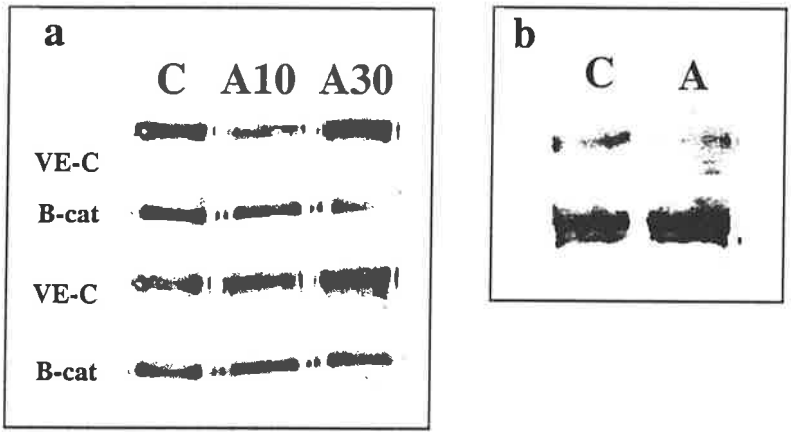
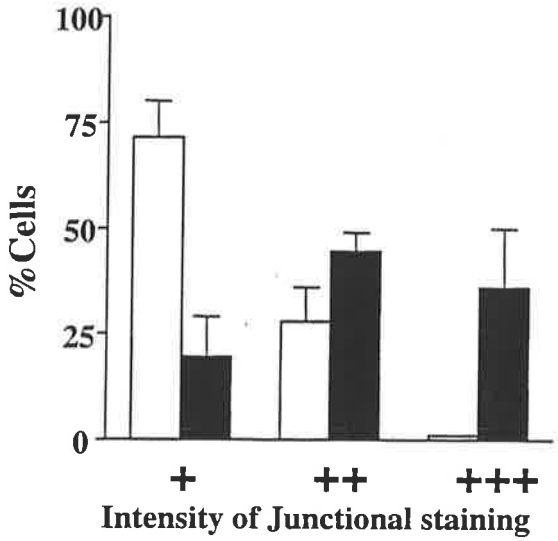


Figure4



Online Figure 1



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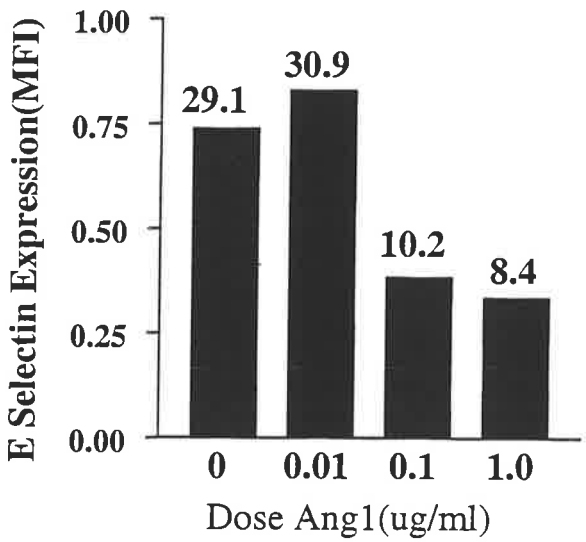

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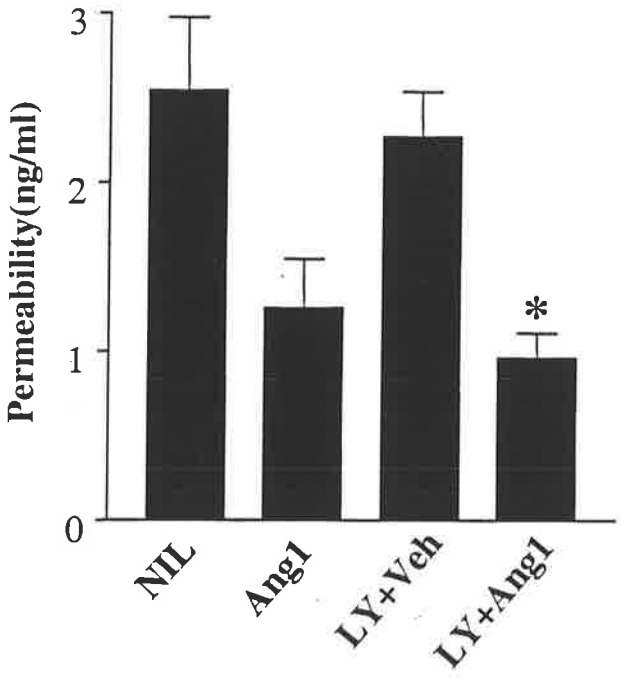

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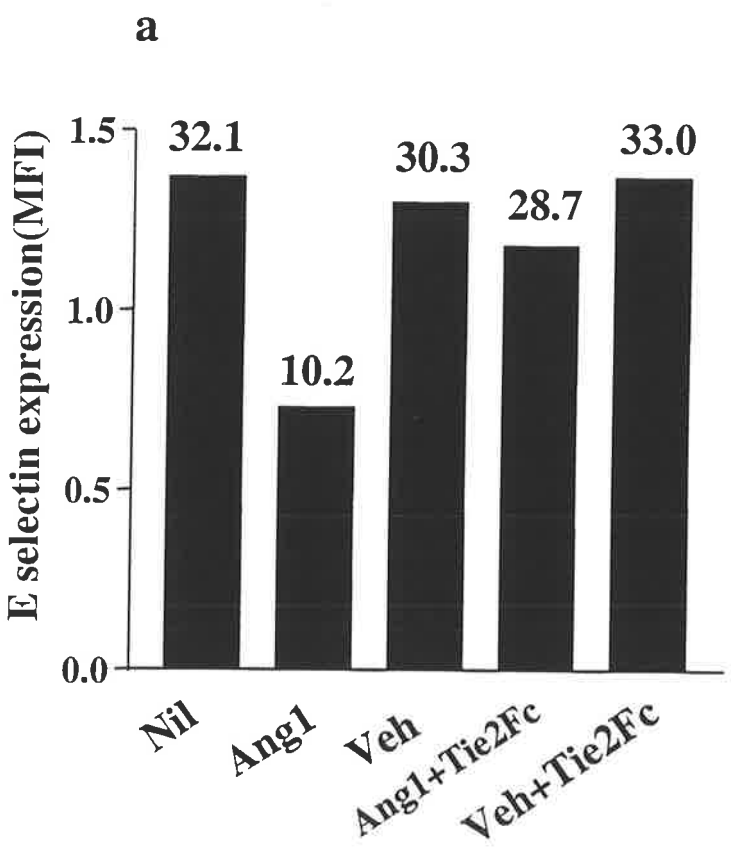

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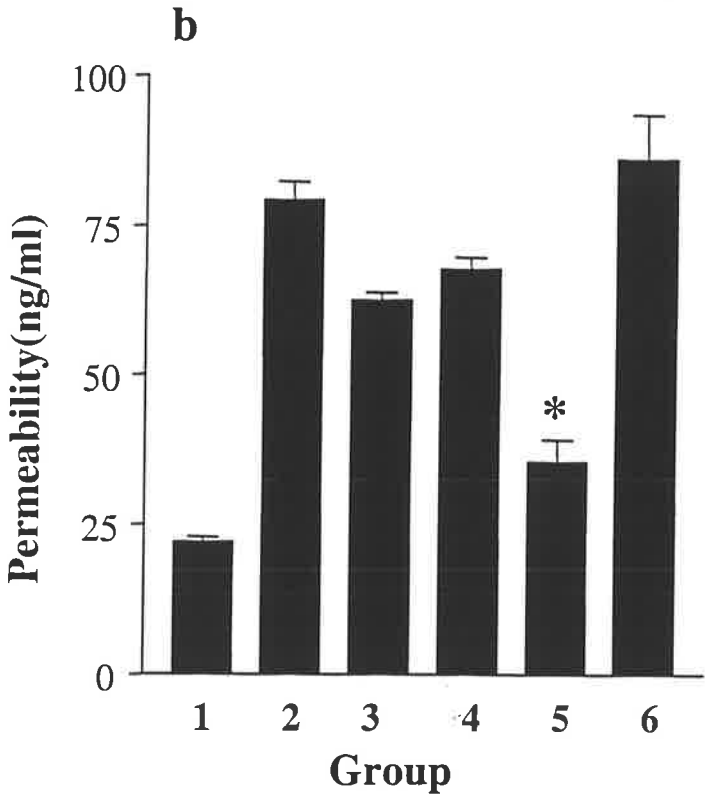

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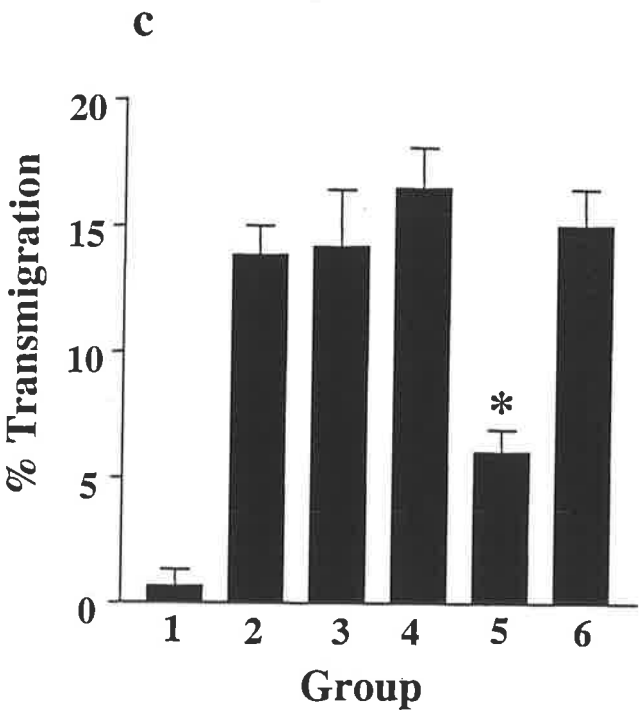

TGF-β and Endothelial Cells Inhibit VCAM-1 Expression on Human Vascular Smooth Muscle Cells

Jennifer R. Gamble, Sandy Bradley, Leanne Noack, Mathew A. Vadas

Abstract Vascular smooth muscle cells (VSMCs) are normally devoid of the adhesion protein vascular cell adhesion molecule–1 (VCAM-1), which has, however, been observed on human VSMCs in atheroma. We now show that cultured human saphenous vein VSMCs express small amounts of VCAM-1 and that the cytokine tumor necrosis factor–α (TNF-α) induces, in a time- and dose-dependent fashion, a significant increase in its expression. Interleukin (IL)-4, IL-1, and to a lesser extent interferon gamma have similar effects. TNF-α–stimulated human VSMCs demonstrate increased binding of T lymphocytes that is totally VCAM-1 mediated. The cytokine transforming growth factor–β (TGF-β) at 2.0 ng/mL inhibited basal VCAM-1 expression by 84±8% and the induction by TNF-α by between 56±16% and 77±15% depending on the dose of TNF. Furthermore, coculture on opposing sides of a polycarbonate filter of human VSMCs with human umbilical vein endothelial cells also inhibited the induction of VCAM-1 by 47±6%. As active TGF-β is produced upon the coculture of VSMCs and endothelial cells, we suggest that the close physical proximity of these cells in vivo is responsible for the lack of expression of VCAM-1 on VSMCs and that the interruption of this contact in atheroma is an important pathogenic event. As VCAM-1 not only serves as an adhesion molecule but also as a costimulator of immune cells, its expression may be crucial in the propagation of vascular lesions. (*Arterioscler Thromb Vasc Biol.* 1995;15:949-955.)

Key Words • atherogenesis • T cell adhesion • tumor necrosis factor • interleukin-4

Smooth muscle cells (SMCs) normally form a contractile and relatively homogeneous sheath of cells in the media of larger blood vessels. Although SMCs are separated from endothelial cells (ECs) by a basement membrane, there is both morphological[1] and functional[2,3] evidence that these two cell types work together and may exist as a physiological unit.

In certain disease states such as atherogenesis or inflammation, the media is invaded by blood cells such as neutrophils, monocytes, and T lymphocytes. It is possible that these blood cells interact directly with medial SMCs by making cell-cell contacts. A consequence of these cellular interactions may be the elaboration of soluble products, eg, cytokines, which can have a profound influence on the pathogenesis of disease. To better understand the adhesion molecules involved in the interaction of SMCs with the T lymphocytes and monocytes that invade the media of blood vessels in atherogenesis, we investigated the expression of vascular cell adhesion molecule–1 (VCAM-1) on SMCs.

VCAM-1 was originally described as an inducible molecule on ECs that mediated the adhesion of lymphocytes, monocytes, and eosinophils via the α4β1 integrin on these blood cells.[4-8] The special significance of VCAM-1 in mediating the adhesion of blood cells to endothelium is derived from its specificity for a subset of leukocytes, its capacity to provide an integrin-mediated adhesion pathway not involving the β2 integrins,[5,9] and its early expression at sites where there is an accumulation of monocytes in fatty steaks.[10,11] Subsequently, the cellular expression of VCAM-1 has broadened to include follicular dendritic cells in lymph nodes,[12] skeletal muscle cells,[13] and SMCs of atheromatous lesions.[14] Indeed, VCAM-1 protein and message have been shown to be inducible on rabbit[15] and human[16] aortic SMCs, respectively.

We show here that cultured human SMCs derived from saphenous veins express low levels of VCAM-1 in cell culture and can be induced to express VCAM-1 by the inflammatory cytokines tumor necrosis factor–α (TNF-α), interleukin (IL)-1β, IL-4, and interferon gamma (IFN-γ) and that VCAM-1 on SMCs serves as an adhesion molecule for lymphocytes. Furthermore, we show that coculture of human umbilical vein ECs (HUVECs) with SMCs suppresses the expression of VCAM-1, a suppression that is reproduced by the cytokine transforming growth factor–β (TGF-β). As active TGF-β is made upon SMC-EC contact[2,3] and is present in blood vessels,[17] our findings suggest that in normal blood vessels VCAM-1 expression on SMCs is chronically depressed by their proximity to ECs and that this depression may be mediated by TGF-β or another unidentified factor.

Methods

SMC Preparation

SMCs were extracted from human saphenous veins by a method modified from Chamley-Campbell et al.[18] Briefly, the endothelial layer was removed by scraping with a scalpel blade, and the vessel was cut into small pieces and digested with 3 μg/mL collagenase (Worthington Biochemicals) and 10.5 ng/mL soybean trypsin inhibitor (Sigma Chemical Co) for 1

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hour. The segments were then incubated in 1 mg/mL elastase for 1 hour and again with collagenase for 1 to 3 hours prior to centrifugation. The resultant cells were seeded into 25-cm² flasks (Corning) in M199 medium with Earle's salts, 10% fetal calf serum (GIBCO BRL), 4 mmol/L glutamine, essential amino acids, sodium pyruvate, sodium bicarbonate, and 10 mmol/L HEPES (Cytosystems). The medium was changed every 2 to 3 days, and cells were used after 4 to 6 weeks of growth. The cells were confirmed to be of the smooth muscle phenotype by staining with anti–α-SMC actin (Sigma). For the investigation of expression of cell surface molecules, vascular SMCs (VSMCs) were plated at 5×10^4 cells/100 μL into fibronectin (Boehringer Mannheim)-coated, 24-well trays (NUNC) and grown overnight. TGF-β, if used, was incubated for 20 hours prior to the addition of the other cytokines.

EC Preparation

ECs were prepared from human umbilical veins by a modified method of Wall et al.[19] The medium for growth was M199 with Earle's salts containing 20 mmol/L HEPES, 20% fetal calf serum (GIBCO BRL), 2 mmol/L glutathione, nonessential amino acids, sodium pyruvate, and sodium bicarbonate (Cytosystems). Cells were used at the first passage without the addition of growth factor or heparin.

Monoclonal Antibodies

Anti–VCAM-1 (51-10C9) and anti–E-selectin (49-1B11) were IgG_1 monoclonal antibodies raised by the authors that reacted specifically with Chinese hamster ovary cells transfected with VCAM and E-selectin cDNA, respectively. 28F11 is an isotype-matched monoclonal antibody that does not bind to unactivated or cytokine-activated VSMCs or HUVECs. Monoclonal anti–TGF-β, a gift of Genentech Inc, neutralizes TGF-β1, TGF-β2, and TGF-β3.

Cytokines

TNF-α (2×10^7 U/mg; lot No. 3901DAX), IFN-γ (2×10^7 U/mg; lot No. BN9327AX), and TGF-β (lot No. W9806AX) were the generous gifts of Genentech Inc. IL-1β (10^8 thymocyte mitogenic units per milligram) and IL-4 were the generous gifts of Immunex Inc.

Coculture Experiments

For the coculture of HUVECs and VSMCs, Millipore Transwells (Millipore) of 0.4-μm pore size and 12-mm diameter were used. Wells were first coated with 100 μg/mL fibronectin for 2 hours and allowed to dry overnight. To the inverted lower surface 4×10^4 VSMCs in 100 μL were added and allowed to adhere for 4 hours. The chamber was then inverted into media present in the Transwell container, and 3×10^4 HUVECs in 100 μL were added to the upper surface. The cells were cocultured for 36 hours prior to stimulation with cytokines.

Flow Cytometry

The appropriate antibody was added to cells in situ for 30 minutes at room temperature. The cells were then washed, and fluorescein isothiocyanate–coupled anti-mouse immunoglobulin (Silenus) was added for a further 30 minutes at room temperature. The cells were removed by trypsin, washed once, and fixed with fluorescence-activated cell sorter fix (paraformaldehyde, glucose, azide, and phosphate-buffered saline, pH 7.5) prior to analysis on an Epics Profile IV. The fluorescence was quantified as the mean channel $\times10^{-2}$ of 10 000 cells counted. The shift in fluorescence was uniform, and the median channel gave identical trends in the results. Background fluorescence refers to the mean channel fluorescence of cells stained with control antibody and fluorescein isothiocyanate–antimouse immunoglobulin.

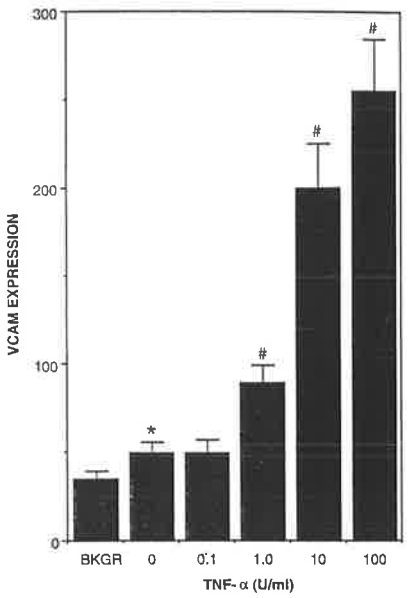

FIG 1. Bar graph showing effect of various doses of tumor necrosis factor–α (TNF-α) on vascular cell adhesion molecule–1 (VCAM) expression on human smooth muscle cells. The cells were incubated with TNF-α for 4 hours before harvesting. VCAM expression is expressed as the mean fluorescence intensity $\times10^{-2}$ of the mean of nine separate experiments. Vertical bars span 2 SEM. BKGR indicates background, ie, fluorescence without anti–VCAM-1 antibody. *$P<.05$ vs BKGR, #$P<.001$ vs no TNF-α.

Cell Adhesion Experiments

VSMCs were plated at 1.5×10^4/well in 96-well microtiter plates (NUNC) and grown overnight. T cells from peripheral blood were purified by Ficoll-Hypaque sedimentation followed by nylon wool separation.[20] The cells were labeled with ^{51}Cr,[21] and 5×10^5 cells were added to the VSMC monolayers for 45 minutes at 37°C. The nonadherent cells were removed by washing, and the adherent cells were extracted by NH_4OH. The percentage of adherent cells was calculated from the total number of cells added.

Statistics

The two-tailed *t* test was used to obtain probability values for statistical significance.

Results

TNF-α Increases VCAM-1 Expression in Human SMCs

Nine different isolates of SMCs were incubated with 0 to 100 U/mL TNF-α for 4 hours, and the expression of VCAM-1 was determined by flow cytometry. There was a small, statistically significant expression of VCAM-1 in the absence of TNF-α (Fig 1). TNF-α (1 to 100 U/mL) caused a dose-dependent increase in VCAM-1 expression. Although there were differences in the absolute amount of VCAM-1 induced between SMC lines, the same trend was seen in each isolate (data not shown).

Time Course of TNF-α–Stimulated VCAM-1 Expression in Human SMCs

A time course of VCAM-1 expression was performed in four different SMC lines by adding 100 U/mL TNF to aliquots of each cell line at various times before simultaneous harvest. The results (Fig 2) show peak expres-

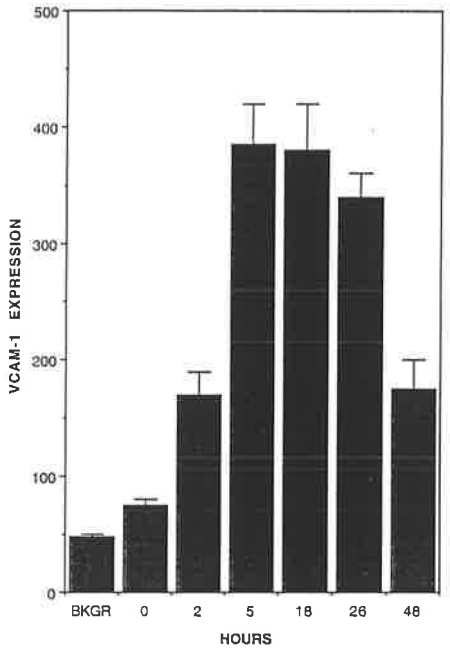

FIG 2. Bar graph showing time course of expression of vascular cell adhesion molecule–1 (VCAM-1) on human smooth muscle cells. Values are mean±SEM and represent at least four separate experiments. $P<.001$ time 0 vs BKGR (background, ie, fluorescence without anti–VCAM-1 antibody); $P<.004$ time 0 vs 2 hours and 2 hours vs 5 hours; $P<.008$ time 0 vs 48 hours.

sion at 5 hours after addition of TNF-α, with waning after 18 hours but still significant levels at 48 hours.

IL-4 Increases VCAM-1 Expression on Human SMCs

Human SMCs were incubated for 4 to 5 hours with a range of doses of IL-4. The results (Fig 3) show the dose-dependent increase in VCAM-1 expression. The addition of 10 U/mL TNF, which by itself had a moderate effect on VCAM-1 expression, was at least additive with IL-4. We found that IFN-γ and IL-1 also increased VCAM-1 expression on SMCs (Table 1) and that the effects of TNF and IFN-γ and of IL-4 and IFN-γ were also synergistic (data not shown). A combination of cytokines may be much more effective in stimulating strong VCAM-1 expression than one cytokine alone.

TGF-β Inhibits Basal and TNF-α–Stimulated VCAM-1 Expression in Human SMCs

TGF-β inhibits E-selectin expression in HUVECs.[21] Human SMCs were incubated with 2 ng/mL TGF-β for 24 hours before basal VCAM-1 was measured. As shown in Fig 4, a summary of six separate experiments, TGF-β diminished basal VCAM-1 expression to near background levels (by a mean of 84±8%). The effect of 2 ng/mL TGF-β on VCAM-1 responses to a series of doses of TNF is also shown in Fig 4. At each dose there was a significant inhibition of VCAM-1 expression that ranged from a mean decrease of 77±15%, 71±17%, 70±6%, and 56±16% at TNF doses of 0.1, 1, 10, and 100 U/mL, respectively. The effect of a range of doses of TGF-β on 100 U/mL TNF–stimulated VCAM-1 (Fig 4, right) demonstrates a dose-dependent increase of inhibition.

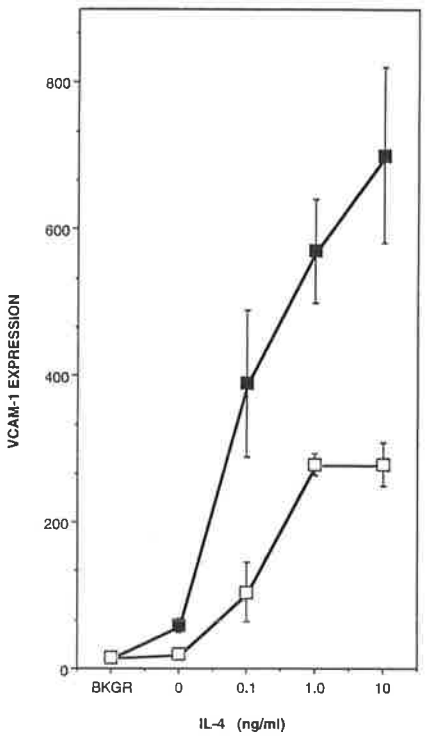

FIG 3. Line graph showing effect of interleukin-4 (IL-4) and tumor necrosis factor (TNF) and IL-4 combined on vascular cell adhesion molecule–1 (VCAM-1) expression on vascular smooth muscle cells. IL-4–stimulated VCAM-1 expression (□) differed from no stimulation (0) by $P=.02$, .0005, and .0005 at 0.1, 1, or 10 ng/mL, respectively. The effect of 10 U/mL TNF (■) by itself or in combination with 0.1, 1, or 10 ng/mL IL-4 is also shown. Values represent arithmetic mean±SEM of four experiments. BKGR indicates background. $P<.02$ IL-4 vs TNF+IL-4.

ECs Inhibit VCAM-1 Expression on Human SMCs

Human SMCs were grown on the bottom of Transwell inserts with 0.4-μm pores. HUVECs were plated in the top half of some of the inserts 24 hours before the addition of TNF-α. VCAM-1 expression was determined on the SMC population 5 hours after the addition of TNF-α, and as a control the effect of TGF-β was also examined on SMCs cultured without ECs. E-selectin expression was also measured on the SMC and EC populations. The results of a single typical experiment are given in Table 2. No E-selectin expression was seen in the SMC population, although it was highly expressed

TABLE 1. Effects of IFN-γ and IL-1 on VSMC VCAM-1 Expression

Treatment, 10 U/mL	VCAM-1 Expression*		
	Experiment 1	Experiment 2	Experiment 3
NIL	8	11	36±4
TNF	578	83	51±5
IFN-γ	73	59	ND
IL-1	ND	ND	162±36

IFN-γ indicates interferon gamma; IL-1, interleukin-1; VSMC, vascular smooth muscle cell; VCAM-1, vascular cell adhesion molecule–1; NIL, no treatment; TNF, tumor necrosis factor; and ND, not done. Experiment 3 is the mean of eight separate experiments. Numbers represent arithmetic mean±SEM.

*Mean fluorescence intensity (×100) with anti–VCAM-1 antibody+ fluoresceinated anti-mouse immunoglobulin−mean fluorescence intensity with fluoresceinated anti-mouse immunoglobulin.

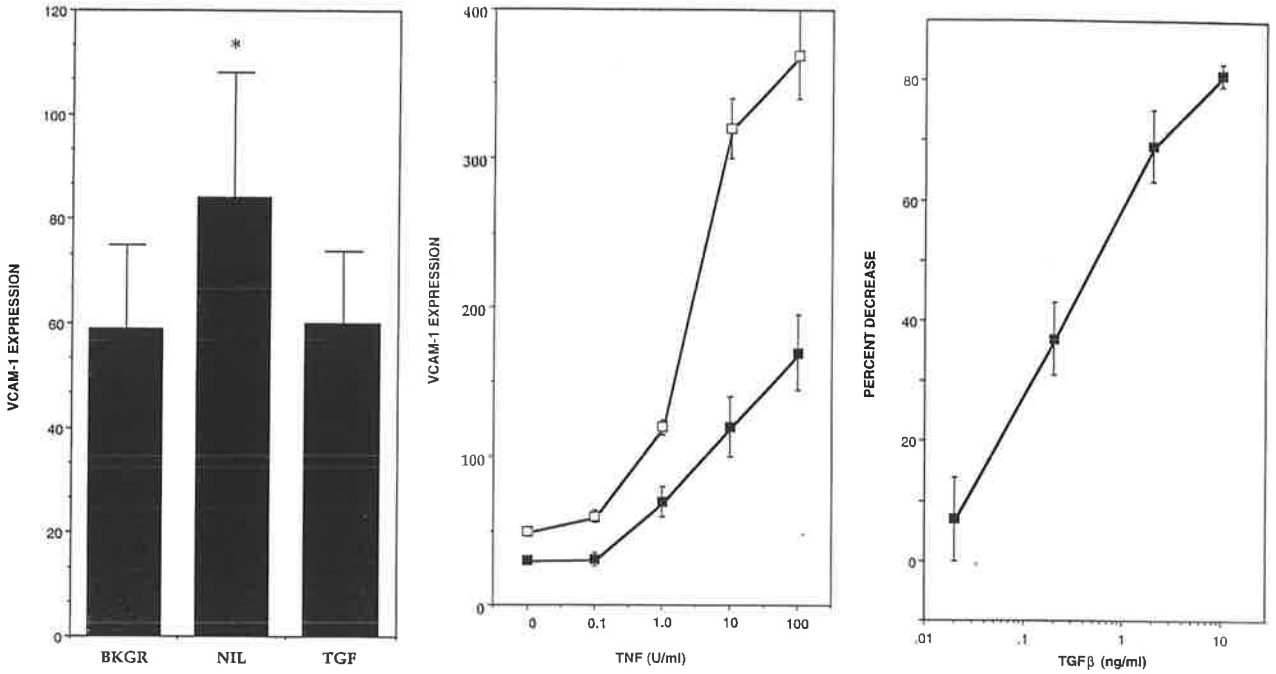

FIG 4. Left, Bar graph showing effect of transforming growth factor–β (TGF) on basal vascular cell adhesion molecule–1 (VCAM-1) expression on human vascular smooth muscle cells. Cells were exposed to 2 ng/mL TGF-β for 24 hours before VCAM-1 expression (mean fluorescence intensity of $\times 10^{-2}$) was measured. BKGR indicates background, ie, fluorescence without anti–VCAM-1 antibody; NIL, no treatment. *$P<.02$ NIL vs BKGR and TGF-β. Middle, Line graph showing effect of 2 ng/mL TGF-β (■) and no TGF-β (□) on VCAM-1 expression stimulated by a range of tumor necrosis factor (TNF) doses. Results are expressed as arithmetic mean±SEM of mean fluorescence intensity of $\times 10^{-2}$ of four separate experiments. BKGR fluorescence of no anti–VCAM-1 antibody was subtracted from each value in each experiment. $P=.01$, .01, .005, and .04 for TGF-β vs no TGF-β at TNF doses of 0.1, 1, 10, and 100 U/mL, respectively. Right, Line graph showing effect of a range of TGF-β doses on 100 U/mL TNF–stimulated VCAM-1 expression in up to seven experiments (n=2, 7, 5, and 2 for TGF-β doses of 10, 2, 0.2, and 0.02 ng/mL, respectively). Results are expressed as percentage decrease to minimize the effect of the variability of cell lines in their response to TNF. Percent decrease equals $([S-T]/S)\times 100$, where S is mean fluorescence in the presence of TNF−BKGR, and T is mean fluorescence in the presence of (TNF and TGF-β)−BKGR.

in the EC population. These results suggested that EC contamination was not an issue and that we were measuring SMC-associated VCAM-1 and not EC-associated VCAM-1. In SMC-EC coculture a decrease in the basal level of E-selectin on these primary ECs was seen, although the TNF-induced E-selectin expression was not altered. This regulation of E-selectin expression by coculture is under further investigation.

The experiment in Table 2 shows that both the basal and TNF-induced VCAM-1 expression on SMCs was inhibited by coculture with ECs. The pooled results of three experiments (Fig 5) show that VCAM-1 expression on VSMCs was reduced by 47%, a figure comparable to the 63% reduction by TGF-β in this series of experiments and to the figures reported in Fig 4. HUVECs cultured in the same dish but not on opposite sides of the filter with VSMCs had no effect on VCAM-1 expression (data not shown).

TABLE 2. Effect of EC-VSMC Coculture on VCAM-1 Expression on VSMCs

	VCAM-1*		E-Selectin*	
Stimulation	**NIL**	**TNF**	**NIL**	**TNF**
ECs only	390	3690	170	3080
VSMCs only	62	396	4	0
VSMCs from VSMC+EC coculture	51	191	3	0
ECs from VSMC+EC coculture	310	4430	20	3380

EC indicates endothelial cell; VSMC, vascular smooth muscle cell; VCAM-1, vascular cell adhesion molecule–1; NIL, no treatment; and TNF, tumor necrosis factor. VSMCs (4×10^4) were cultured on fibronectin-coated Transwells (lower membrane). After 4 hours the chambers were inverted, and either 4×10^4 human umbilical vein ECs or medium only was added to the upper surface. The cells were cultured for 36 hours prior to the addition of 10 U/mL TNF-α. Five hours later all wells were washed, and the cells were stained for either VCAM-1 or E-selectin expression. The VSMC and EC populations were then removed by trypsin treatment and analyzed for fluorescence.

*Mean fluorescence intensity (×100) with anti–VCAM-1 antibody or anti–E-selectin antibody+fluoresceinated anti-mouse immunoglobulin−mean fluorescence intensity with fluoresceinated anti-mouse immunoglobulin.

TNF-α– or IL-4–Stimulated SMCs Support the Adhesion of T Lymphocytes

To show the functional nature of induced VCAM-1 expression on SMCs, the adhesion of T lymphocytes, a cell type known to adhere to VCAM-1, was examined. Treatment of SMCs with TNF-α or IL-4 increased adhesion (Fig 6, left). This adhesion was inhibited by antibody to VCAM-1 (but not to E-selectin; data not shown) and, in the case of TNF-α, also by TGF-β (Fig 6, right).

Discussion

This article makes several important points that relate to the regulation of the interaction of SMCs with the blood cell types that invade the media of blood vessels in pathological states. First, VCAM-1 expression is strongly induced on SMCs by cytokines secreted by monocytes (TNF-α and IL-1) or T lymphocytes (IL-4 and IFN-γ). These findings are in partial agreement with Li et al[15] and Couffinhal et al.[16] Li et al[15] found that IFN-γ and lipopolysaccharide increased VCAM-1 message and protein in rabbit aortic SMCs and IL-4 and IFN-γ in

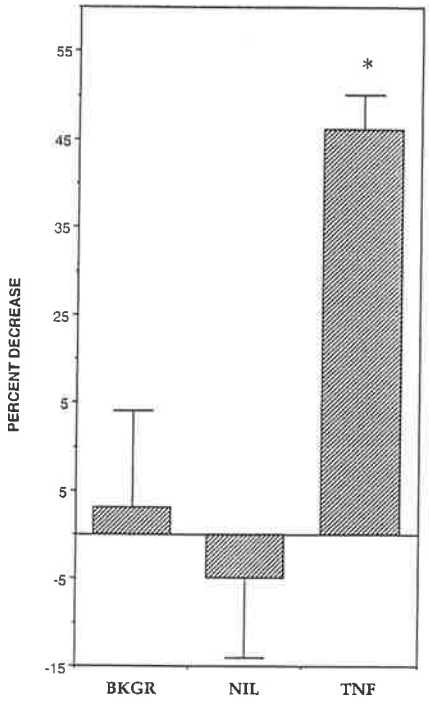

FIG 5. Bar graph showing effect of human umbilical vein endothelial cells (HUVECs) on vascular cell adhesion molecule–1 (VCAM-1) expression on vascular smooth muscle cells (VSMCs). Percent decrease equals ([N−H]/N)×100, where N is mean fluorescence without HUVEC−BKGR, and H is mean fluorescence with HUVEC−BKGR. Mean±SEM of three separate experiments is shown. BKGR indicates background, ie, fluorescence without anti–VCAM-1 antibody; NIL, no tumor necrosis factor (TNF) stimulation; and TNF, VSMCs that were stimulated with 10 U/mL TNF. *P=.02.

human aortic SMCs. These investigators failed to detect upregulation by either IL-1 or TNF-α. In contrast, Couffinhal et al[16] show a strong effect of TNF-α and a weak one of IFN-γ on VCAM-1 expression on human aortic SMCs. No synergy was observed in these experiments, and IL-1 was found to be without effect. Such differences may be a result of SMC source, extraction procedures, or cell-culture conditions.

In our experience the level of expression of VCAM-1 on SMCs induced by the cytokines TNF and IL-4 was highly significant and was reflected by an increased adhesion of T lymphocytes (Fig 6) that was totally VCAM-1 dependent. The role of VCAM-1 on VSMCs may extend beyond that of being a passive adhesion structure for cells expressing α4β1 or α4β7 integrins.[5,9] There exists evidence that VCAM-1 also serves as a costimulatory molecule of resting or short-term–stimulated T cells.[22-25] Interestingly, VCAM-1 induces apoptosis in a proportion of long-term–stimulated T cells.[26] Thus, VCAM-1 appears to have a key role in regulating T cell responses, and as T cells from atheromatous lesions express high levels of β1 integrins,[27] it is likely that this pathway operates in the microenvironment of atheromata. The cytokine profile of atheromatous lesions may thus be critical in maintaining the inflammatory-type response. The role of IL-4 is notable in this regard as it appears to act as a switching cytokine in the expression of adhesion molecules. On HUVECs it inhibits E-selectin expression[28] but enhances, as it does on VSMCs, VCAM-1 expression. Interestingly, IL-4 inhibits monocyte adhesion to HUVECs by mechanisms that remain to be defined.[29] These findings place special relevance on finding the TH2 type of T cells, a chief source of IL-4, in atheromatous lesions. However, to our knowledge the T cells in atheromatous lesions have not been characterized beyond expressing an activated phenotype.

The synergistic effect of TNF and IL-4 on VCAM-1 expression (Fig 3) suggests that the mixture of cytokines found in lesions such as atheroma may be more inducive to VCAM-1 expression than individual cytokines by

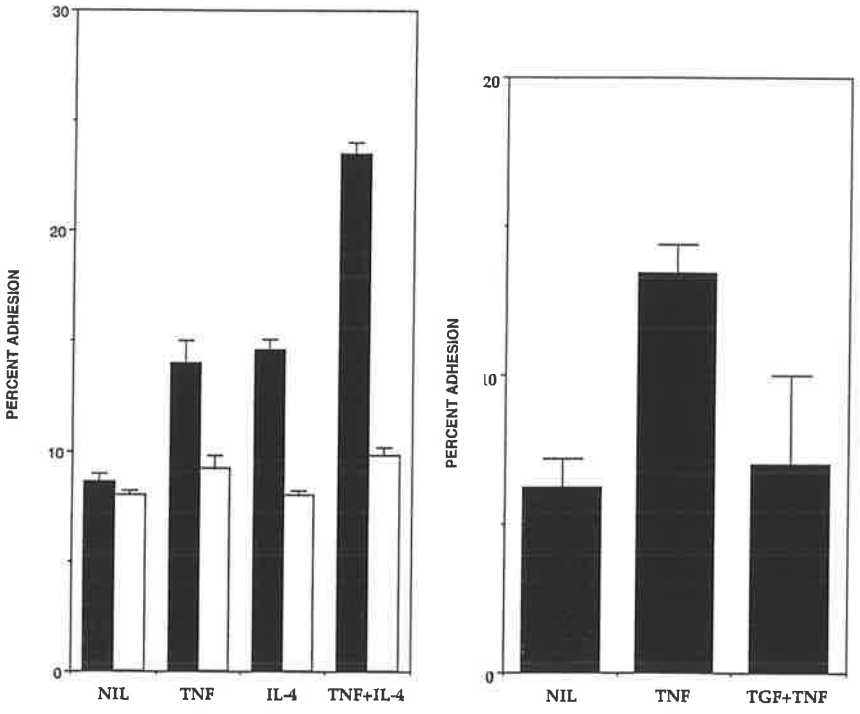

FIG 6. Bar graphs. Left, Adhesion of purified T lymphocytes to vascular smooth muscle cells (VSMCs) after treatment with tumor necrosis factor (TNF), interleukin-IL (IL-4), or TNF+IL-4 (TNF 10 U/mL, IL-4 10 ng/mL). All agents caused a significant (P=.04 for NIL [no treatment], P≤.001 for other groups) increase in adhesion that was inhibited by a blocking anti–vascular cell adhesion molecule–1 antibody (open bar). This is a typical experiment of three similar experiments. Each group was performed in quadruplicate and is expressed as mean±SEM. Right, Inhibition of adhesion by transforming growth factor–β (TGF) of purified T lymphocytes to TNF-α–stimulated VSMCs. VSMCs were treated with 2 ng/mL TGF-β for 20 hours prior to TNF-α stimulation for 4 hours. This is a representative experiment of three similar experiments. Mean±SEM of each group performed in triplicate is given. P<.0005 TNF-α–induced adhesion vs NIL; P<.01 TGF-β–inhibited adhesion to TNF-α–stimulated VSMCs.

themselves. TNF has been demonstrated by immunocytochemical techniques to be present in atheromatous lesions,[30] and IL-4 is a product of a subset of the activated T cells that form approximately 30% of the cellular infiltrate of atheromatous lesions.[27,31] Thus, the type of synergy demonstrated in Fig 3 may well take place in vivo. Interestingly, O'Brien et al[14] have demonstrated VCAM-1 expression on intimal SMCs only in areas of inflammatory cell infiltration, suggesting the possibility that factors released by these inflammatory cells result in the induction of VCAM-1 expression. We would postulate that on the basis of the phenotypes of T cells and macrophages in these lesions, TNF and IL-1 are likely candidates. The local production of cytokines would also explain the finding of SMC activation in isolated, specific regions and not throughout the total neointima.

The second chief finding is the strong suppression of VCAM-1 expression that is exerted by TGF-β on both basal and TNF-stimulated VCAM-1 expression (Fig 4). TGF-β inhibits endothelial E-selectin expression, but it did not significantly alter the expression of VCAM-1 on ECs.[22] The effect of TGF-β on VSMCs differed from HUVECs in other aspects as well. On VSMCs the dose-response curve was linear (Fig 4) compared with the inverted U-shaped curve seen on HUVECs.[21] This difference cannot be explained by but may be related to the types of receptors expressed on the two cell types or intracellular signaling events that may follow ligand binding. The inhibition of TGF-β was more complete on VSMCs and operated almost equally at all doses of TNF, whereas the inhibition seen on HUVECs was mainly at low doses of TNF.

The third important observation presented here is that ECs cocultured with VSMCs also inhibit VCAM-1 expression (Fig 5). The nature of this inhibitory effect upon coculture has yet to be defined. One possibility is that it is mediated through TGF-β. Active TGF-β is made when ECs are cocultured and in close association with another cell type, most relevantly VSMCs.[2,3] However, in our system, the anti–TGF-β antibodies (which are known to neutralize TGF-β1, TGF-β2, and TGF-β3) that neutralized the effect of exogenously added TGF-β did not reproducibly neutralize the inhibitory effect on SMC–VCAM-1 expression by EC-SMC coculture (data not shown). It is possible that the exogenously added antibody has a variable access to the sites of endogenous production of TGF-β in a Transwell system. Addition of aprotinin and mannose 6-phosphate, both of which inhibit the conversion of latent to active TGF-β,[3,32] failed to reverse the inhibitory effect of EC-SMC coculture on VCAM-1 expression. An alternate possibility for the inhibitory effect is that a factor other than TGF-β may be produced upon EC-SMC coculture. As for TGF-β1, this factor is nondiffusible and requires close cell growth, since ECs grown in the same well but not on opposing sides of a filter did not regulate VCAM-1 expression on SMCs. The nature of this factor is currently being investigated.

A central role of TGF-β in the proper functioning of the vasculature is supported by a number of observations. First, TGF-β1–null mice[33,34] show spontaneous multifocal inflammatory disease, widely disseminated leukocyte infiltration, and excess production of the proinflammatory cytokines TNF, IFN-γ, and macrophage inflammatory protein–1α. Our previous observations[20,32,35] suggest that disregulated expression of adhesion molecules on the endothelium may account for some of these effects. Second, the high-risk factors for atherosclerosis, lipoprotein(a) and plasminogen activator inhibitor–1, block the activation of latent TGF-β by competitively inhibiting tissue plasminogen activator, an enzyme essential in the conversion of latent to active TGF-β.[36,37] TGF-β inhibits the migration and proliferation of VSMCs in cell culture[36,37] and, as we show here, the expression of VCAM-1. Interestingly, at sites of apo(a) accumulation (the active moiety of lipoprotein[a]), VSMCs display an activated phenotype, and there is a high propensity for the development of vascular lesions at these sites.[14,38] Third, patients with advanced atherosclerosis show a significantly lower concentration of active TGF-β in their serum than do normal control subjects.[39] The correlation of active TGF-β with atherosclerosis is stronger than any of the known risk factors.

Our data also suggest the importance of EC-SMC interaction in vascular function. A physical or functional separation of these cells, such as may happen as a result of the thickening of the basal lamina[40] during the early events in models of atheroma, may result in profound effects on the function of SMCs and ECs. As we have shown, the adhesiveness of VSMCs through regulation of VCAM-1 expression is controlled by ECs. Furthermore, the migration, proliferation, and production of plasminogen activator inhibitor–1 by ECs is inhibited by VSMCs.[2,3,41] At least one of the controlling factors produced as a result of EC-SMC apposition has been identified as TGF-β.[2,3]

Acknowledgments

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High-Density Lipoproteins Inhibit Cytokine-Induced Expression of Endothelial Cell Adhesion Molecules

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Abstract While an elevated plasma concentration of HDLs is protective against the development of atherosclerosis and ensuing coronary heart disease (CHD), the mechanism of this protection is unknown. One early cellular event in atherogenesis is the adhesion of mononuclear leukocytes to the endothelium. This event is mediated principally by vascular cell adhesion molecule-1 (VCAM-1) but also involves other molecules, such as intercellular adhesion molecule-1 (ICAM-1) and E-selectin. We have investigated the effect of isolated plasma HDLs and reconstituted HDLs on the expression of these molecules by endothelial cells. We show that physiological concentrations of HDLs inhibit tumor necrosis factor-α (TNF-α) or interleukin-1 (IL-1) induction of these leukocyte adhesion molecules in a concentration-dependent manner. Steady state mRNA levels of TNF-α–induced VCAM-1 and E-selectin are significantly reduced by physiological concentrations of HDLs. At an HDL concentration of 1 mg/mL apolipoprotein A-I, the protein expressions of VCAM-1, ICAM-1, and E-selectin were inhibited by 89.6±0.4% (mean±SD, n=4), 64.8±1.0%, and 79.2±0.4%, respectively. In contrast, HDLs have no effect on the expression of platelet endothelial cell adhesion molecule (PECAM) or on the expression of the p55 and p75 subunits of the TNF-α receptor. HDLs were effective when added from 16 hours before to 5 minutes after cytokine stimulation. HDLs had no effect on TNF-α–induced expression of ICAM-1 by human foreskin fibroblasts, suggesting that the effect is cell-type restricted. This study provides the first evidence that HDLs may protect against CHD by inhibiting the expression of adhesion molecules, which are required for the interaction between leukocytes and the endothelium. (*Arterioscler Thromb Vasc Biol.* **1995;15:1987-1994.**)

Key Words • inflammation • coronary heart disease • VCAM-1 • E-selectin

Atherosclerotic CHD is still one of the major causes of death in the western world.[1] Epidemiological studies have shown a strong inverse correlation between the concentration of plasma HDLs and the incidence of CHD.[2,3] Direct evidence of a protective effect of HDLs has come from the studies of transgenic mice, in which high levels of expression of human apoA-I, the major apolipoprotein in HDLs, increased the HDL concentrations and gave protection against diet-induced atherosclerosis.[4] It is not yet known whether the protection relates to the involvement of HDLs in reverse cholesterol transport or whether HDLs protect against CHD by a mechanism unrelated to their lipid transport function.

An early event in atherogenesis is the adhesion of monocytes to the endothelium via adhesion molecules such as VCAM-1, ICAM-1, and E-selectin, all of which are rapidly synthesized in response to cytokines. VCAM-1 (CD106) is a member of the immunoglobulin-like superfamily and is primarily involved in the adhesion of mononuclear leukocytes to the endothelium. VCAM-1 is rapidly induced by the inflammatory cytokines IL-1 and TNF-α, and its induction is sustained for 48 to 72 hours. ICAM-1 (CD54) is expressed on many cell types and is involved in both monocyte and lymphocyte adhesion to activated endothelium. E-selectin (CD62E) is an endothelial-specific adhesion molecule important in the adhesion of polymorphonuclear leukocytes, monocytes, and lymphocytes to cytokine-treated HUVECs.[5-14] It has also been shown to be important in capturing leukocytes from the axial stream to roll along the endothelium.[15]

There is considerable evidence for the involvement of adhesion molecules in the development of early atherosclerotic lesions[16] and in mature atherosclerotic plaques.[17] The expression of VCAM-1 is coincident with early foam cell lesions in hypercholesterolemic rabbits.[16] Variable and low levels of E-selectin and VCAM-1 have been detected in the arterial endothelium over plaques.[17,18] VCAM-1 has also been observed in areas of neovascularization and inflammatory infiltrates at the base of plaques, suggesting that intimal neovascularization may be an important site of inflammatory cell recruitment into advanced coronary lesions.[19] ICAM-1[20] and P-selectin, another member of the selectin family also involved in the rolling of leukocytes, have also been shown to be expressed on the endothelium overlaying atheromatous plaques, suggesting that leukocyte adhesion and recruitment into the plaque may be mediated through a synergy between the selectins and members of the immunoglobulin-like superfamily of adhesion molecules.[21]

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Selected Abbreviations and Acronyms

apo = apolipoprotein
CHD = coronary heart disease
FACS = fluorescence-activated cell sorting
HUVEC = human umbilical vein endothelial cell
ICAM-1 = intercellular adhesion molecule-1
IL = interleukin
PECAM = platelet endothelial cell adhesion molecule
TNF = tumor necrosis factor
VCAM-1 = vascular cell adhesion molecule-1

The concept of the critical role of adhesion molecules in atherogenesis has been strengthened by reports showing that LDLs,[22] especially if minimally oxidized,[23] increase monocyte adhesion to endothelial cells. Furthermore, it has been shown that lysophosphatidylcholine, a major component of oxidatively modified LDLs, induces expression of VCAM-1 and ICAM-1.[24,25] These observations suggest that LDLs promote atherogenesis at least in part by this mechanism. In this study we test the hypothesis that HDLs may have a direct effect on the endothelium by inhibiting the induced expression of adhesion molecules.

We show that physiological levels of native and reconstituted HDLs inhibit the induction of adhesion molecules on endothelial cells. These observations strongly point to a specific role for HDLs in preventing the adhesion-dependent early events in atherogenesis and in preventing the progression of atheromata by a similar mechanism. Therefore, this study demonstrates a function of HDL beyond those previously described.

Methods

Cell Culture

HUVECs were isolated as described previously.[26] All cells were cultured on gelatin-coated culture flasks in medium M199 with Earle's salts (Cytosystems) supplemented with 20% fetal calf serum (GIBCO), 20 mmol/L HEPES, 2 mmol/L glutamine, 1 mmol/L sodium pyruvate, nonessential amino acids, penicillin, streptomycin, 50 μg/mL endothelial growth supplement (Collaborative Research), and 50 μg/mL heparin (Sigma). Confluent HUVECs were incubated for 16 hours in varying concentrations of HDLs; then TNF-α or IL-1 (100 U/mL) was added to the culture medium and incubated for an additional 4 hours, at which time the cell-surface expression of VCAM-1, E-selectin, and ICAM-1 were measured by flow cytometry. In the case of TNF-α receptor and PECAM, cells were incubated for 20 hours in HDLs but were not cytokine stimulated before assay of their expression by flow cytometry.

Flow Cytometry Analysis

Cells were harvested by trypsinization, and then washed in PBS. Levels of cell-surface expression of proteins were measured by incubation of the cell suspension in 50 μL of primary antibody for 30 minutes at 4°C. Cells were then washed in FACS wash (PBS containing 0.02% azide and 5% newborn calf serum) at 4°C, resuspended in 50 μL of FACS wash containing the appropriate FITC-conjugated secondary antibody, and incubated at 4°C for an additional 30 minutes. After another wash, the cell pellet was resuspended in FACS fixative (PBS containing 2% glucose, 0.02% azide, and 2.5% formaldehyde), and expression of cell-surface molecules was measured as fluorescence intensity by use of a Coulter Epics Profile II flow cytometer. Each sample counted 1×10^4 cells. Controls for each assay included the absence of primary antibody and the incubation of the cells with an isotype-matched, nonrelevant antibody.

Antibodies Used

Mouse monoclonal antibody to VCAM-1 (10C9) and mouse monoclonal antibody to E-selectin (49-1B11) were generated in our laboratory and characterized by their ability to bind to VCAM-1– and E-selectin–transfected Chinese hamster ovary cells, respectively. The TNF receptor antibodies for the p75 subunit (utr-1) and the p55 subunit (htr-9) were a gift from Dr Brockhaus (Roche). The rabbit polyclonal antibody against PECAM was a gift from Dr Micheal Berndt (Baker Institute).

Northern Blot Analysis

Total RNA was prepared as described by Chomczynski and Sacchi.[27] Equal aliquots of total RNA (10 μg) were electrophoresed in a 1% formaldehyde gel and transferred to nylon membrane (Hybond N, Amersham). RNA was fixed in a UV strata-linker (Stragagene). The blots were prehybridized according to Church and Gilbert[28] and hybridized with 10 ng/mL α^{32}P-labeled cDNA probes. After washing, the blots were exposed on Kodak XAR5 film (Eastman Kodak). Relative amounts of RNA per lane were normalized to the ethidium bromide–stained ribosomal RNAs.

Isolation of HDLs

Blood samples from normal healthy donors under 40 years of age were collected in EDTA-Na_2 (final concentration, 1 mg/mL). Plasma was separated by centrifugation at 4°C. HDLs were isolated by sequential ultracentrifugation in the 1.07- to 1.21-g/mL density range, as described elsewhere.[29] Resulting preparations of HDLs were dialyzed against four changes of PBS before filter sterilization with 0.2-mm acrodiscs (Gelman Sciences).

The concentrations of apoA-I and apoB in the preparations of HDLs were determined immunoturbidimetrically on a Cobas-Fara centrifugal analyzer (Roche Diagnostic). The apoA-I and apoB antibodies and standards were obtained from Boehringer Mannheim. HDL particle size distribution was evaluated by electrophoresis on a 3% to 35% nondenaturing gradient gel according to manufacturer's recommendations (Pharmacia LKB Biotechnology). All preparations of HDLs contained two main populations: one with particles of Stokes' diameter 10.45 nm (HDL_{3b}) and one with particles of diameter 8.6 nm (HDL_{3a}). There was no evidence of contamination by particles in the size range of LDLs, and no apoB was detected in the HDL preparations.

Preparation of Reconstituted HDL Particles

Discoidal reconstituted A-I HDL was prepared by the cholate dialysis method from egg-yolk phosphatidylcholine, unesterified cholesterol, and apoA-I.[30] ApoA-I was prepared from human plasma, as described elsewhere.[31] Egg-yolk phosphatidylcholine, unesterified cholesterol, and sodium cholate were obtained from Sigma and used without further purification. Particle size was measured by nondenaturing gradient gel electrophoresis, and concentration of apoA-I was measured immunoturbidimetrically, as described above.

Results

HDLs Inhibit Both TNF-α–Induced and IL-1–Induced VCAM-1 Expression on Endothelial Cells

Both IL-1 and TNF-α have been shown to induce the expression of VCAM-1 on endothelial cells.[32] To test the hypothesis that HDLs inhibit cytokine-induced expression of VCAM-1, confluent HUVEC monolayers were incubated for 16 hours in the presence of HDLs at an apoA-I concentration of 1 mg/mL before the addition of

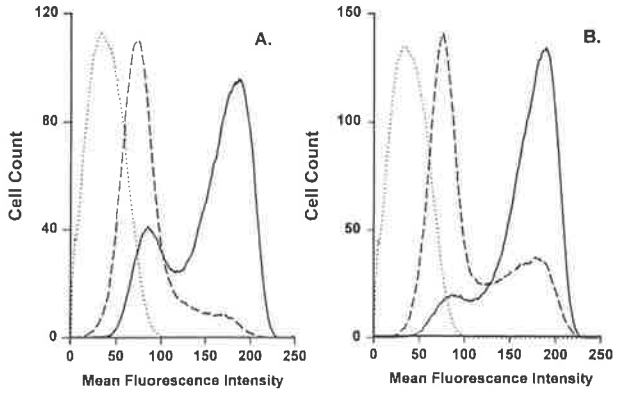

FIG 1. Flow cytometry profiles showing the effect of HDLs on cytokine induction of VCAM-1. After a 16-hour preincubation of confluent HUVECs with HDLs (1 mg/mL of apoA-I), either TNF-α or IL-1 was added to the culture medium (100 U/mL). Cells were then cultured for an additional 4 hours. After this treatment, cell-surface expression of VCAM-1 was measured by flow cytometry. Graphs show the expression of VCAM-1 after IL-1 activation (A) compared with that after TNF-α activation (B) in the presence of HDLs (dashed lines) and the absence of HDLs (solid lines). A negative control profile with the isotype-matched, nonrelevant antibody (dotted lines) is also shown. These data are representative of three such experiments.

IL-1 or TNF-α. After an additional 4 hours of incubation, cell-surface expression of VCAM-1 was determined by flow cytometry. Fig 1 shows that both IL-1 (A) and TNF-α (B) induce the expression of VCAM-1 on HUVECs (solid line), and that HDLs (1 mg/mL apoA-I, dashed line) inhibit the expression of VCAM-1 in both IL-1–stimulated (A) and TNF-α–stimulated (B) HUVECs. In similarly designed experiments, preincubation of HUVECs with native LDL or oxidized LDL did not alter the level of cytokine-induced expression of adhesion molecules (data not shown). Consistent with the findings of Khan et al,[33] we showed no induction of endothelial cell adhesion molecule expression by native or oxidized LDL per se (data not shown).

Concentration-Dependent Effects of HDLs on TNF-α–Induced Expression of Adhesion Molecules

Cytokine stimulation of HUVECs also leads to the induced expression of ICAM-1 and E-selectin, as well as VCAM-1.[32] To compare the effects of HDLs on VCAM-1 with those on ICAM-1 and E-selectin expression, we incubated endothelial monolayers for 16 hours with a range of concentrations of HDLs before the addition of TNF-α. HDLs inhibited the TNF-α–induced expression of VCAM-1 in a concentration-dependent manner (Fig 2). At a concentration of 1 mg/mL apoA-I, HDLs reduce the expression of VCAM-1 by 89.6±0.4% (mean±SD, n=4). TNF-α–induced expression of ICAM-1 and E-selectin was also inhibited in a concentration-dependent manner, with inhibition of 64.8±1.0% and 79.2±0.4%, respectively, at an apoA-I concentration of 1 mg/mL. To exclude the possibility that the decrease in cytokine-induced expression of adhesion molecules was an artifact resulting from residual HDLs sterically hindering access of the antibodies to their respective antigens, flow cytometry was performed in the presence and absence of exogenous HDLs. Monolayers of HUVECs were stimulated with TNF-α (100 U/mL for 4 hours). After removal of the cell monolayer by trypsinization, the cells were resuspended in the respective primary antibodies in the presence of HDLs over a range of concentrations. The Table shows that HDL concentrations of up to 1.0 mg/mL apoA-I did not change the detectability of cell-surface molecules.

HDLs Reduce Steady State mRNA Levels of VCAM-1 and E-Selectin

The effects of HDLs on steady state levels of mRNA of VCAM-1 and E-selectin in endothelial cells were investigated. Confluent endothelial cells were incubated (1) for 20 hours with no addition, (2) for 16 hours with no addition followed by 4 hours in the presence of TNF-α (100 U/mL), and (3) for 16 hours with HDLs at a physiological concentration (1 mg/mL apoA-I) before adding TNF-α for an additional 4 hours of incubation. After completion of the incubations, total RNA was extracted, and the levels of mRNA specific for VCAM-1 and E-selectin were measured by Northern blot analysis with cDNA probes (Fig 3). In the uninduced samples there was no detectable message for either VCAM-1 or E-selectin. In the samples induced by TNF-α in the absence of HDL, the level of message for both adhesion molecules was substantial. The levels of message for both VCAM-1 and E-selectin were markedly reduced by HDLs.

HDLs Have No Effect on Expression of PECAM and TNF Receptor

PECAM is constitutively expressed on endothelial cells.[34] To investigate the possibility that incubation of endothelial cells with HDLs had a nonspecific effect on the expression of all molecules, we examined the effect of HDLs on the expression of PECAM. There was no change in PECAM expression after a 20-hour incubation of HUVEC monolayers with a range of concentrations of HDLs (Fig 4).

The p55 and p75 subunits of the TNF-α receptor are also constitutively expressed on endothelial cells.[35] We examined whether incubation of HUVECs with HDLs could result in a reduction in cell-surface expression of TNF-α receptor. We incubated confluent HUVECs with HDLs (1 mg/mL apoA-I) for 20 hours. TNF-α receptor expression was then assayed by flow cytometry. Fig 5 shows flow cytometric profiles of HUVECs stained with antibodies against the p55 (dashed line) and p75 (dotted line) subunits of the TNF-α receptor in the presence versus the absence of HDLs. The presence of HDLs had no effect on the levels of expression of these molecules.

Time Course of Inhibition of VCAM-1 Expression by HDLs

To establish the time dependence of the inhibitory effect of HDLs on HUVECs, we examined the level of VCAM-1 expression 4 hours after TNF-α treatment in cells to which HDLs (1 mg/mL apoA-I) were added from 16 hours before to 4 hours after TNF-α. Fig 6 shows that the inhibitory effect of HDLs does not require preincubation, since significant inhibition (86.4±0.6%) is obtained when HDLs (1 mg/mL apoA-I) are added at the same time or even 5 minutes after the TNF-α. However, when added 1 or more hours after TNF-α activation, the level of VCAM-1 expression is comparable with the control cultures to which HDLs have not been added

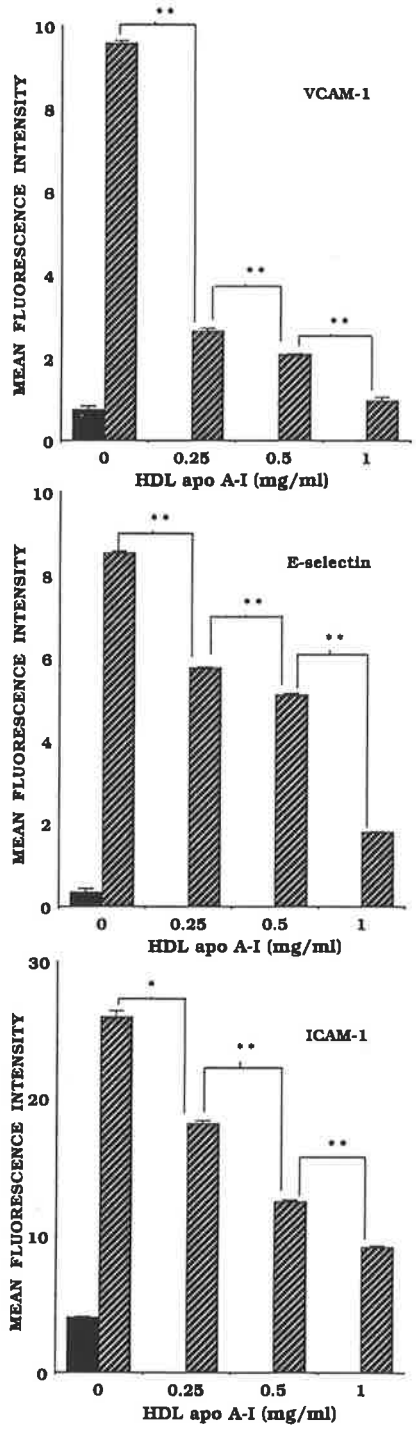

FIG 2. Bar graphs showing the concentration-dependent effect of HDLs on TNF-α–induced expression of adhesion molecules. Confluent monolayers of endothelial cells were incubated in the presence of a range of HDLs for 16 hours. At this time TNF-α (100 U/mL) was added to the culture medium. The incubation was then continued for an additional 4 hours. The cell-surface expression of adhesion molecules was measured by using flow cytometry. The values represent the mean fluorescence intensity detected with antibodies directed against the respective proteins (as indicated) in response to TNF-α (hatched), relative to unstimulated basal levels (solid). The results represent data from one experiment but are representative of four experiments conducted with four different HUVEC donors and four different HDL preparations. Each experiment was conducted in quadruplicate. Means and SD are shown. Differences between means were evaluated using unpaired *t* tests. **P*<.005, ***P*<.001.

Effect of HDLs on the Assay of Adhesion Molecules

HDL (apoA-I, mg/mL)	ICAM-1	E-Selectin	VCAM-1
	Mean Fluorescence Intensity		
0.0	36.52	10.21	8.09
0.25	36.78	11.10	8.15
0.50	33.65	12.92	9.07
1.00	40.41	12.87	10.53

Confluent HUVECs were incubated for 4 hours in the presence of TNF-α (100 U/mL) and then assayed for adhesion molecules by using flow cytometry (see "Methods"). Levels of surface expression of adhesion molecules are represented as mean fluorescence intensity measured when the assay is conducted in the presence of varying concentrations of HDLs (as indicated).

(NA). When cell cultures were incubated for 16 hours with HDLs followed by two washes in PBS, which removes >95% of the HDL (as determined by measuring the recovered HDL according to the method described in "Methods"), maximal inhibition of expression of TNF-α–induced VCAM-1 expression was observed (R).

Since the inhibition of cytokine-induced VCAM-1 expression could be sustained when HDLs were removed before TNF-α stimulation, we investigated the kinetics of this acquired inhibition. HDLs (1 mg/mL apoA-I) were added over a range of times to confluent monolayers of HUVECs before being removed from the cultures by two washes in PBS. Fresh medium containing TNF-α was then added, and the VCAM-1 expression was determined by flow cytometry 4 hours later. Fig 7 shows that the acquisition of inhibition was time dependent, with 50% maximal inhibition requiring ≈60 minutes of incubation. With 10 minutes of preincubation with HDLs, no significant difference in VCAM-1 expression is observed. After 30 minutes and 1 hour of preincubation, VCAM-1 expression was suppressed by 42.3±1.31% and 56.5±0.6%, respectively. In cultures in which HDLs have been preincubated for 2 hours, the

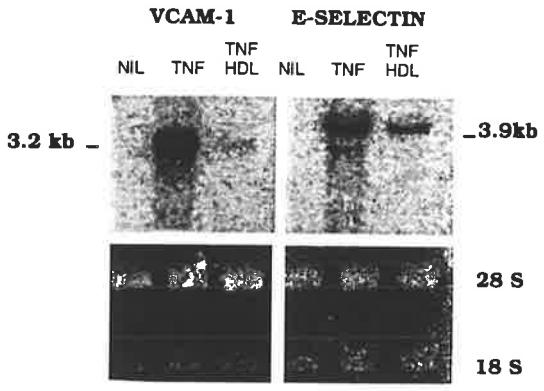

FIG 3. Autoradiograph showing the effect of HDLs on the steady state mRNA levels of VCAM-1 and E-selectin in endothelial cells. Confluent HUVECs were incubated (1) for 20 hours with no addition (NIL), (2) for 16 hours with no addition followed by 4 hours in the presence of TNF-α (100 U/mL) (TNF), and (3) for 16 hours with HDLs (1 mg/mL apoA-I) before adding TNF-α for an additional 4 hours of incubation (TNF HDL). After the above treatment, total RNA was extracted and 10 mg per sample size fractionated in a 1% formaldehyde gel. Equivalent loading was assessed by ethidium-bromide staining of the 28S and 18S ribosomal bands (as indicated). Levels of mRNA for VCAM-1 and E-selectin (as indicated) after autoradiography were detected by hybridization to specific radiolabeled cDNA probes ("Methods").

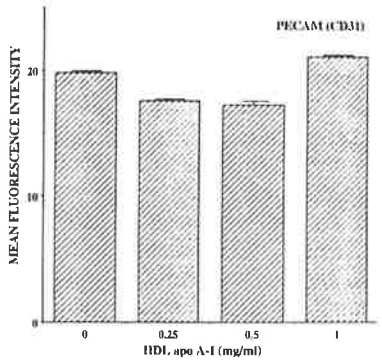

FIG 4. Bar graph showing the effect of HDLs on PECAM expression. Confluent endothelial-cell monolayers were incubated for 20 hours in the presence of a range of concentrations of HDLs (as indicated). Cells were then assayed for cell-surface PECAM by flow cytometry by using a rabbit polyclonal anti-PECAM antibody. The mean and SDs shown are data from one experiment representative of three experiments. There was no significant effect of HDLs in any of these experiments.

level of TNF-α–induced expression of VCAM-1 was inhibited by 88.4±0.76%.

HDLs Do Not Inhibit TNF-α–Induced Induction of ICAM-1 in Fibroblasts

ICAM-1 is expressed and regulated on fibroblasts. We examined the ability of HDLs to inhibit the TNF-α–induced expression of this molecule on fibroblasts. Fig 8 shows that after a 4-hour stimulation with TNF-α (100 U/mL), ICAM-1 expression was significantly ($P<.005$) induced from a basal level of 20.46±0.43 to 60.06±11.4 mean fluorescence intensity. When incubated with HDLs (1 mg/mL apoA-I), the level of TNF-α–induced expression of ICAM-1 was not significantly altered (66.46±12.3).

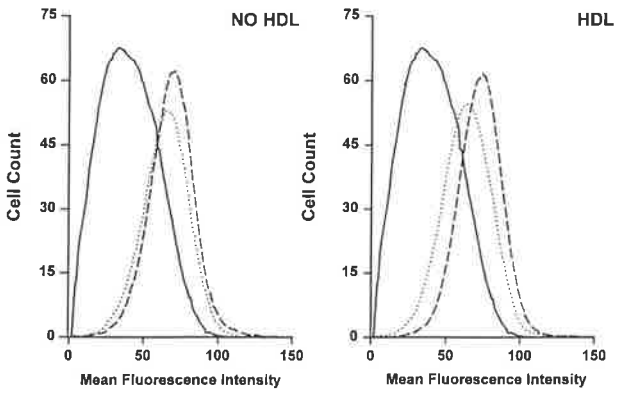

FIG 5. Flow cytometric profiles showing the effect of HDLs on expression of TNF receptor. Confluent cultures of HUVECs were incubated for 20 hours in the absence or in the presence of HDLs (1 mg/mL of apoA-I). The level of TNF-α–receptor expression was subsequently measured by flow cytometry by using antibodies against the p55 (utr-1) (dashed lines) and the p75 (htr-9) (dotted lines) subunits. Profiles of HUVECs stained with antibodies against the p55 (dashed lines) and the p75 (dotted lines) subunits of the TNF-α receptor and an isotype-matched control antibody (solid lines) were obtained after incubations conducted in the absence (NO HDL) and presence (HDL) of HDLs.

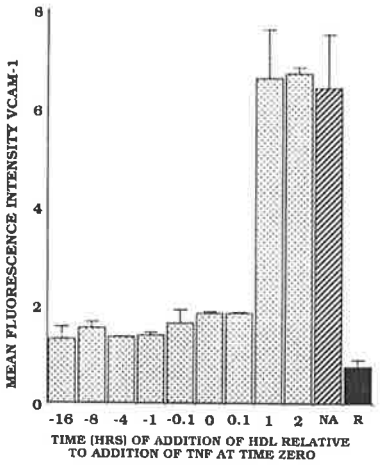

FIG 6. Bar graph showing time dependence of inhibition of VCAM-1 expression by HDLs. Confluent cultures of HUVECs were incubated for varying lengths of time with HDLs (1 mg/mL apoA-I) both before (–) and after the addition of TNF-α (100 U/mL) at time zero. The cells were trypsinized 4 hours after the addition of TNF-α and levels of VCAM-1 were measured by flow cytometry. Samples to which HDLs were not added are indicated by the bar labeled NA. The bar labeled R indicates the level of expression of VCAM-1 when cultures were incubated with HDLs for 16 hours and then washed twice in PBS to remove the HDLs before being incubated for an additional 4 hours in fresh growth medium containing TNF-α (100 U/mL). Results represent the means and ranges of two experiments, using two different HUVEC donors of similar passage. The same preparation of HDLs was used in both experiments.

Reconstituted HDLs Inhibit the TNF-α–Induced Expression of VCAM-1

To exclude the possibility that the inhibiting effects of plasma HDLs were mediated by contaminating plasma components that co-isolated with the HDLs, we investigated the effects of reconstituted HDLs. Reconstituted discoidal HDLs containing purified apoA-I as their sole

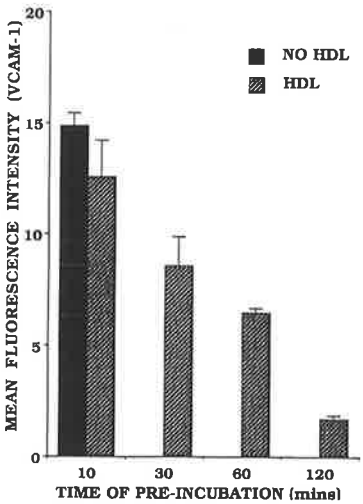

FIG 7. Bar graph showing time dependence of the inhibitory effect of HDLs. Confluent HUVECs were preincubated with HDLs (1 mg/mL apoA-I) for varying lengths of time and then washed twice in PBS to remove the HDLs before being incubated for an additional 4 hours in fresh medium containing TNF-α (100 U/mL). Results, representing levels of VCAM-1 expression measured by flow cytometry, show the mean and SD of quadruplicates of one experiment and are representative of three such experiments.

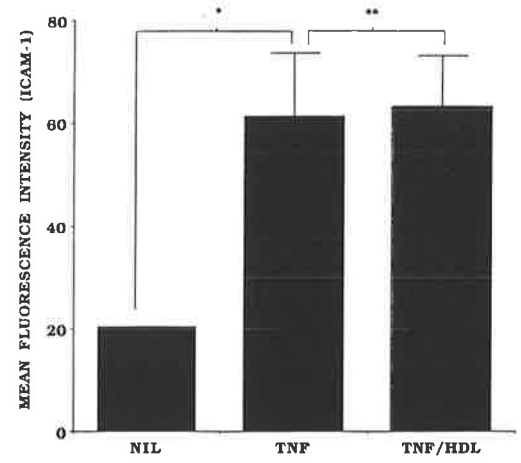

FIG 8. Bar graph showing the effect of HDLs on TNF-α–induced ICAM-1 expression on human foreskin fibroblasts. Confluent cultures of human foreskin fibroblasts were incubated for 4 hours either with nothing (NIL), TNF-α (100 U/mL) (TNF), or TNF-α and HDL (1 mg/mL apoA-I) (TNF/HDL), after which the cells were trypsinized and levels of ICAM-1 measured by flow cytometry ("Methods"). The data represent one experiment representative of three experiments and show means and SDs of triplicate samples. Differences between means were evaluated using an unpaired *t* test. *$P<.005$, **P=NS. NS indicates not significant.

protein, egg-derived phosphatidylcholine as their sole phospholipid, and unesterified cholesterol[30] were incubated with HUVEC monolayers before the addition of TNF-α. We found that these reconstituted HDLs inhibit the expression of VCAM-1 in a concentration-dependent manner comparable with that of plasma HDLs (Fig 9).

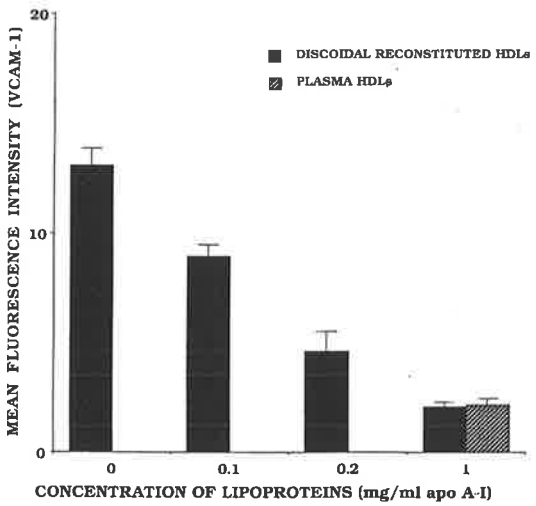

FIG 9. Bar graph showing the effect of reconstituted HDLs containing apoA-I on TNF-α–induced VCAM-1 expression. Confluent monolayers of HUVECs were incubated at 37 °C either with no added lipoproteins, with native HDLs (1 mg/mL apoA-I), or with reconstituted discoidal HDLs that contained apoA-I as their sole apolipoprotein (concentrations as shown). After 10 minutes, TNF-α was added (100 U/mL) to the culture medium, and the incubation was continued for an additional 4 hours. Expression of cell-surface VCAM-1 was then measured by flow cytometry. Means and SDs shown represent the cumulative data of three experiments, using three HUVEC donors and the same batch of plasma HDLs and reconstituted HDLs.

Discussion

Our studies show an effect of HDLs on endothelial cells that is consistent with the proposition that HDLs have a direct inhibitory effect on one of the earliest events in atherogenesis. Their ability to inhibit cytokine-induced cell-surface expression of adhesion molecules raises the possibility that HDLs may inhibit atherogenesis at an early stage by preventing monocyte adhesion to the endothelium. The inhibitory effects of HDLs may also influence the progression of atheromata by altering the expression of adhesion molecules shown to be important in trafficking T lymphocytes to these lesions.

HDLs have been shown to have other functions unrelated to their role in plasma cholesterol transport; these include acting as an antioxidant,[36] acting as a mitogen,[37] and having the ability to bind lipopolysaccharide.[38] A mitogenic effect of HDLs on endothelial cells may protect against vascular disease by maintaining an intact endothelium. Finally, the binding of lipopolysaccharide to HDLs protects against septic shock by rendering the toxin inaccessible to its receptor (CD14)[39] on the monocyte/macrophage, thus preventing the subsequent release of cytokines, such as TNF-α, IL-1, and IL-6.[40] Our current findings that physiological concentrations of HDLs are able to significantly inhibit the cytokine activation of adhesion molecules on the endothelial cell suggest that HDLs may also contribute to the protective effects of an acute inflammatory response.

Experiments were conducted to exclude the possibility that the effects of HDLs on the adhesion protein expression had a trivial explanation. The possibility of artifact as a result of HDLs inhibiting the detection of cell-surface proteins was excluded because we found no interference of detection in the flow cytometry assay. Our results also show that it is unlikely that HDLs are preventing access to the cytokine receptor or are affecting the level of receptor expression on the endothelial cell. We demonstrate no difference in the ability to detect TNF-receptor subunit levels on endothelial cells after 20 hours of incubation in HDLs. It is also unlikely that HDLs are restricting availability of the cytokine by binding it, as we show that removal of HDLs after a 2-hour preincubation followed by TNF-α stimulation resulted in maximal inhibition of VCAM-1 expression. Provided endothelial cells had undergone a 2-hour incubation with HDLs before cytokine stimulation, HDLs were not required during the cytokine stimulation to achieve maximal inhibition. The possibility that the inhibition of adhesion molecule expression may be due to cytotoxic effects was excluded by the observation that cell viability, as determined by the standard dye exclusion method and plating efficiency of the cultures, remained >95% after a 20-hour incubation in the presence of HDLs (1 mg/mL apoA-I). In addition, confluent cultures maintained a healthy "cobblestone" morphology in the presence of HDLs (data not shown), further suggesting that the general metabolic state of the cell cultures was not compromised. Inhibition was not donor specific and is highly reproducible, as evidenced by the fact that all the experiments reported have been completed on more than 20 donors of HUVECs with different batches of HDLs.

In addition, all the above experiments with TNF-α have been performed and identical results obtained with

the spontaneously transformed HUVEC line C11STH,[41] demonstrating that this cell line is an appropriate model for future studies. In contrast to these results with endothelial cells, the cytokine induction of ICAM-1 on fibroblasts was unaffected by the presence of HDLs, suggesting that the inhibitory effect of HDLs is restricted to certain cell types.

We show that the addition of HDLs to endothelial cell cultures at the same time or 5 minutes after the addition of TNF-α can inhibit cytokine-induced expression of adhesion molecules. However, when HDLs were added to the cultures 1 hour after TNF-α, they were no longer able to inhibit the cytokine-induced VCAM-1 expression, suggesting that HDLs are operating to inhibit an early cell-signaling response to the cytokine. TNF-α has many overlapping actions with IL-1, which has led to a suggestion of shared signal transduction mechanisms. We show a qualitative similarity in the ability of HDLs to suppress IL-1–induced or TNF-α–induced adhesion molecule expression, indicating that the mechanism of action is not receptor specific but may be through a common signaling pathway.

It is noteworthy that HDLs inhibit the expression of all the adhesion proteins shown to support monocyte adhesion. Although there are other cytokines that also inhibit adhesion protein expression, these operate only on some proteins. Transforming growth factor-β, for example, suppresses E-selectin but not VCAM-1 expression,[42] and IL-4 increases VCAM-1 while decreasing E-selectin.[43] Thus, HDLs appear unique in the breadth of their actions. Demonstration that the selectins and the immunoglobulin-like class of adhesion molecules may synergize to enable the recruitment and adhesion of leukocytes into the plaques is important in understanding the development of the atheroma.[20,21] We demonstrate that HDLs are able to reduce the protein and mRNA levels of both classes of molecules; in this capacity they may be able to prevent the progression of existing atherosclerotic lesions. This suggestion is consistent with studies in which regression of atherosclerotic lesions occurs when animals are infused with HDLs.[44]

The demonstration that reconstituted HDLs that contain only apoA-I are able to mimic the effects of native HDL is very important because it suggests that the most protective apolipoprotein has a function ascribable to the whole plasma HDL. In addition, it makes it less likely that unidentified molecules copurified along with native HDL particles, rather than known components of HDL, are responsible for these effects. Clarification of the mechanism by which HDLs inhibit the expression of adhesion molecules and elucidation of the HDL subpopulations that are responsible may ultimately lead to the development of novel strategies for the prevention and treatment of atherosclerosis.

Acknowledgments

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Tumor necrosis factor-α induces adhesion molecule expression through the sphingosine kinase pathway

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ABSTRACT **The signaling pathways that couple tumor necrosis factor-α (TNFα) receptors to functional, especially inflammatory, responses have remained elusive. We report here that TNFα induces endothelial cell activation, as measured by the expression of adhesion protein E-selectin and vascular adhesion molecule-1, through the sphingosine kinase (SKase) signaling pathway. Treatment of human umbilical vein endothelial cells with TNFα resulted in a rapid SKase activation and sphingosine 1-phosphate (S1P) generation. S1P, but not ceramide or sphingosine, was a potent dose-dependent stimulator of adhesion protein expression. S1P was able to mimic the effect of TNFα on endothelial cells leading to extracellular signal-regulated kinases and NF-κB activation, whereas ceramide or sphingosine was not. Furthermore, *N,N*-dimethylsphingosine, an inhibitor of SKase, profoundly inhibited TNFα-induced extracellular signal-regulated kinases and NF-κB activation and adhesion protein expression. Thus we demonstrate that the SKase pathway through the generation of S1P is critically involved in mediating TNFα-induced endothelial cell activation.**

Tumor necrosis factor-α (TNFα) was originally described for its antitumor activity, but is now recognized to be one of the most pleiotropic cytokines in mediating systemic inflammatory and immune responses (1, 2). A major site for these TNFα actions is the vascular endothelium, where TNFα triggers endothelial cells to secrete various cytokines and induces or enhances the expression of adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 and E-selectin (3). The regulated expression of these adhesion molecules is essential for the recruitment of circulating blood cells to the endothelium during the inflammatory and immune responses (3–5).

TNFα activity is exerted through binding two distinct membrane receptors, p55 (TNFα-R1) and p75 (TNFα-R2). Engagement of the TNFα receptors results in recruitment of two distinct classes of receptor-associated proteins, one the TRADD, FADD/MORT1 and RIP family, and the other, the TRAF family (6–8). Both of these appear to couple TNFα receptors to downstream signaling cascades such as cysteine proteases and NF-κB activation to regulate cell proliferation, differentiation, and programmed cell death (6). Recently, the lipid second messenger, ceramide, has also received attention in TNFα signaling (6, 9). TNFα stimulates the activation of sphingomyelinase, yielding ceramide that, in turn, can induce apoptosis and may play a role in apoptotic signaling in various cell types (6, 9). In addition, ceramide can be subsequently metabolized to sphingosine and sphingosine 1-phosphate (S1P), via ceramidase and sphingosine kinase (SKase) activation, respectively (10). These sphingomyelin metabolites were also proposed to play a variety of roles in regulation of cellular activities such as calcium mobilization, cell motility, and mitogenesis (9, 10). In this study, we demonstrate that TNFα promoted generation of ceramide that was unable to mediate the TNFα proinflammatory action in human umbilical vein endothelial cells (HUVEC). By contrast S1P was a potent inducer of adhesion molecule expression. We show that TNFα stimulated SKase activity and S1P generation in HUVEC, and blockage of SKase by its inhibitor markedly reduced the TNFα-dependent extracellular signal-regulated kinase (ERK) and NF-κB activation and adhesion molecule expression. Thus, we show that SKase activation is an important signaling event in the TNFα-induced endothelial activation, a major effect of TNFα *in vivo*.

EXPERIMENTAL PROCEDURES

Cell Culture and Flow Cytometry Analysis. HUVEC were isolated as described (11). The cells were cultured on gelatin-coated culture flasks in medium M199 with Earle's salts supplemented with 20% fetal calf serum, 25 μg/ml endothelial growth supplement (Collaborative Research), and 25 μg/ml heparin. The cells were used between passages 2 and 6 for all experiments. To measure the adhesion molecule expression, cells were plated in 24-well dishes and reached confluency. After the indicated treatment, cells were washed with medium M199 and incubated with primary monoclonal antibodies to VCAM-1, E-selectin, or an isotype-matched nonrelevant antibody for 30 min. These antibodies were generated in our laboratory and their characteristics were described previously (12). Cells were then incubated with fluorescein isothiocyanate-conjugated secondary antibody and fixed in 2.5% formaldehyde. The expression of cell-surface adhesion molecules was measured as fluorescence intensity by use of a Coulter Epics Profile XL flow cytometer.

Metabolic Labeling, Sphingolipids, and SKase Assay. To measure sphingomyelin and S1P levels, the HUVEC were labeled with [^{3}H]serine (5 μCi/ml; 1 Ci = 37 GBq) for 48 h. After TNFα stimulation for the indicated times, cellular lipids were extracted and resolved by TLC with two different solvent systems: (*i*) chloroform/methanol/acetic acid/water (50:30:8:5, vol/vol) and (*ii*) 1-butanol/acetic acid/water (3:1:1, vol/vol). Sphingolipid spots were visualized by fluorography, quantified by scintillation spectrometry, and normalized by radioactivity recovered in total cellular lipids.

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Abbreviations: DMS, *N,N*-dimethylsphingosine; ERK, extracellular signal-regulated kinase; HUVEC, human umbilical vein endothelial cells; SKase, sphingosine kinase; S1P, sphingosine 1-phosphate; TNFα, tumor necrosis factor-α; VCAM-1, vascular adhesion molecule-1; JNK, c-JUN N-terminal kinase.

†J.R.G. and M.A.V. contributed equally to this work.

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Cellular ceramide was extracted and quantified with the diacylglycerol kinase reaction (13). To exclude a possible error caused by some factors in the extracts affecting diacylglycerol kinase (14), synthetic C_2-ceramide was added in assays as an internal control. There were no changes in the phosphorylated C_2-ceramide in this assay system.

SKase activity was measured *in vitro* by incubating the cytosolic fraction with 10 μM sphingosine-BSA complex and [γ-^{32}P]ATP (1 mM, 0.5 mCi/ml) for 15 min at 37°C as described previously (15). For kinetic study, cell extract was prepared from the HUVEC treated with TNFα for 5 min. The kinase assay was performed with various concentrations of sphingosine (1.25, 2.5, 5, and 10 μM) in the absence or presence of *N,N*-dimethylsphingosine (DMS) at 5 or 10 μM, respectively. The K_m value (16.4 μM sphingosine) was obtained from a double-reciprocal plot. The formation of S1P *in vivo* was measured in the permeabilized cells as described previously (15).

ERK and c-JUN N-Terminal Kinase (JNK) Activity Assays. The cells were treated with the indicated agents for 30 min, ERK activities were then assayed with myelin basic protein (MBP) as substrate after immunoprecipitation with antibodies against p42/p44^{ERK} (Santa Cruz Biotechnology). The kinase reaction products were separated on SDS/10% PAGE. In parallel, an aliquot of the same cell lysates was blotted with anti- p42/p44^{ERK} antibodies to ensure equal ERK expression. JNK activity was measured by a solid-phase assay using glutathione *S*-transferase-jun-(1–79) fusion protein as substrate as described (16).

Electrophoretic Mobility-Shift Assay. Nuclear extracts were prepared from HUVEC treated for 30 min with vehicle or the indicated agents. The double-stranded oligonucleotides used as a probe in these experiments included 5′-GGATGCCATTGGGGATTTCCTCTTTACTGGATGT-3′, which contains a consensus NF-κB binding site in the E-selectin promoter that is underlined (17). Gel mobility shift of a consensus NF-κB oligonucleotide was performed by incubating a ^{32}P-labeled NF-κB probe with 4 μg of nuclear proteins. The specific DNA–protein complexes were abolished by addition of a 50-fold molar excess of unlabeled E-selectin NF-κB oligonucleotides. The specificity of NF-κB binding complex was further identified by supershift analyses. Anti-p50 and anti-p65 polyclonal antibodies (Santa Cruz Biotechnology) were added before addition of radiolabeled NF-κB probe.

RESULTS

TNFα Stimulates Sphingomyelin Turnover in HUVEC. As ceramide has been implicated in TNFα signaling (9), we examined the effect of TNFα on the hydrolysis of sphingomyelin to ceramide in HUVEC. TNFα stimulation of HUVEC rapidly reduced sphingomyelin content to 40% of control within 30 min, with return to near basal levels by 2 h (Fig. 1). In parallel, the cellular ceramide levels were rapidly increased (≈2-fold), peaking at 30 min after TNFα treatment (Fig. 1*B*). Our data indicate that TNFα induces sphingomyelin turnover and ceramide generation in HUVEC.

Ceramide Does Not Mediate TNFα-Induced Adhesion Molecule Expression. To clarify whether sphingomyelin turnover is involved in TNFα-induced adhesion molecule expression, we determined the effect of ceramide in HUVEC by using either a cell-permeant form of ceramide (C_2-ceramide) or sphingomyelinase, which generates endogenous ceramide. As shown in Fig. 2, C_2-ceramide was a poor stimulator of E-selectin and VCAM-1 expression, reaching levels that were <10% of that stimulated by TNFα. In parallel, the cells treated with sphingomyelinase or D-*erythro*-(*N*-myristoylamine)-1-phenyl-1-propanol, a ceramidase inhibitor (18) that induced endogenous ceramide accumulation by 3-fold (data not shown), failed to stimulate the expression of E-selectin or VCAM-1 (Fig. 2*B*). Thus, ceramide is unlikely to be a second messenger in mediating TNFα-induced adhesion protein expression.

S1P Is a Mediator in TNFα-Induced Adhesion Molecule Expression. Because not only ceramide but its metabolites, especially S1P, have been proposed to serve as modulators or signaling molecules in a variety of cellular activities, we next explored the role of S1P in endothelial cells. In contrast to ceramide, S1P profoundly stimulated cell-surface expression of E-selectin and VCAM-1 and their mRNA levels in HUVEC (Fig. 2). Data showed that S1P was a potent and dose-dependent inducer of E-selectin and VCAM-1, with an EC_{50} at ≈1 μM and reaching levels at 5 μM that were approximately

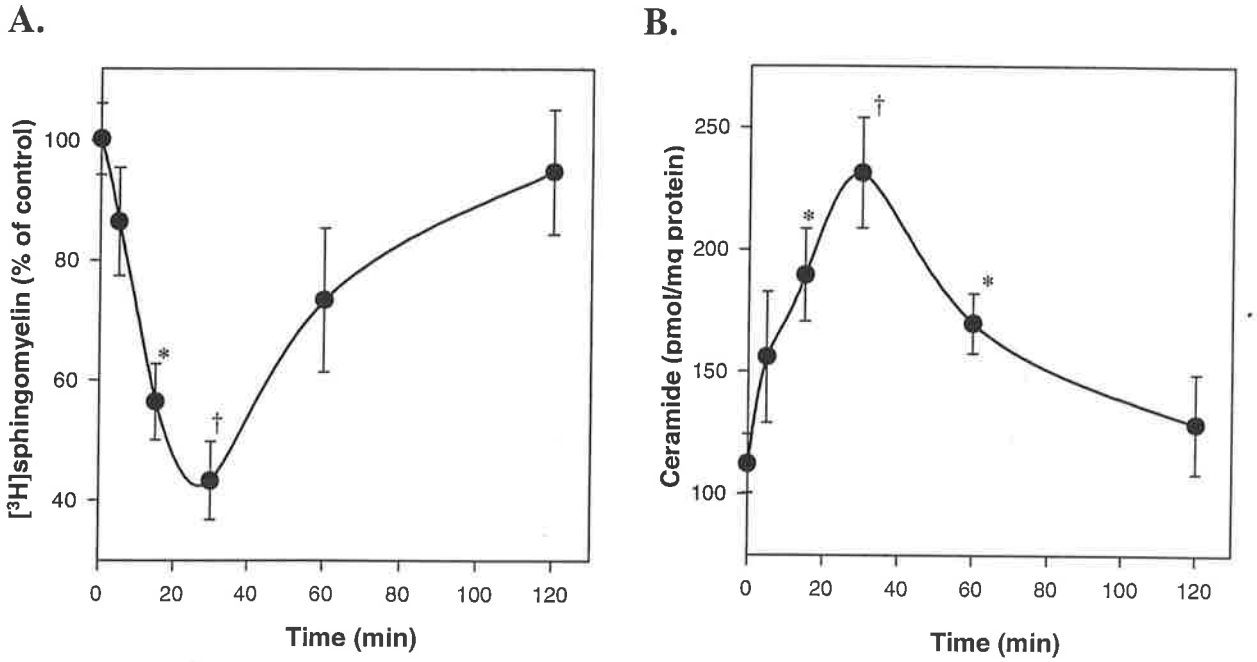

FIG. 1. TNFα-induced sphingomyelin turnover in HUVEC. (*A*) HUVEC were labeled with [^{3}H]serine for 48 h and treated with TNFα (100 units/ml), total cellular lipids were extracted, and [^{3}H]sphingomyelin was then resolved by TLC at the desired time point. (*B*) The unlabeled cells were treated with TNFα as indicated above, and cells were lysed to measure ceramide levels by using the diacylglycerol kinase assay. The results represent mean values ± SD from three independent experiments. *, $P < 0.01$; †, $P < 0.001$, vs. the basal levels.

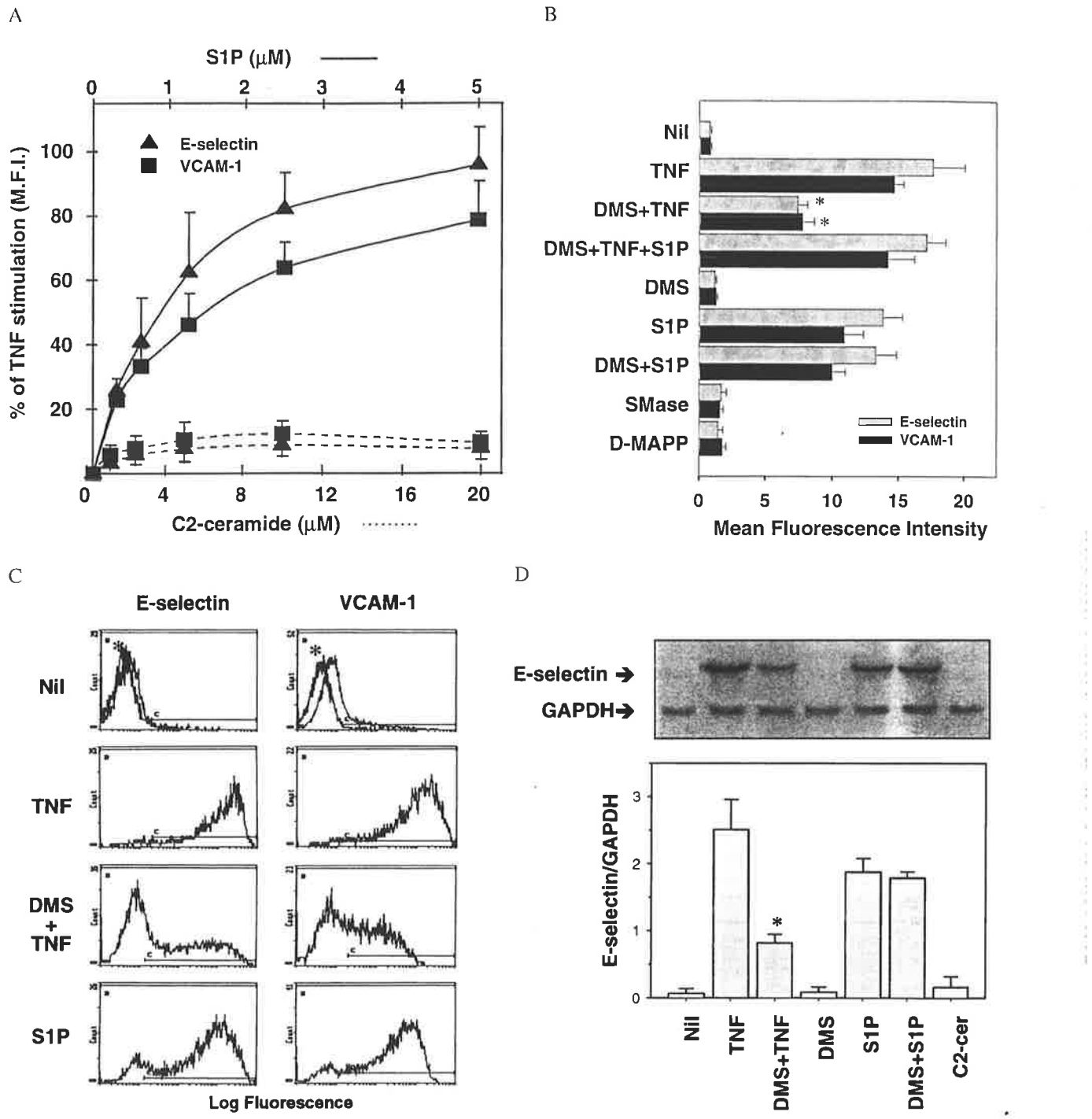

FIG. 2. Effects of C_2-ceramide and S1P on adhesion molecules expression. (*A*) HUVEC were treated with an increasing concentration of C_2-ceramide or S1P for 4 h. The cell-surface expression of E-selectin or VCAM-1 was measured by flow cytometry. The data are expressed as percent of TNFα (100 units/ml)-stimulation in the mean fluorescence intensity (M.F.I.). (*B*) The endothelial cells were treated with vehicle (Nil), S1P (5 μM), C_2-ceramide (10 μM), DMS (5 μM), sphingomyelinase (SMase, 1 unit/ml), D-*erythro*-(*N*-myristoylamine)-1-phenyl-1-propanol (D-MAPP, 5 μM), and/or TNFα (TNF, 100 units/ml), respectively, for 4 h, then the cell-surface E-selectin or VCAM-1 was measured. (*C*) Flow cytometry profiles showed the effect of S1P on expression of E-selectin (*Left*) and VCAM-1 (*Right*). Asterisks indicate a negative control profile with the isotype-matched nonrelevant antibody. (*D*) After the indicated treatment for 4 h, E-selectin mRNA levels were measured by Northern blot assay with α-^{32}P-labeled cDNA probes (12). Bar graph (*Bottom*) depicts relative levels of E-selectin mRNA quantified by the PhosphorImager and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Values in *A*, *B*, and *D* represent mean ± SD from at least three independent experiments. *, $P < 0.01$, compared with TNFα stimulation.

equivalent to 100 units/ml TNFα (Fig. 2 *A–C*). As a control, sphingosine had no effect on the expression of adhesion molecules (data not shown), suggesting a specific effect of S1P in HUVEC. To further examine the role of S1P in TNFα-mediated activation, the formation of intracellular S1P was inhibited by a competitive inhibitor of SKase, DMS. DMS markedly decreased the TNFα-induced adhesion protein expression and mRNA levels by 50–70% (Fig. 2 *B–D*), indicating that SKase activation is an important signaling event in the TNFα action. Under the same conditions, DMS had no cytotoxic effect on HUVEC measured by cell viability (MTT assay) and morphological analysis (data not shown). Con-

versely, the DMS-inhibited TNFα action was reversed by the addition of S1P, and DMS did not prevent S1P-induced adhesion protein expression (Fig. 2*B*), further suggesting the linkage between S1P and TNFα signaling in the endothelial cell activation.

TNFα Induces SKase Activation and S1P Generation in HUVEC. Given the potent stimulatory role of S1P for the signaling in TNFα-induced adhesion protein expression, we sought to determine the effect of TNFα on S1P generation. As S1P is generated from sphingosine by SKase (19), we first measured SKase activity both *in vitro* and *in vivo*. TNFα stimulation of HUVEC caused a rapid and transient increase in cytosolic SKase activity, reaching a maximum of 165% ± 13% ($P < 0.01$) of basal within 5 min (Fig. 3*A*). Kinetic study of the SKase indicated that it followed Michaelis–Menten kinetics. The K_m value of activation was 16.4 μM sphingosine, obtained from *in vitro* determination performed with various concentrations of sphingosine in the presence of an increasing concentration of DMS (Fig. 3*A*, *Inset*). Fig. 3*B* shows that TNFα induced a time-dependent increase in the formation of S1P *in vivo*, which was in parallel with the SKase activity *in vitro*. To confirm the effect of TNFα on SKase activation, the levels of S1P in intact cells measured from the [^{3}H]serine-labeled HUVEC clearly demonstrated the activation of SKase by TNFα stimulation (Fig. 3*B*, *Inset*). Addition of DMS at a concentration of 5 μM completely normalized the TNFα-induced increase in S1P levels, indicating a specificity of inhibition on SKase activity in HUVEC.

SKase Activation Is Involved in TNFα-Induced Activation of ERK and NF-κB. Members of the mitogen-activated protein kinase family are important mediators of signals transduced from the cell surface to intracellular responses and gene expression. The ERK, an archetypal member of this family, has been proposed to mediate endothelial activation by a variety of stimuli (20). We thus examined the role of SKase in ERK activation. Fig. 4*A* shows that both TNFα and S1P were approximately equipotent in stimulating ERK activities, whereas C_2-ceramide did not (data not shown). Treatment with DMS inhibited TNFα-activated ERK by 51% ± 14% ($P < 0.02$), showing a role for SKase in the TNFα-activated ERK signal cascade. Fig. 4*B* shows that S1P failed to activate JNK and DMS did not interrupt TNFα-induced JNK activation, suggesting that JNK may not be involved in SKase pathway.

Because the transcription factor NF-κB is essential for regulation of TNFα-induced adhesion protein gene transcription (21), the role of activated SKase pathway in activation of NF-κB was investigated. Electrophoretic mobility-shift assay showed that treatment of cells with DMS markedly inhibited the TNFα-induced activation of NF-κB by 62% ± 16% ($P < 0.01$, Fig. 5 *A* and *D*), but did not inhibit other transcription factors such as Oct-1 activation (Fig. 5*B*), indicating the specificity of SKase role in TNFα-promoted NF-κB activation. In confirming the effect of SKase activity in NF-κB activation, Fig. 5*C* shows that treatment of HUVEC with S1P induced a significant nuclear NF-κB accumulation. The composition of S1P-induced NF-κB specific protein–DNA complexes were identical to that induced by TNFα, which was revealed to be p50/p65 heterodimer by antibody supershift assay and by competition analyses. Taken together, these results indicate that the activation of SKase is necessary in human endothelial cells for TNFα-stimulated NF-κB activation, a critical component in TNFα-induced adhesion protein expression.

DISCUSSION

The expression of adhesion proteins on activated endothelial cells plays a major role in the recruitment of blood cells to the endothelium during the inflammatory responses (3–5). The nature of the inflammatory signals and associated molecular mechanisms that activate adhesion molecule expression in endothelial cells are unknown. Factors commonly found in inflammatory lesions, such as TNFα and interleukin 1, induce the expression of adhesion molecules in cultured endothelial cells. Thus, TNFα-stimulated adhesion molecule expression on HUVEC provided a useful model to investigate the signal pathway in the regulation of endothelial cell activation. Using

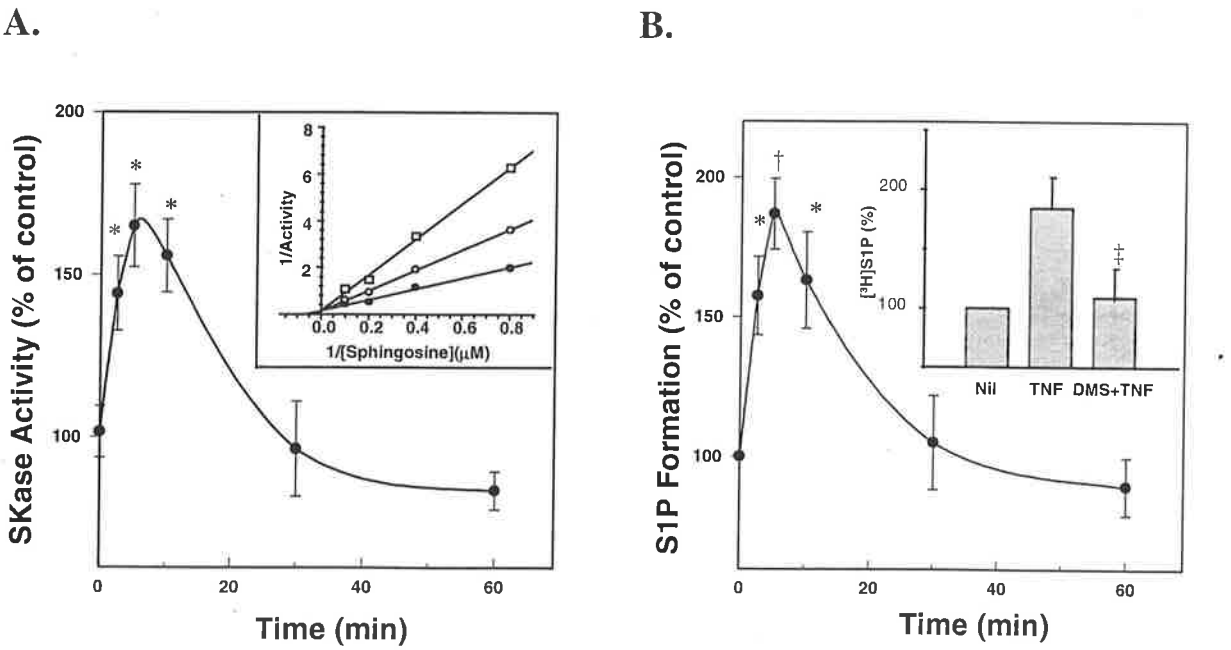

FIG. 3. TNFα-induced SKase activation. (*A*) HUVEC treated with TNFα (TNF, 100 units/ml) at the desired time points, the cytosolic fractions were extracted to measure SKase activity. (*Inset*) Kinetic study of SKase. The cell extract was prepared from the cells treated with 100 units/ml TNFα for 5 min. The kinase assay was performed with various concentrations of sphingosine in the absence (●) or presence of 5 μM (○) or 10 μM (□) DMS. (*B*) After treatment with TNFα as described above, the cells were permeabilized to measure the production of S1P *in vivo*. (*Inset*) S1P levels in intact cells measured by labeling with [^{3}H]serine. The data in *A* and *B* are mean values ± SD of three individual experiments. *, $P < 0.01$; †, $P < 0.001$, vs. the basal levels; ‡, $P < 0.001$, vs. TNFα stimulation.

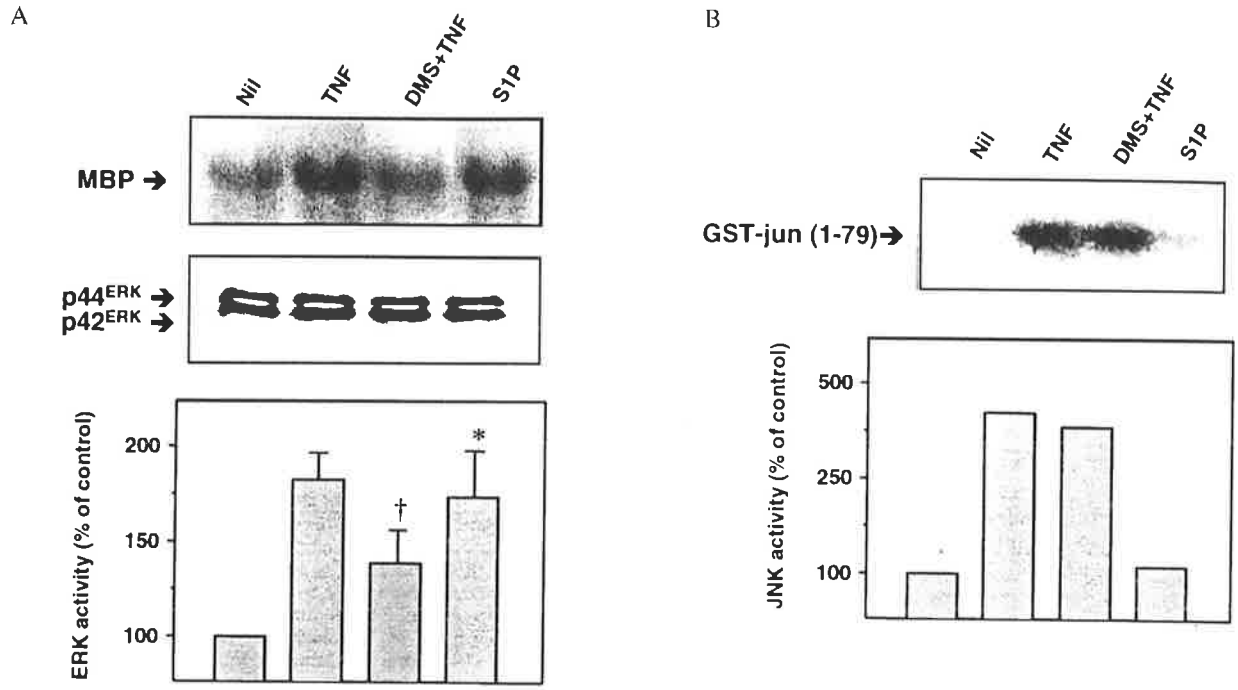

FIG. 4. Effect of SKase activity on ERK and JNK activation. (*A*) The cells were treated with the indicated agents for 30 min, and ERK activities were then assayed with myelin basic protein (MBP) as substrate after immunoprecipitation with antibodies against p42/p44^{ERK}. The kinase reaction products were separated on SDS/10% PAGE. In parallel, an aliquot of the same cell lysates was blotted with anti-p42/p44^{ERK} antibodies to ensure equal ERK expression. Bar graph (*Bottom*) depicts ERK activity quantified by the PhosphorImager. Values represent mean ± SD in percent of control from three independent experiments. *, $P < 0.01$, compared with control; †, $P < 0.02$, vs. TNFα (TNF) stimulation. (*B*) JNK activity was measured with glutathione *S*-transferase-jun-(1–79) as substrate after the treatment as described above. Bar graph (*Bottom*) shows JNK activity quantified by the radioactivity incorporated into glutathione *S*-transferase (GST)-jun-(1–79). Data represent mean of two separate experiments.

this experimental model, we demonstrated that SKase pathway is critically involved in TNFα proinflammatory action.

Ceramide has been suggested to couple the TNFα receptors to certain downstream events by activated sphingomyelinase (9, 22). However, the role of ceramide has been controversial. Indeed, we showed that whereas TNFα induced sphingomyelin turnover and ceramide generation in human endothelial cells (Fig. 1), ceramide failed to mimic the effect of TNFα on stimulation of adhesion molecule expression. This finding concurs with other observations that questioned the physiological significance of ceramide, with the evidence suggesting its effect unlikely to be relevant (9, 23, 24). In fact, we have shown that ceramide inhibits the expression of adhesion molecules by TNFα stimulation (unpublished results). Thus, it is unlikely that ceramide can serve as a second messenger to couple TNFα receptors and adhesion molecules expression in endothelial cells.

S1P is now shown to be a potent molecule in stimulating adhesion protein expression in human endothelial cells (Fig. 2). The finding that S1P up-regulated adhesion molecules not only in cell-surface expression but also in mRNA levels and activation of NF-κB, an essential transcription factor for these proteins, suggests a potential role of S1P linking signal transduction to gene expression. The strong signaling role of S1P has been reported in mediating a variety of cellular functions such as calcium mobilization (25, 26), cell motility, and proliferation (27–29). S1P is believed to serve both as an intracellular second messenger (10, 15) and as an extracellular ligand for the G-protein-coupled receptor Edg-1 (30, 31). These two conceptually different signaling roles have been claimed for this single lipid molecule in a cell type- and phenomenon-specific manner, making S1P a unique bioactive signal molecule (29). At this stage the site of S1P action in the induction of adhesion protein expression is not known. However, two sets of observations suggest that S1P acts at least in part intracellularly. (*i*) The effective concentrations of S1P were on the order of micromolar ($EC_{50} \approx 1$ μM), which is in contrast with the nanomolar amounts needed for effects of S1P through Edg-1 (30). Interestingly, it has been previously noted that the effective dose of S1P required for proliferation was micromolar, whereas the dose for cell motility regulation was nanomolar (29). (*ii*) Neither pertussis toxin (a G protein inhibitor) nor suramin (a nonspecific inhibitor of phospholipid receptors; ref. 32) caused a >30% of inhibition on the effects of S1P in the induction of adhesion molecules (data not shown).

In this report we demonstrated that S1P is a mediator in TNFα-induced endothelial cell activation. (*i*) TNFα induced a rapid and transient activation of SKase, with corresponding increases in S1P levels in HUVEC. (*ii*) The addition of S1P mimicked TNFα effect on the induction of adhesion proteins. (*iii*) The blockage of S1P production by DMS, a competitive inhibitor of SKase, significantly decreased TNFα-induced adhesion protein expression. In contrast, DMS did not inhibit the expression of adhesion molecules induced by S1P (Fig. 2*B*) or endotoxin (lipopolysaccharide, data not shown), indicating a specific inhibition on TNFα-dependent SKase activation. (*iv*) The inhibitory effect of DMS was reversed by addition of exogenous S1P. It is thus likely that SKase activation and S1P generation are involved in the TNFα-promoted endothelial cell activation.

In an attempt to define the intracellular targets through which S1P modulates TNFα signaling, we examined the activation of MEK/ERK and NF-κB. Previous studies have established that NF-κB is essential for regulating adhesion protein expression (21). Mitogen-activated protein kinases containing multiple subgroups such as ERK, JNK, and p38 kinases were also shown to be involved in endothelial cell gene expression in responses to TNFα stimulation (17, 20). Our preliminary data (not shown) show that the inhibition of ERK by a MEK-specific inhibitor (PD098059) significantly reduces TNFα-induced adhesion molecule expression, in agreement with previous reports showing the inhibition of TNFα-dependent gene expression by this inhibitor

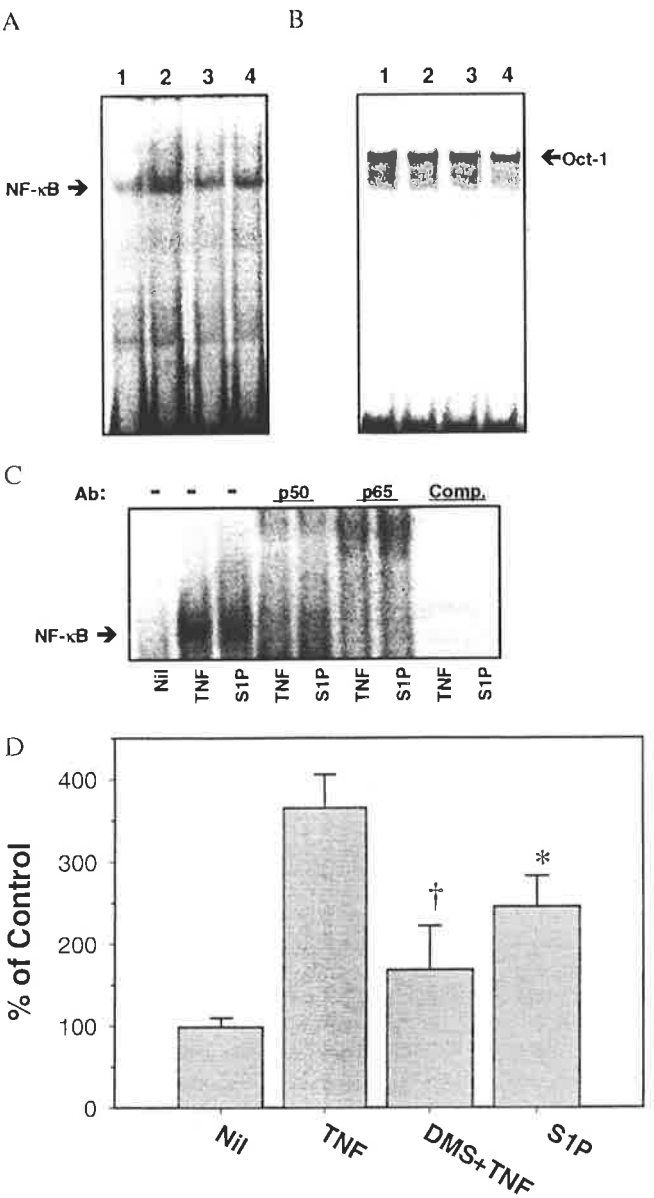

FIG. 5. Effect of SKase activity on NF-κB activation. NF-κB (*A*) and Oct-1 (*B*) binding activity were measured by electrophoretic mobility-shift assay after 30-min treatment with a vehicle (lane 1), TNFα (TNF, 100 units/ml, lane 2), DMS (5 μM) plus TNFα (lane 3), and S1P (5 μM, lane 4), respectively. (*C*) The specific NF-κB binding complexes were identified by the supershift gel assay with anti-p50 and anti-p65 antibodies and by competition analyses with the addition of a 50-fold molar excess of unlabeled NF-κB oligonucleotides. (*D*) Bar graph depicts relative binding activity of NF-κB quantified by the PhosphorImager from *A*. Values represent mean ± SD in percent of control from three independent experiments. *, $P < 0.01$, compared with control; †, $P < 0.01$, vs. TNFα stimulation.

(33, 34). We found that S1P mimicked the effect of TNFα in stimulating ERK but not JNK activation, and again DMS markedly reduced TNFα effect on ERK and NF-κB signaling cascades (Figs. 4 and 5). By contrast, neither ceramide nor sphingosine could activate these signals and induce adhesion molecule expression. Taken together, our data demonstrated an important role of SKase pathway in mediating TNFα stimulation on endothelial cells. It is noted that SKase activation may not be the only pathway to mediate endothelial cell activation by TNFα, as the blockage of TNFα effects by a saturating dose of DMS was not complete, and stimulation of ERK and NF-κB can certainly be achieved by other pathways (20, 21). Further understanding of the precise role of the SKase pathway in regulating endothelial cellular functions is expected, as this critical enzyme has just been cloned (35).

In conclusion, we have demonstrated that TNFα, a pleiotropic cytokine, induces activation of SKase and generation of S1P, which in turn may serve as a second messenger to mediate TNFα-induced endothelial cell activation and adhesion molecule expression. This could provide a mechanism for the regulation of endothelial activation during the systemic inflammatory and immune responses.

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An oncogenic role of sphingosine kinase

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Sphingosine kinase (SphK) is a highly conserved lipid kinase that phosphorylates sphingosine to form sphingosine-1-phosphate (S1P). S1P/SphK has been implicated as a signalling pathway to regulate diverse cellular functions [1–3], including cell growth, proliferation and survival [4–8]. We report that cells overexpressing SphK have increased enzymatic activity and acquire the transformed phenotype, as determined by focus formation, colony growth in soft agar and the ability to form tumours in NOD/SCID mice. This is the first demonstration that a wild-type lipid kinase gene acts as an oncogene. Using a chemical inhibitor of SphK, or an SphK mutant that inhibits enzyme activation, we found that SphK activity is involved in oncogenic H-Ras-mediated transformation, suggesting a novel signalling pathway for Ras activation. The findings not only point to a new signalling pathway in transformation but also to the potential of SphK inhibitors in cancer therapy.

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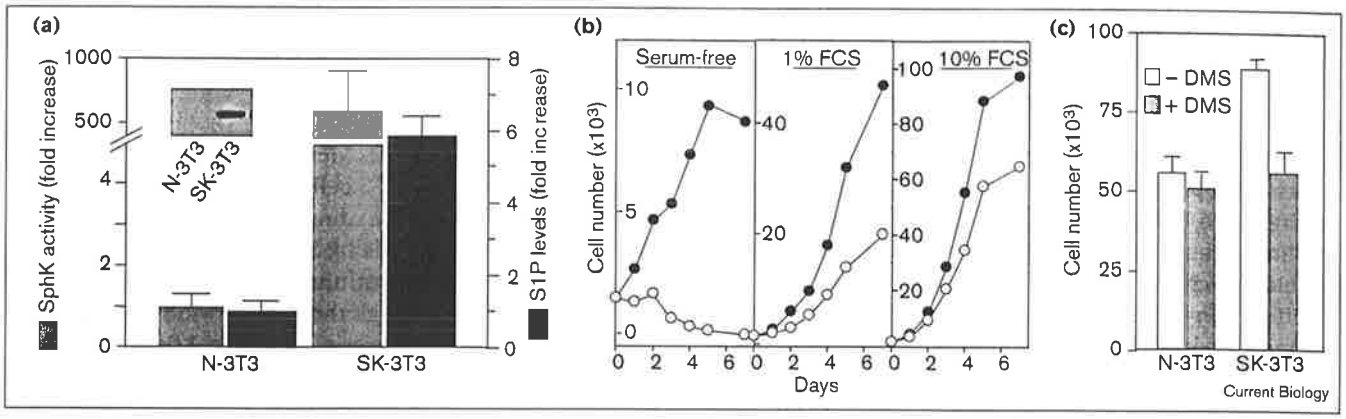
Results and discussion

To investigate the oncogenic role of SphK, non-transformed NIH3T3 fibroblasts were transfected with human SphK cDNA, which was recently cloned in our laboratory [9]. Pooled stable transfectants (referred to as SK-3T3) were used to avoid the phenotypic artefacts that may arise from the selection and propagation of individual clones from single transfected cells. In the SK-3T3 cell pools, SphK activity was increased by over 600-fold (Figure 1a) in comparison to NIH3T3 cells that were transfected with the empty vector (N-3T3). Immunoblot analysis showed a specific protein band with an apparent molecular weight consistent with the predicted size of FLAG epitope-tagged human SphK, which was detected only in the SK-3T3 pools but absent in N-3T3 cells (Figure 1a, inset). Intracellular levels of S1P, the direct product of SphK, were increased in SK-3T3 cells by 5.9 ± 0.9-fold, indicating that SphK was activated constitutively in the stable transfectants (Figure 1a). Although there was a wide range of SphK expression levels (increases of between 12-fold and ~800-fold) in different clones of transfected cells, intracellular levels of S1P were increased by only between 4-fold and ~8-fold. Thus, S1P levels did not correlate with the expression levels of SphK, assayed by *in vitro* enzyme activity. This may have been an influence of the availability and/or subcellular location of sphingosine, and rapid degradation of S1P by S1P phosphatase or S1P lyase. Despite notable increases in intracellular levels of S1P, there was no detectable S1P secretion into the extracellular media (data not shown), which is consistent with a previous report showing that overexpression of SphK in NIH3T3 or HEK293 cell lines were unable to secrete S1P [8].

The growth curves of SK-3T3 cells were significantly different from those of control cells (Figure 1b). Overexpression of SphK dramatically enhanced cell growth in media containing either 1% or 10% serum. Even in serum-free medium for up to 7 days, SK-3T3 cells survived and grew whereas the N-3T3 cells were dying. Furthermore, when the cells reached saturation density, SK-3T3 cells continued to proliferate (see Supplementary material), suggesting an escape from contact inhibition. Treatment of SK-3T3 cells with a specific inhibitor of SphK, *N,N*-dimethylsphingosine (DMS), significantly diminished the enhanced proliferation induced by overexpression of SphK, whereas DMS had no effect on proliferation of N-3T3 cells (Figure 1c). These results suggest that the constitutive activation of SphK in cells stably transfected with SphK reduces two key growth-limiting properties: serum dependence and contact inhibition.

The transforming activity assayed by focus formation in NIH3T3 cells showed that cells transfected with SphK but not empty vector induced numerous foci (Table 1 and Figure 2a). Both SphK and control vectors displayed similar efficiency in the generation of G418-resistant colonies (data not shown), indicating that the transforming activity was not a non-specific effect of transfection. Furthermore, SK-3T3 cells formed vigorous colonies in soft agar (Table 1 and Figure 2b), revealing the acquisition of anchorage-independent growth. Although N-3T3 cells exhibited a low background level of colony formation, which may be due to spontaneous transformation,

Figure 1



Overexpression of SphK stimulates proliferation in NIH3T3 cells. **(a)** Cytosolic SphK activity (light grey bars) and intracellular S1P levels (dark grey bars) were measured in the cells stably transfected with a plasmid expressing FLAG epitope-tagged SphK (SK-3T3) or empty vector alone (N-3T3) as described previously [9]. SphK activity was measured by incubating the cytosolic fraction with 5 μM sphingosine dissolved in 0.1% triton X-100 and [γ-^{32}P]ATP (1 mM, 0.5 mCi/ml) for 15 min at 37°C as described previously [16]. For assays of intracellular S1P levels, cells were labelled with [^{3}H]sphingosine (1 μM, 2 μCi/ml) for 30 min, and radioactivity incorporated into cellular lipids was extracted; [^{3}H]S1P was then resolved by thin-layer chromatography (TLC) with 1-butanol/methanol/acetic acid/water (8:2:1:2, volume/volume), visualised and quantified as described previously [8]. The inset shows expression of FLAG-tagged SphK in transfected cells by an immunoblot assay probed with anti-FLAG monoclonal antibodies (M2, Kodak). **(b)** Stably transfected SK-3T3 (shaded circles) or N-3T3 cells (unshaded circles) were plated in 48-well plates (1,000 cells per well) in DMEM containing 10% FCS. After 8 h, the cells were washed twice with DMEM and then grown in DMEM containing 1% or 10% FCS, or serum-free medium (DMEM containing 0.1% BSA). At the indicated times, cells were counted in a haemocytometer or by a thiazolyl blue (MTT) assay as described previously [7]. **(c)** Equal numbers of N-3T3 and SK-3T3 cells were plated. After a 5 day incubation in the presence or absence of DMS (2.5 μM), cell number was counted. Medium was replaced every day. Values are the mean ± SD from more than three experiments.

overexpression of SphK resulted in a 20–50-fold increase in the number of colonies and an obvious increase in colony size. Two randomly selected clones of SphK-transfected cell lines, KT-2 and KT-5, showed a similar transforming capacity although KT-2 had a 50-fold higher expression level than KT-5 (see Supplementary material). Importantly, DMS inhibited the transforming capacity of SphK in a dose-dependent manner, with 2.5 μM DMS resulting in total reversion to the normal phenotype (Figure 2b), suggesting that the activity of the enzyme is essential for the transforming process. Moreover, overexpression of a mutant SphK, SphK^{G82D}, which lacks enzymatic activity [10] failed to induce transformation (Figure 2a). Taken together, these results suggest that it is the activation of SphK, which results in increased S1P levels, rather than the overexpression itself that is responsible for the transforming capacity of this enzyme.

In addition to the intracellular function of S1P, the signalling role of SphK is also mediated by the binding of S1P to membrane G-protein-coupled receptors (Edg family), mainly in a pertussis-toxin-sensitive manner [1,2].

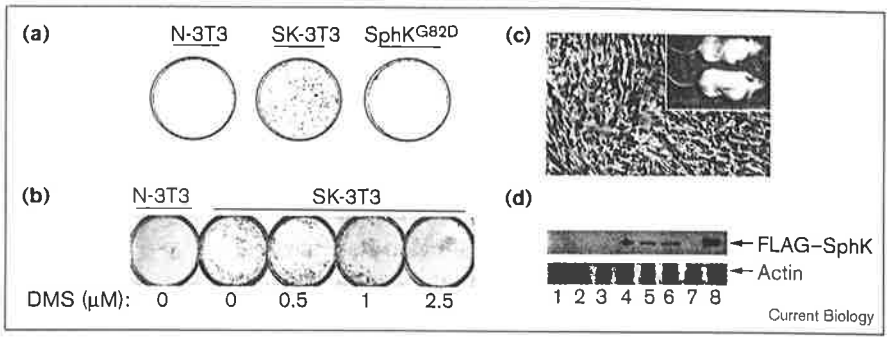
Table 1

Transforming activities and tumorigenicity in transfected NIH3T3 cells.

	Foci		Colonies in soft agar		Tumorigenicity
Cell line	– PTX	+ PTX	Number	Size (mm)	(tumours per injection)
N-3T3	1.8 ± 1.5	0.8 ± 0.6	3.3 ± 2.1	< 0.1	0/3
SK-3T3	49 ± 7.5	41.2 ± 13.2	122.3 ± 17.6	0.1–0.45	9/9

For the focus-formation assay, low-passage NIH3T3 cells were transfected with SphK (SK-3T3) or empty vector (N-3T3); 2 days later, the transfected cells were seeded to six-well plates. After reaching confluence, the cells were cultured for 3 weeks in DMEM containing 5% FCS in the presence or absence of pertussis toxin (PTX, 50 ng/ml). The foci were scored after staining with 0.5% crystal violet. For the soft-agar assay, suspensions of 1×10^4 cells from the stably transfected SK-3T3 or N-3T3 cells in a growth medium containing 0.33% agar were overlaid onto 0.6% agar gel. After 21 days incubation, colonies were stained with 0.1 mg/ml MTT and scored. Results shown are the mean ± SD from 3–5 experiments done in duplicate or triplicate. For the tumourigenesis assay, 4–6-week-old NOD/SCID mice were injected subcutaneously with 5×10^5 cells from various lines (see Supplementary material), in 200 μl sterile PBS. Animals were examined twice per week and tumours were measured after 4 weeks.

Figure 2



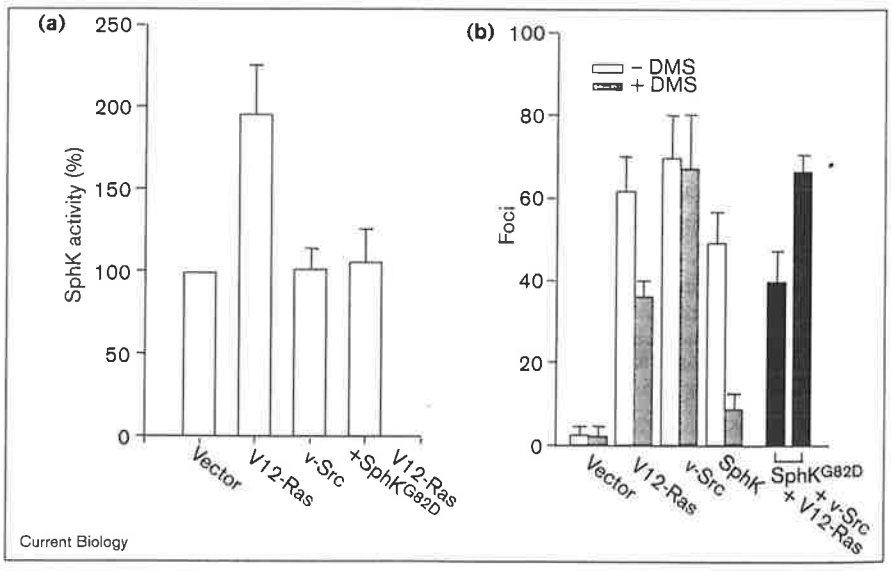
Cells overexpressing SphK are transforming and tumourigenic. **(a)** Focus-formation assay in NIH3T3 cells transfected with SphK, SphK^{G82D}, or vector alone as described in Table 1. **(b)** N-3T3 and SK-3T3 cells were cultured on soft agar and fed with medium containing various concentrations of DMS every 2 days. Colonies were stained with MTT and photographed after 3 weeks of incubation. **(c)** Morphology of a paraffin-fixed tumour section stained with hematoxylin and eosin (100 × magnification) and a photograph of tumours (inset) in NOD/SCID mice injected with SK-3T3 cells. **(d)** Whole cell extracts from three individual tumours (lanes 4–6) and respective peripheral tissues (lanes 1–3) and N-3T3 (lane 7) or SK-3T3 cells (lane 8) were analysed by western blotting. The upper blot was probed with anti-FLAG and the bottom with anti-actin antibodies.

Indeed, some of the biological responses to S1P have been reported to be inhibited by pertussis toxin [5,11,12]. However, the presence of pertussis toxin (50 ng/ml) had no effect on the transforming activity of SphK (Table 1), which is consistent with the previous report that pertussis toxin does not suppress cell growth in SphK-transfected fibroblasts [8]. Furthermore, the inhibitory effect of DMS on the transformation, along with the observation that SK-3T3 cells were unable to secrete S1P into media, do not support an extracellular role for S1P in mediating cell transformation induced by overexpression of SphK.

When SphK-transfected NIH3T3 cells from either stable transfectant pools or selected clones were injected subcutaneously into NOD/SCID mice, tumours became apparent at the site of injection within 3–4 weeks (Table 1 and Figure 2c). No mice injected with the control cells induced tumours during 10 weeks of observation. The histological appearance of tumour sections displayed the morphology of fibrosarcoma with many mitotic figures (Figure 2c). Western blot analysis of extracts derived from tumours showed high levels of FLAG-tagged protein (Figure 2d), revealing that the neoplastic cells retain and express the SphK transgenes. Thus, the tumours developed from the injected SphK-transfected cells but not from spontaneously transformed NIH3T3 cells.

Given the ability of SphK to transform cells, the potential role of SphK in the well-documented oncogene-induced transformation was explored. When NIH3T3 cells were transfected with an activated mutant H-Ras (V12-Ras), SphK activity was significantly increased to 198 ± 32% in comparison with the parental cells (Figure 3a). In contrast, cells transfected with v-Src (Figure 3a) or dominant-negative

Figure 3



SphK activation is involved in Ras transformation. **(a)** NIH3T3 cells were transfected with the indicated vectors and SphK activity was measured 48 h after transfection. **(b)** Focus-formation assays were performed in the presence or absence of DMS (2.5 μM), or in cells cotransfected with V12-Ras plus SphK^{G82D}, and v-Src plus SphK^{G82D}. Values are the mean ± SD from more than three experiments.

Ras (N17-Ras, data not shown) had no changes in SphK activity, suggesting a specific involvement of SphK in the Ras-activated pathway. Moreover, when the cells were treated with DMS, the focus formation was reduced by $41 \pm 4\%$ ($p < 0.0001$) in V12-Ras-transfected cells, but not in v-Src-transfected cells (Figure 3b), indicating that Ras transformation requires SphK activation. The inability of DMS to inhibit v-Src transformation rules out non-specific effects or a general toxicity resulting from the inhibition of SphK. To further confirm the role of SphK in Ras transformation, we used $SphK^{G82D}$, which is a dominant-negative mutant that not only lacks kinase activity but also blocks agonist-stimulated activation of endogenous SphK [10]. When V12-Ras was cotransfected with $SphK^{G82D}$, the Ras-induced SphK activation was completely inhibited (Figure 3a) and the transforming ability of Ras was also significantly reduced (Figure 3b, $p < 0.0001$). Again v-Src-induced transformation was not inhibited by $SphK^{G82D}$ (Figure 3b). Thus, SphK not only possesses transforming potential of its own right, as demonstrated by overexpression, but is also involved in Ras-mediated transformation.

The events downstream from activation of SphK that promote transformation are unclear but could include protection from apoptosis and acceleration of cell-cycle progression. Previous work has shown that increased intracellular S1P by overexpression of SphK expedites the G_1–S transition and promotes cell growth in low-serum media [8]. Agonist-stimulated endogenous SphK activation protects against cell death [5–7]. Inhibition of DEVDase activity and caspase-3 activation by SphK is likely to relate to its anti-apoptotic effect [5,6]. The effect of SphK on cell survival and growth is evolutionarily conserved [13,14], indicating the importance of SphK in regulating cell growth from yeast to mammals.

The oncogenic effect of SphK and its involvement in Ras transformation indicate a novel signalling pathway in cellular transformation and provide a potential link between sphingolipids and mammalian tumour pathogenesis. Interestingly, there is a recent report that S1P levels are significantly increased in the ascitic fluids of patients with ovarian cancer [15]. The importance of constitutive SphK activation in transformation also raises the possibility of using SphK inhibitors in cancer therapy.

Supplementary material

Supplementary material including growth of transfected cells at saturation density, transforming activity of SphK-transfected clones and a table showing tumorigenesis of NOD/SCID mice after injection of transfected cells is available at http://current-biology.com/supmat/supmatin.htm.

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Activation of Sphingosine Kinase by Tumor Necrosis Factor-α Inhibits Apoptosis in Human Endothelial Cells*

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Human umbilical vein endothelial cells (HUVEC), like most normal cells, are resistant to tumor necrosis factor-α (TNF)-induced apoptosis in spite of TNF activating sphingomyelinase and generating ceramide, a known inducer of apoptosis. Here we report that TNF activates another key enzyme, sphingosine kinase (SphK), in the sphingomyelin metabolic pathway resulting in production of sphingosine-1-phosphate (S1P) and that S1P is a potent antagonist of TNF-mediated apoptosis. The TNF-induced SphK activation is independent of sphingomyelinase and ceramidase activities, suggesting that TNF affects this enzyme directly other than through a mass effect on sphingomyelin degradation. In contrast to normal HUVEC, in a spontaneously transformed endothelial cell line (C11) TNF stimulation failed to activate SphK and induced apoptosis as characterized by morphological and biochemical criteria. Addition of exogenous S1P or increasing endogenous S1P by phorbol ester markedly protected C11 cell line from TNF-induced apoptosis. Conversely, *N,N*-dimethylsphingosine, an inhibitor of SphK, profoundly sensitized normal HUVEC to killing by TNF. Thus, we demonstrate that the activation of SphK by TNF is an important signaling for protection from the apoptotic effect of TNF in endothelial cells.

Tumor necrosis factor-α (TNF),[1] originally defined by its tumoricidal activity, is a pleiotropic cytokine that has strikingly different biologic effects in different cell types (1, 2). Although TNF elicits a cytocidal effect on numerous tumor cells or virally infected cells, most normal cells are resistant (3, 4). For example, human endothelial cells that play a crucial role in maintaining normal vasculature and modulating the inflammatory response are not directly killed by TNF (4, 5). However, endothelial cells as well as other cells can be rendered sensitive to TNF in the presence of protein or RNA synthesis inhibitors such as cycloheximide or actinomycin D (3, 4). Conversely, it has been demonstrated that sensitive cells can be made resistant to TNF challenge by prior sublethal exposure to TNF (6). These findings have led to the hypothesis that TNF-inducible signals confer a protective effect from the cytocidal activity of TNF in normal cells.

A major advance in understanding TNF signaling was the identification of protein molecules that are recruited to TNF receptor-1 (p55) and receptor-2 (p75) following ligand-induced trimerization (7–13). Engagement of the TNF receptor results in recruitment of a complex of proteins to the cell membrane including TRADD, FADD/MORT1, and RIP, which may lead to the further recruitment and activation of various caspases and, subsequently, to cell death (9, 10). On the other hand, TNF induces the interaction of its receptor with a second class of adaptor protein TRAFs and recruits downstream signals such as NF-κB-inducing kinase to activate NF-κB, which protects many different cell types from death (10–13). Additional signaling molecules, sphingolipids, have recently emerged as regulators of cell growth, differentiation, diverse cell phenotypes, and cell death (14–20). Signaling via sphingolipid turnover is exemplified by two distinct pathways: the formation of ceramide resulting from the activation of sphingomyelinase by TNF and a variety of other stimuli (14–16) and the formation of sphingosine-1-phosphate (S1P) upon sphingosine kinase (SphK) activation by several growth factors such as platelet-derived growth factor and phorbol ester (17–19). Thus, it has been proposed that cells activate sphingomyelinase in response to cytokines, whereas growth factors activate SphK and thereby choose between the formation of ceramide that favors cell death *versus* S1P that inhibits death (19). However, how the activities of these key enzymes are controlled in response to various stimuli is still incompletely understood.

In this study we report that in human endothelial cells TNF itself can activate SphK independent of sphingomyelinase activation to generate S1P that acts as a protective factor against the cytocidal effect of TNF. In addition, we identify a transformed endothelial cell line (C11) that undergoes apoptosis in response to TNF stimulation and that exhibits a specific defect in SphK activation. Thus, our data demonstrate that in human endothelial cells TNF simultaneously and independently activates two antagonistic biochemical signaling pathways, sphingomyelinase and SphK pathways, the balance of which could regulate the fate of cell in response to TNF stimulation.

EXPERIMENTAL PROCEDURES

Materials—TNF was purchased from R & D Systems Inc. (Minneapolis, MN). C2-ceramide, S1P, sphingosine, *N,N*-dimethylsphingosine (DMS), and dihydrosphingosine were from Biomol (Plymouth Meeting, PA). [^{3}H]Serine and [choline-methyl-^{14}C]sphingomyelin were from NEN Life Science Products. γ-[^{32}P]ATP was purchased from Bresatec (Adelaide, Australia). *Escherichia coli* diacylglycerol kinase was from Calbiochem (La Jolla, CA). Anti-CPP32 antibody was purchased from Transduction Laboratories. Other chemicals were from Sigma.

Cell Culture—HUVEC were isolated as described previously (21).

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[1] The abbreviations used are: TNF, tumor necrosis factor-α; HUVEC, human umbilical vein endothelial cell(s); SphK, sphingosine kinase; S1P, sphingosine-1-phosphate; TNF, tumor necrosis factor-α; DMS, *N,N*-dimethylsphingosine; PBS, phosphate-buffered saline; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; Bicine, *N,N*-bis(2-hydroxyethyl)glycine; PMA, phorbol 12-myristate 13-acetate; NOE, *N*-oleoylethanolamine; MAPP, (1*S*,2*R*)-D-*erythro*-2-(*N*-myristoylamino)-1-phenyl-1-propanol; NF-κB, nuclear factor κB.

The C11 cell line was generated in our laboratory and characterized as previously reported (22). All cells were cultured on gelatin-coated culture flasks in medium M199 with Earle's salts supplemented with 20% fetal calf serum, endothelial growth supplement (Collaborative Research), and heparin. Niemann-Pick type A skin fibroblasts and normal skin fibroblasts were kindly provided by Dr. J. Hopwood (Women's and Children's Hospital, Adelaide, Australia) and cultured in medium M199 with Earle's salts supplemented with 10% fetal calf serum.

Apoptosis Assays—Oligonucleosomal banding was demonstrated by harvesting total cellular DNA. After the indicated treatment both adherent and detached cells were harvested, washed with phosphate-buffered saline (PBS), and lysed in 50 mM Tris, pH 7.5, 10 mM EDTA, 0.5% Triton X-100, and 0.5 mg/ml proteinase K for 16 h at 50 °C. Samples were then extracted with phenol/chloroform/isoamyl alcohol and precipitated with ethanol. The pellet was resuspended in Tris/EDTA and 10 μg/ml RNase A, and the DNA was separated by electrophoresis on a 1.8% agarose gel stained with ethidium bromide.

Apoptosis was also assessed by *in situ* staining cells based on terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) using a cell death detection kit (Roche Molecular Biochemicals). The labeling procedure was performed in Lab-Tek 8-well chamber slides following the supplier's instructions. After staining, cells were analyzed under fluorescence microscopy (Olympus BH2).

Cell Viability Assay—Cell viability was measured using MTT dye reduction assay. Cells were seeded on 48-well plates at a density of 30,000 cell/well for 24 h, and the cells reached confluence. After various treatments, cells were incubated with 1 mg/ml MTT for 4 h. The medium was then aspirated, and the formazan product was solubilized with 10% SDS in 10 mM HCl. Cell viability was assessed by spectrophotometry at 570 and 650 nm absorbance in a 96-well enzyme-linked immunosorbent assay reader plate.

Metabolic Labeling, Extraction, and Analysis of Cellular Sphingolipids—For sphingolipids labeling, the cells were incubated with regular growth medium containing [^{3}H]serine (10 μCi/ml) in the presence of 0.5 mM 4-deoxypyridoxine and 0.1 mM L-canaline. After 48 h of incubation, the radioactive medium was removed, and cells were incubated for another 4 h in culture medium. Cellular lipids were extracted and resolved by TLC with two different solvent systems as described previously (20). Radioactive sphingolipid spots, identified by comparison with concomitantly run standards, were scraped and quantitated by scintillation spectrometry. The levels of sphingolipids were normalized by radioactivity recovered in total cellular lipids.

Ceramide Measurement—After treatment for the indicated times, cells were harvested, and lipids were extracted with chloroform/methanol/hydrochloric acid (1 N) (100:100:1) and buffered saline solution containing 15 mM EDTA. Cellular ceramide was quantified with the diacylglycerol kinase reaction as described previously (14). Briefly, the lipids in the organic phase extract were dried and resuspended into sample buffer containing 7.5% *n*-octyl-β-D-glucopyranoside, 5 mM Cardiolipin, and 1 mM DTPA. The samples were reacted with diacylglycerol kinase and [γ-^{32}P]ATP in enzyme buffer containing 20 mM Tris/HCl, pH 7.4, 10 mM dithiothreitol, and 15% glycerol. After 30 min at 22 °C, the reaction was stopped by extraction of lipids. The product of the phosphorylation reaction, ceramide-1-phosphate was resolved by TLC using $CHCl_3/CH_3OH/HAc$ (65:15:5) as solvent, detected, and quantified by the Phosphoimage system (Molecular Dynamics). To exclude a possible error caused by some factors in the extracts affecting diacylglycerol kinase (23), synthetic C_2-ceramide was added in assays as an internal control. There were no changes in the phosphorylated C_2-ceramide in this assay system.

Measurement of SphK Activity—As described previously (18, 20), cells were washed with ice-cold PBS and homogenized in lysis buffer (100 mM phosphate buffer, pH 7.2, 10 mM $MgCl_2$, 20% glycerol, 1 mM dithiothreitol, 1 mM EDTA, 20 μM $ZnCl_2$, 1 mM Na_3VO_4, 15 mM NaF, 10 μg/ml leupeptin and aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 0.5 mM 4-deoxypyridoxine). After ultracentrifugation at 100, 000 × *g* for 30 min, SphK activity was measured in the supernatant by incubation with 20 μM sphingosine-bovine serum albumin complex and [γ-^{32}P]ATP for 20 min at 37 °C. The labeled lipids were extracted and resolved two times by TLC in the solvent of $CHCl_3/CH_3OH/NH_4OH$ (65:35:8) and $CHCl_3/CH_3OH/HAc$ (9:1:1), respectively. The formation of S1P was visualized and quantified by the Phosphoimage system. For assay of SphK *in vivo*, the formation of S1P was measured in the permeabilized cells as described previously (18).

Sphingomyelinase Activity Assay—The sphingomyelinase assay was performed as described previously by Wiegmann *et al.* (24). Cells were stimulated with or without TNF for various times and stopped by removing medium and freezing in liquid nitrogen. Cells were then scraped in ice-cold PBS. To measure acidic sphingomyelinase activity, cell pellets were resuspended in 0.1% Triton X-100 and incubated for 15 min at 4 °C before homogenization. An aliquot of cellular lysate protein (100 μg) was incubated for 1 h at 37 °C in a buffer containing 250 mM NaAc, 1 mM EDTA, and [choline-methyl-^{14}C] sphingomyelin (100,000 cpm/assay). To measure neutral sphingomyelinase activity, cell pellets were resuspended in 0.1% Triton X-100, 20 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 5 mM dithiothreitol, 2 mM EDTA, 20 μM $ZnCl_2$, 1 mM Na_3VO_4, 15 mM NaF, 10 mM glycerophosphate, 0.5 mM ATP, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin and aprotinin. After incubation for 5 min at 4 °C, cells were homogenized, and 100 μg of cellular lysate protein were incubated for 1 h at 37 °C in a buffer containing 20 mM HEPES, pH 7.4, 1 mM $MgCl_2$, and [choline-methyl-^{14}C]SM (100,000 cpm/assay). Radioactive phosphocholine produced from [choline-methyl-^{14}C]SM was then extracted with $CHCl_3/CH_3OH$ (2:1) and quantified by scintillation counting.

Ceramidase Assay—Ceramidase activity was measured by a modification of the method of Gatt and Yavin (25, 26). Cells were washed with ice-cold PBS and homogenized in a buffer containing 0.1 M Bicine, pH 7.4, 1 mM $CaCl_2$, 1 mM dithiothreitol, 1 mM Na_3VO_4, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin and aprotinin. The lysates were then centrifuged at 500 × *g* for 5 min to remove the debris and then ultracentrifuged at 100,000 × *g* for 1 h at 4 °C to pellet the membrane fraction. The membrane pellets were resuspended in a reaction buffer supplemented with 1% Triton X-100 containing either 0.1 M CAPSO (3-(cyclohexylamino)-2-hydroxyl-1-propanesulfonic acid), pH 9, or 0.1 M acetate buffer, pH 4.5. After incubation with 0.2 μCi of [^{14}C]*N*-oleoyl-sphingosine (ceramide) for 1 h at 37 °C, the reaction was terminated by extraction of oleic acid. The labeled oleic acid was resolved by TLC and quantitated by liquid scintillation counting.

Immunoblot Analysis—Cytosolic proteins (30 μg) were resolved by 15% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose filters (Schleicher & Schuell). Members were blocked by incubation with TPBS (PBS and 0.1% (v/v) Tween 20) containing 5% (w/v) skim milk for 2 h at room temperature. Antibodies were applied in TPBS at the appropriate dilutions for overnight at 4 °C. The complexed IgGs were detected by incubation with secondary antibodies conjugated to horseradish peroxidase and developed using ECL system (Amersham Pharmacia Biotech).

RESULTS

TNF Does Not Induce Apoptosis in Normal HUVEC but It Does in C11 Cell Line—In agreement with previous reports that HUVEC are resistant to the cytotoxic effect of TNF (4, 5), treatment of HUVEC with up to a saturating dose (10 ng/ml) of TNF resulted in no morphological changes of death (Fig. 1*A*, *a* and *b*). However, TNF-induced cell death was observed morphologically as early as 6 h poststimulation (Fig. 1*A*, *c* and *d*) in a spontaneously transformed cell line generated from HUVEC, named C11, which has maintained many other functional endothelial characteristics (22). The electrophoretic pattern of the DNA extracted from the treated C11 cell line demonstrated the typical DNA internucleosomic fragmentation of apoptotic cells (Fig. 1*B*). To quantify the number of cells undergoing apoptosis, the MTT dye reduction assay was performed. Again, HUVEC displayed resistance to TNF cytotoxic effects, although these cells underwent apoptosis with serum deprivation (Fig. 1*C*). By contrast, in the C11 cell line TNF-induced cell death in a dose-dependent manner in the cultures containing various concentration of serum (Fig. 1*C*). Thus, the difference in phenotype between HUVEC and C11 cell line provided a useful model to investigate the mechanism for endothelial cell death in responses to TNF.

TNF-induced Ceramide Generation Is Not Sufficient to Trigger Apoptosis in Endothelial Cells—Ceramide, generated from sphingomyelin hydrolysis, has emerged as a second messenger in mediating apoptosis induced by various stimuli including TNF (14–16), although the role of ceramide is still controversial. As shown in Fig. 2*A*, the addition of exogenous ceramide, cell-permeable C_2-ceramide, was able to induce apoptosis in both HUVEC and C11 cell line in a dose-dependent manner. In addition, treatment with sphingomyelinase (1 unit/ml) to gen-

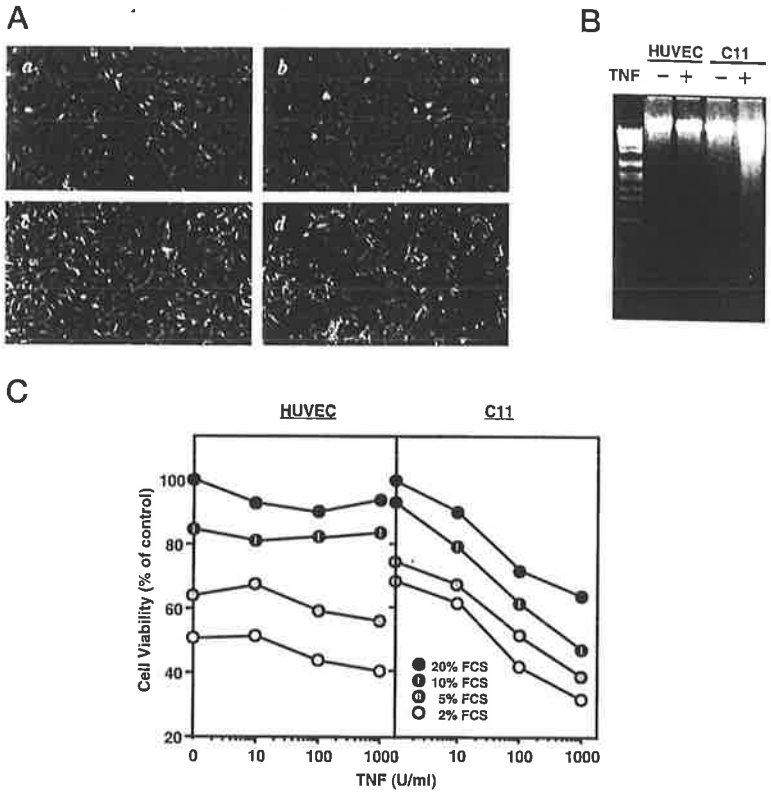

FIG. 1. **The differences between normal and transformed HUVEC in responses to TNF killing effect.** *A*, morphology of normal HUVEC (*a* and *b*) and C11 cell line (*c* and *d*) after 16 h of incubation in culture medium (*a* and *c*) or the medium containing 1 ng/ml of TNF (*b* and *d*) (magnification, ×200). *B*, C11 cell line but not normal HUVEC shows oligonucleosomal banding after 16 h of treatment with TNF (1 ng/ml). *C*, confluent HUVEC and C11 cells were treated with an increasing concentration of TNF for 16 h in the culture medium containing the indicated concentration of fetal calf serum. Cell viability assessed by an MTT assay is expressed as a proportion of cells maintained in culture medium containing 20% of fetal calf serum. Data are the means of three individual experiments, and each experiment was done in triplicate.

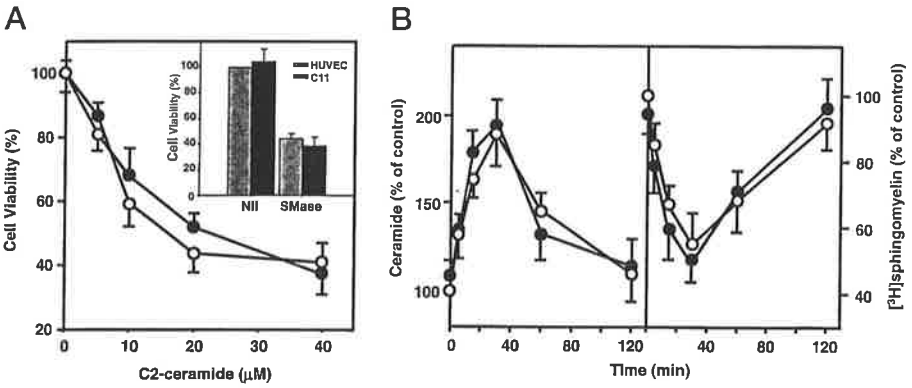

FIG. 2. **Endogenous ceramide is not sufficient to trigger TNF-induced apoptosis.** *A*, confluent HUVEC (○) and C11 cell line (●) were treated with an increasing concentration of C2-ceramide for 16 h, and viability was assessed by an MTT assay. The inset shows effect of sphingomyelinase (*SMase*, 1 unit/ml) treatment on cell viability. Data are the means ± S.E. of three individual experiments, and each experiment was done in triplicate. *B*, after cells were treated with TNF (1 ng/ml) for various time, ceramide levels (*left panel*) were measured by using the diacylglycerol kinase assay, and sphingomyelin levels (*right panel*) were measured in the [^{3}H]serine-labeled cells. ○, HUVEC; ●, C11 cell line. The results represent mean values ± S.D. from three independent experiments.

erate endogenous ceramide by hydrolysis of sphingomyelin also induced apoptosis in both endothelial cell types (Fig. 2*A*, *inset*). These data raise the hypothesis that the resistance to TNF cytotoxic effect in HUVEC may be due to a lack of ceramide generation in response to TNF. However, our previous report showed that TNF rapidly induced an increase in intracellular ceramide levels (~2-fold increase) peaking at 30 min after stimulation and a concomitant decrease in sphingomyelin content (20). To confirm this finding, Fig. 2*B* shows that TNF induced an identical sphingomyelin-ceramide turnover in both HUVEC and C11 cell line. There was also no difference in the basal levels of sphingomyelin and ceramide in these two cell lines. Thus, ceramide itself could not explain the differential responses to TNF between HUVEC and C11 cell line.

Resistance to TNF Cytotoxic Effect Is Associated with SphK Activation—S1P, another sphingomyelin metabolite generated from sphingosine through SphK activation, has been implicated as a signal molecule in mediating cell growth, proliferation, and protecting cell death (17–19). To explore the role of SphK in regulating TNF cytotoxic effect, the activity of SphK was measured. Treatment of HUVEC with TNF caused a rapid and transient increase in SphK activity reaching a maximum of 174 ± 16% ($p < 0.01$) of basal within 10 min (Fig. 3), which was consistent with our previous report (20). In contrast, TNF failed to induce any increases in SphK activity in C11 cell line. There were no significant differences in the basal levels of SphK activity between HUVEC and C11 cell line (21.4 ± 1.8 and 19.7 ± 1.9 pmol/min/mg protein, respectively). As a control, PMA (phorbol 12-myristate 13-acetate), an activator of SphK through protein kinase C activation (19), induced a similar increase in SphK activity in C11 cell line and HUVEC (Fig. 3), suggesting a specific defect in activation of SphK by TNF in C11 cell line. In parallel with the SphK activity, the production of S1P *in vivo* and its levels in intact cells were increased by

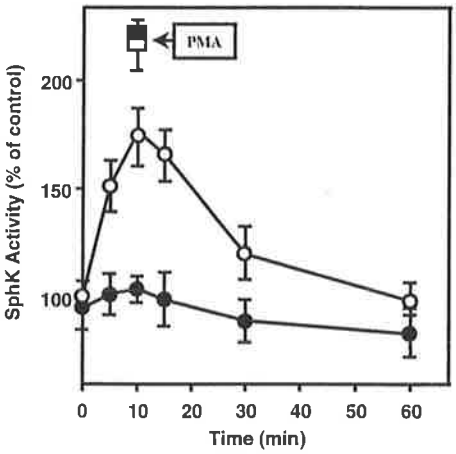

FIG. 3. **TNF-induced SphK activation.** HUVEC (*open symbols*) or C11 cell line (●,■) were treated with TNF (1 ng/ml, *circles*) or PMA (100 ng/ml, *squares*). The cytosolic fractions were then extracted at the desired time points to measure SphK activity. The data represented are the means ± S.D. of three individual experiments.

TNF stimulation in HUVEC (20), but not in C11 cells (data not shown), confirming the differential responses in SphK activation by TNF between these two types of endothelial cells. These data suggested that the responses of endothelial cells to the TNF cytotoxic effect might be associated with the SphK activation.

TNF-induced SphK Activation Is Independent of Sphingomyelinase and Ceramidase Activities—Because S1P is a downstream metabolite in ceramide metabolic pathway, the TNF-induced increases in S1P generation could be related to the activation of sphingomyelinase by TNF stimulation. To test whether the TNF-induced S1P generation is dependent or independent of sphingomyelinase activity, we studied the skin fibroblasts from a patient with Niemann-Pick disease type A, a lysosomal storage disease characterized by a complete lack of acidic sphingomyelinase activity and sphingomyelin accumulation (27). The phenotype of this cell line was identified by measuring sphingomyelinase activity in neutral and acidic pH ranges in post-nuclear extracts from the Niemann-Pick fibroblasts and from age-matched normal skin fibroblasts. In comparison with normal skin fibroblasts, the Niemann-Pick fibroblast line completely lacked acidic sphingomyelinase activity and had only a small amount of neutral sphingomyelinase activity (Fig. 4*A*). Despite a complete lack of ceramide generation in response to TNF in the Niemann-Pick fibroblasts, the TNF-induced SphK activation and S1P generation remained unimpaired as compared with controls (Fig. 4*B*). Thus, it is unlikely that TNF-induced S1P generation is a downstream metabolic event of ceramide.

To further define the TNF-induced generation of S1P, we investigated ceramidase, another key metabolic enzyme upstream of S1P, which catalyzes the metabolism of ceramide to sphingosine, the precursor of S1P production. Because two main isoforms of ceramidase, acid and alkaline forms, exist in most tissues and cells (25), we used the two isoform inhibitors, *N*-oleoylethanolamine (NOE) and (1*S*,2*R*)-D-*erythro*-2-(*N*-myristoylamino)-1-phenyl-1-propanol (MAPP). NOE is a more potent inhibitor of acid ceramidase (28), whereas MAPP predominantly inhibits the alkaline form (29). Neither NOE nor MAPP has a detectable effect in interfering with the TNF-induced increase in S1P formation in HUVEC (Fig. 5*A*), whereas the acid or alkaline ceramidase activity was inhibited by ~60 or ~70%, respectively (data not shown). As a control, TNF-induced S1P generation was completely inhibited by the addition of the competitive inhibitors of SphK, DMS or dihydrosphingosine (19, 20), suggesting a direct effect of TNF on SphK activation. In addition, Fig. 5*B* shows that TNF treatment did not stimulate ceramidase activity at either acidic or alkaline pH values, which are consistent with a previous report on mesangial cells (26). These data further indicate that the activation of SphK by TNF is independent of ceramidase activity.

An Inhibitor of SphK Sensitizes HUVEC to Apoptosis while S1P Protects C11 Cells from Death—Given the potent relationship between SphK activation and cellular responses to TNF killing effect, we determined the role of SphK in resistance to TNF by using a competitive inhibitor of SphK, DMS. As shown *in situ* TUNEL-stained cells, treatment with DMS, blocking S1P formation, profoundly sensitized HUVEC to apoptosis induced by TNF (Fig. 6*e*), although DMS or TNF alone had no apoptotic effect in normal HUVEC (Fig. 6, *a–d*). Conversely, the effect of DMS on sensitizing TNF action was prevented by the addition of S1P (Fig. 6*f*). In contrast to HUVEC, the exposure of C11 cell line to TNF alone induced apoptosis consistent with the observation in Fig. 1, whereas the addition of S1P protected these cells from death (Fig. 6, *g–l*). The DNA fragmentation assay further confirmed the effect of S1P in modulating cellular responses to the TNF cytotoxic effect as shown in Fig. 7*A*: DMS sensitized HUVEC to death, whereas S1P protected C11 cell line from death in a dose-dependent manner in both cases. The cell viability assay (Fig. 7*B*) shows similar results to TUNEL and DNA fragmentation analysis. Furthermore, increasing endogenous S1P by SphK activation with PMA treatment also protected against TNF-induced cell death in C11 cell line (Fig. 7*B*) and prevented DMS-sensitized HUVEC death (data not shown). Taken together, these data indicate a critical role of SphK activation by TNF in protecting against its own apoptotic effect.

Activation of the caspase cascade is believed to be a key phenomenon of apoptotic cell death induced by multiple stimuli (30, 31). As expected, in C11 cell line TNF treatment led to cleavage and hence activation of caspase-3/CPP32 (Fig. 7*C*) that is the pivotal caspase involved in TNF- and Fas ligands-induced apoptosis (32). Co-treatment with S1P profoundly reduced TNF-induced CPP32 activity, which is in agreement with previous observation in Jurkat T cell line (33). In contrast, TNF failed to activate CPP32 caspase in normal HUVEC, whereas inhibition of SphK activity by DMS sensitized TNF-induced activation of CPP32 (Fig. 7*C*). These findings further ascertained the role of TNF-induced SphK activation in regulating cell death in human endothelial cells.

DISCUSSION

The endothelial cell is the interface between blood and tissue and plays an important role in organ physiology and pathology of a variety of disease states. Endothelium in normal tissues, although metabolically active, is generally considered to be quiescent because the turnover is very low (34). This would suggest that normal endothelium in the steady state has mechanisms of maintaining cell numbers by promoting viability independent of proliferation. In noninjured tissue, the endothelium also acts as a barrier to conceal the thrombogenic substratum from circulating platelets and clotting factors (35, 36). Therefore, intact endothelium plays a crucial role as a sensor and effector of signals as well as in maintaining a nonthrombogenic surface. Given the importance of an intact, viable endothelium, we attempted to study the mechanism of resistance to TNF cytotoxicity in normal endothelial cells.

The central finding of this report is that TNF not only induces sphingomyelin hydrolysis resulting in ceramide accumulation but also activates SphK to generate S1P that serves as an anti-apoptotic molecule for cell survival in normal HUVEC. We found that: (i) TNF stimulation caused sphingomyelin

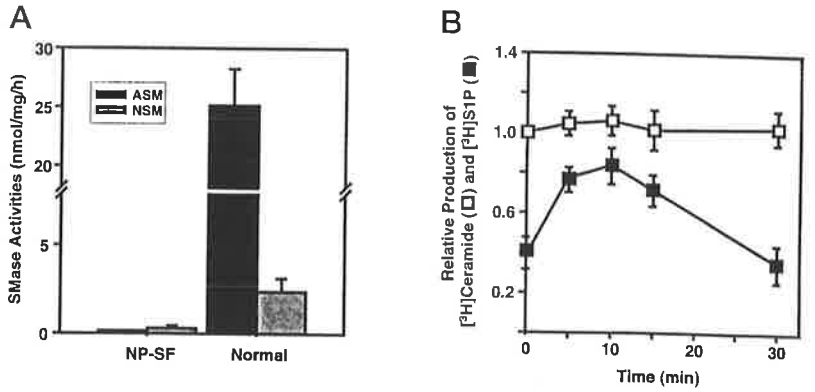

FIG. 4. **TNF activated SphK independently of sphingomyelinase.** *A*, sphingomyelinase activities were measured in acidic (*ASM*) and neutral (*NSM*) pH ranges in post-nuclear extracts from the Niemann-Pick fibroblasts (*NP-SF*) and from age-matched normal skin fibroblasts. *B*, ceramide (□) and S1P (■) levels were measured in the [³H]serine-labeled Niemann-Pick fibroblasts treated with TNF (1 ng/ml) for various times as described under "Experimental Procedures." The results represent mean values ± S.D. from three independent experiments.

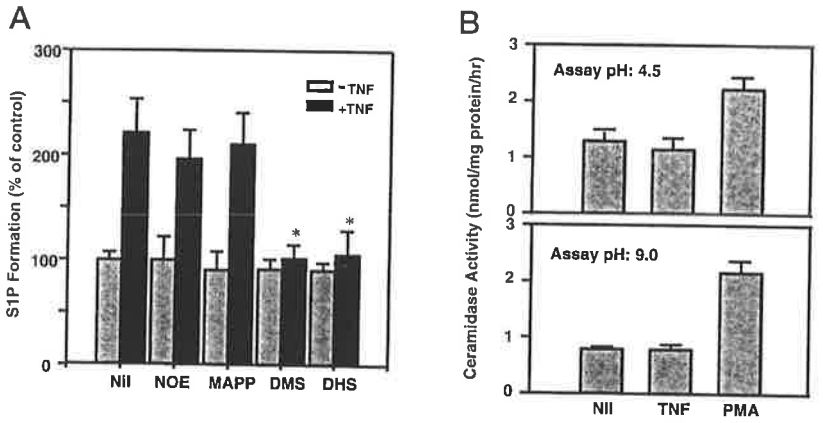

FIG. 5. **TNF activated SphK independently of ceramidase.** *A*, HUVEC were pretreated with a vehicle (*Nil*), NOE (0.5 mM), MAPP (5 μM), DMS (5 μM), and DHS (10 μM) for 30 min, respectively, followed by stimulation with (*black bars*) or without (*gray bars*) TNF (1 ng/ml) for 10 min. S1P formation *in vivo* was measured in the permeabilized cells. *B*, after cells were stimulated with TNF (1 ng/ml) or PMA (100 ng/ml) for 10 min, cellular ceramidase activity was measured at acidic or alkaline pH value. The results represent mean values ± S.D. from three independent experiments. *, $p < 0.001$ compared with TNF stimulation.

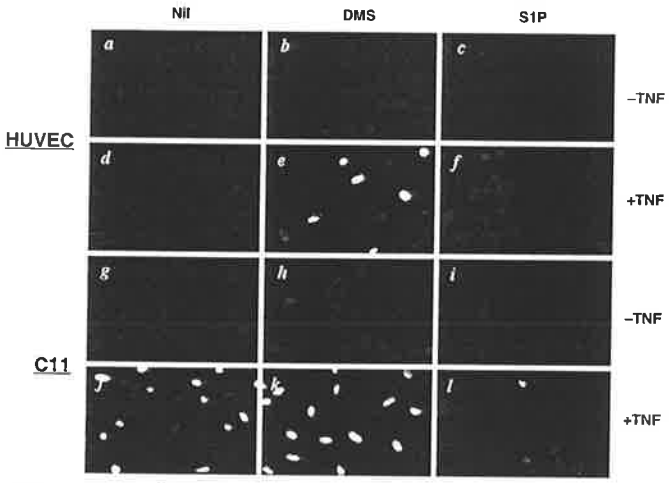

FIG. 6. **Effect of SphK on TNF-induced apoptosis.** HUVEC (*a–f*) or C11 cells (*g–l*) were grown in 8-well chamber slides. After 24-h cultures, cells were treated with or without TNF (1 ng/ml) for 16 h in the presence of a vehicle (*Nil*), DMS (5 μM), S1P (5 μM, *c*, *i*, and *l*), or DMS+S1P (*f*), respectively. Apoptotic cells were assessed by *in situ* staining cells using TUNEL (magnification, ×400).

breakdown with a concomitant increase in ceramide; (ii) TNF induced a rapid and transient activation of SphK, with corresponding increases in S1P levels; (iii) the inhibition of S1P production by DMS, a competitive inhibitor of SphK, significantly sensitized HUVEC to TNF-induced apoptosis; and (iv) the effect of DMS was reversed by addition of S1P. These findings provided new evidence to support the hypothesis of "self-control": that TNF itself can induce the generation of molecules that protect cells from the cytotoxicity of TNF (3).

In contrast to normal endothelial cells, the transformed C11 cell line failed to activate SphK generating S1P and underwent apoptosis in response to TNF stimulation. The defect in SphK activation is not due to the lack of this enzyme because it can be activated by PMA, an established agonist of SphK, through the activation of protein kinase C (19). Addition of exogenous S1P or treatment with PMA to generate endogenous S1P profoundly protected C11 cell line from apoptosis induced by TNF. In agreement with a previous report (19), the effect of PMA on antagonizing the TNF-induced apoptosis was markedly inhibited by the addition of DMS (data not shown), indicating a role of SphK activation in protecting cell death. Taken together, these data suggested that the activation of SphK after TNF-induced sphingomyelin-ceramide turnover "switches on" the signaling that could inhibit cells death.

Ceramide has been described as a mediator of apoptotic cell death in response to a variety of stimuli including TNF (14–16), and this notion has recently been challenged by other observations (see review in Ref. 37). Ceramide is also a substrate for ceramidase to form sphingosine, which can further convert to S1P via the activation of SphK. It has been reported that various mitogenic and growth factors such as PMA and platelet-derived growth factor stimulated proliferation is mediated, at least in part, by activating ceramidase (26) or SphK (17–19). Here we show for the first time that TNF itself is able to activate SphK independently of sphingomyelinase or ceramidase activation as judged by four criteria. First, TNF induced increases in SphK activity and S1P formation earlier than sphingomyelinase activation (Figs. 2 and 3). Second, in the Niemann-Pick fibroblasts TNF failed to induce ceramide generation but retained the ability to activate SphK and produce S1P. Third, the inhibitors of ceramidase did not diminish the TNF-induced SphK activation. Fourth, TNF had no effect to stimulate ceramidase activity. Thus, it is likely that S1P generation post TNF stimulation in HUVEC directly resulted from SphK activation rather than a downstream event in the sphingomyelin metabolic pathways.

The phenomenon that the individual enzymes in sphingomyelin metabolic pathways can be regulated by a given agonist (TNF) concurs with the previous observation that interleukin-1

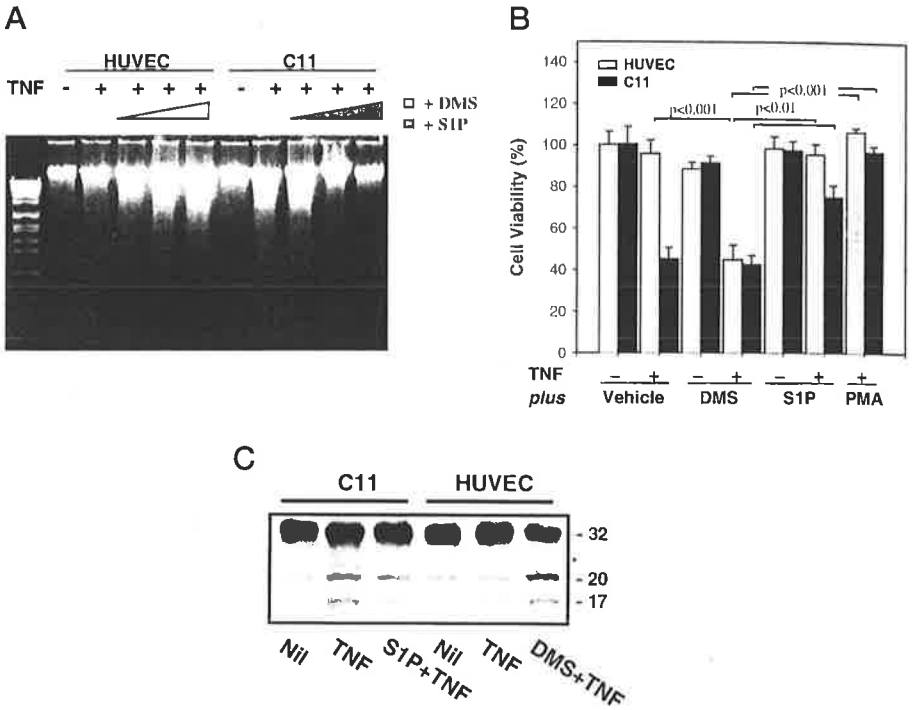

FIG. 7. **DMS sensitizes HUVEC to apoptosis, whereas S1P protects C11 cells from death.** *A*, confluent cells were treated with or without TNF (1 ng/ml) for 16 h in the presence of vehicle or DMS (2.5, 5, and 10 μM; for HUVEC) or S1P (2.5, 5, and 10 μM; for C11). Total cellular DNA was then isolated and analyzed by electrophoresis on a 1.8% agarose gel stained with ethidium bromide. Results are representative of three similar experiments. *B*, HUVEC (*gray bars*) and C11 cell line (*black bars*) were treated with or without TNF (1 ng/ml) for 16 h in the presence of a vehicle, DMS (5 μM), S1P (5 μM), or PMA (100 ng/ml), respectively, cell viability was then assessed by an MTT assay. Data are the means ± S.D. from one experiment done in triplicate, which were repeated three times with similar results. *C*, after cells were treated as indicated, caspase-3/CPP32 was measured by Western blotting assay with anti-CPP32 antibodies. The activated form of the caspase appears as lower molecular mass (20 and 17 kDa) cleavage products.

not only induced sphingomyelin hydrolysis but also activated ceramidase in a highly concentration-dependent manner in hepatocytes (38). Now we show that SphK can be activated by TNF independently of sphingomyelinase or ceramidase activity. The possibility of individual and independent activation of the enzymes in this metabolic pathway exists. This may be an important paradigm because bioactive sphingolipid metabolites have diverse effects, and the particular function of cytokine may be explained by a selectivity for the enzymes in question.

The mechanisms for TNF-promoted activation of SphK is not known although a novel signaling protein named FAN (factor associated with *N*-sphingomyelinase) has recently been described that can directly couple TNF receptor death domain to neutral sphingomyelinase activation (15). TRAF, a member of the adaptor protein family recruited to TNF receptors, has been shown to play a critical role in anti-apoptotic effect after TNF stimulation (10–13). Whether TNF-induced SphK activation needs the recruitment of TRAF to TNF receptor or *vice versa* is currently unknown. Another question raised from our study relates to the exact intracellular targets of S1P in protecting endothelial cells from death. Although they are not completely understood, several lines of evidence suggest the involvement of SphK activation in multiple anti-apoptotic signaling pathways. Previous studies from our laboratory and others have showed that S1P stimulated the extracellular signal-regulated kinase signaling pathway (19, 20) and activated the transcription factor, NF-κB (20). The activation of extracellular signal-regulated kinase and NF-κB have been considered as potent signals in protection against apoptosis in a variety of cell types (5, 39, 40). Activation of caspases is believed to be a central signal in mediating apoptotic cell death, and inhibition of caspases causes a resistance to apoptosis in response to multiple stimuli (30, 31). We found that co-treatment with S1P in C11 cell line protected TNF-induced CPP32 caspase activity. By contrast, in normal HUVEC that are resistant to TNF apoptotic effect, TNF failed to activate CPP32, whereas inhibition of SphK by DMS led the activation of CPP32 and sensitization to apoptosis upon TNF stimulation. These findings suggest an inhibitory effect of S1P on the caspase cascade. Recently, S1P has been reported to inhibit the activation of caspases that cleave poly(ADP-ribose) polymerase and lamins during Fas- and ceramide-mediated apoptosis in Jurkat T lymphocytes (33). Thus, the anti-apoptotic effect of S1P appears to be, at least partially, mediated through inhibition of caspase pathways.

In summary, TNF stimulation results in strikingly different cellular responses such as cell death or survival that were determined by the opposing signaling pathways simplified as Yin and Yang. For example, TNF induces the recruitment of FADD and TRAF (10), the activation of NF-κB and caspases (39), and we showed here the activation of SphK *versus* sphingomyelinase. The balance of such Yin and Yang could have profound effect to regulate the cytocidal effect of TNF. A significant advance in understanding TNF self-control mechanism came recently with the finding that TNF activated NF-κB that provide cells with resistance against death through the induction of inhibitor-of-apoptosis proteins (3, 13, 40). This self-control mechanism could be important for designing a novel strategy to modulate TNF effect on cell death or survival in the systemic immune reaction and anti-cancer therapy.

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Expression of a Catalytically Inactive Sphingosine Kinase Mutant Blocks Agonist-induced Sphingosine Kinase Activation

A DOMINANT-NEGATIVE SPHINGOSINE KINASE*

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Sphingosine kinase (SK) catalyzes the formation of sphingosine 1-phosphate (S1P), a lipid messenger that plays an important role in a variety of mammalian cell processes, including inhibition of apoptosis and stimulation of cell proliferation. Basal levels of S1P in cells are generally low but can increase rapidly when cells are exposed to various agonists through rapid and transient activation of SK activity. To date, elucidation of the exact signaling pathways affected by these elevated S1P levels has relied on the use of SK inhibitors that are known to have direct effects on other enzymes in the cell. Furthermore, these inhibitors block basal SK activity, which is thought to have a housekeeping function in the cell. To produce a specific inhibitor of SK activation we sought to generate a catalytically inactive, dominant-negative SK. This was accomplished by site-directed mutagenesis of Gly^{82} to Asp of the human SK, a residue identified through sequence similarity to the putative catalytic domain of diacylglycerol kinase. This mutant had no detectable SK activity when expressed at high levels in HEK293T cells. Activation of endogenous SK activity by tumor necrosis factor-α (TNFα), interleukin-1β, and phorbol esters in HEK293T cells was blocked by expression of this inactive sphingosine kinase (hSK^{G82D}). Basal SK activity was unaffected by expression of hSK^{G82D}. Expression of hSK^{G82D} had no effect on TNFα-induced activation of protein kinase C and sphingomyelinase activities. Thus, hSK^{G82D} acts as a specific dominant-negative SK to block SK activation. This discovery provides a powerful tool for the elucidation of the exact signaling pathways affected by elevated S1P levels following SK activation. To this end we have employed the dominant-negative SK to demonstrate that TNFα activation of extracellular signal-regulated kinases 1 and 2 (ERK1,2) is dependent on SK activation.

The lipid second messenger sphingosine 1-phosphate (S1P)[1] has been implicated in the regulation of a variety of important mammalian cell processes, including proliferation, differentiation, and apoptosis (1–3). Specifically, some of the diverse signaling roles attributed to elevated cellular S1P levels include prevention of ceramide-induced apoptosis (4, 5), calcium mobilization (6), stimulation of DNA binding activity of the transcription factor activator protein-1 (7), activation of mitogen-activated protein kinase pathways (8, 9), activation of phospholipase D (10), and stimulation of adhesion molecule expression (9).

Cellular levels of S1P are largely mediated by its formation from sphingosine by the activity of sphingosine kinase (SK) and to a lesser extent by its degradation by S1P lyase (11) and S1P phosphatase (12, 13) activities. Cultured mammalian cells, in the absence of stimulating factors, have basal SK activity; however numerous studies have shown that this activity can be rapidly increased by certain cell agonists. Depending on cell type, some of the diverse agonists that stimulate SK activity include tumor necrosis factor-α (TNFα) (4, 9), platelet-derived growth factor (14), nerve growth factor (15, 16), *n*-formyl-methionyl-leucyl-phenylalanine (17), muscarinic acetylcholine agonists (18), serum (14), phorbol esters (19, 20), and engagement of the FcεRI (21) and FcγRI (22) antigen receptors. Stimulation of SK activity by these agonists results in rapid and transient increases in cellular S1P, the basal levels of which are generally low (2, 14), triggering the downstream pathways described earlier.

Although cell agonists stimulate SK activity, there is considerable basal activity found in unstimulated cells. We have previously suggested this may represent dual roles for SK in the cell (23). Basal activity is likely to be required for a "housekeeping" function as part of the sphingomyelin degradative pathway (3, 24) clearing the cell of sphingosine and ceramide, two molecules with apparent pro-apoptotic functions (3, 25). In contrast, the higher levels of SK activity seen upon cell stimulation with various cell agonists appear to confer cell signaling roles for this enzyme through the production of S1P (1, 2).

To date, studying the roles of agonist-induced SK activation and the resultant elevated S1P levels in cell signaling has relied upon the use of SK inhibitors to decrease S1P formation in response to agonist stimulation of cells, as well as the addi-

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[1] The abbreviations used are: S1P, sphingosine 1-phosphate; SK, sphingosine kinase; TNFα, tumor necrosis factor-α; hSK, human SK1; hSK^{G82D}, hSK with Gly^{82} → Asp mutation; hSK^{WT}, wild-type hSK; dnSK, dominant-negative SK; ERK1,2, extracellular signal-regulated kinases 1 and 2; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; IL-1, interleukin-1β; HA, hemagglutinin; DGK, diacylglycerol kinase.

tion of endogenous S1P in these conditions to resurrect S1P-mediated signaling. This system, although providing useful insights into the roles of SK activation and S1P, is not ideal. Three main inhibitors of sphingosine kinase are known, *N,N*-dimethylsphingosine, *N,N,N*-trimethylsphingosine, and DL-*threo*-dihydrosphingosine (23). Although these inhibitors have been used extensively in studies examining the role of S1P and SK, unfortunately they do not show absolute specificity to SK. Instead, they appear to have secondary effects on the cell, including inhibition of protein kinase C (PKC) (26, 27), activation of sphingosine-dependent protein kinases (28), 3-phosphoinositide-dependent kinase 1 (29), and casein kinase II (30), among numerous other effects on the cell (3). Additionally, their use inhibits basal (housekeeping) SK activity in cells, inevitably leading to increases in cellular sphingosine and ceramide levels and resulting in modulation of signaling pathways affected by these molecules (3, 25). To add further complexity, the addition of endogenous S1P to cells not only resurrects intracellular signaling functions of S1P but also triggers numerous other pathways leading from the recently identified EDG family of G protein-coupled S1P-specific cell surface receptors, of which four isoforms are known (31–34). This has made interpretation of the exact roles of S1P in cell signaling difficult.

In this study we have overcome the problems associated with the use of these SK inhibitors and addition of exogenous S1P by the development of a dominant-negative SK that specifically blocks SK activation, while leaving the relatively high basal SK activity unaltered.

EXPERIMENTAL PROCEDURES

Materials—D-*erythro*-Sphingosine and S1P were purchased from Biomol Research Laboratories Inc. (Plymouth Meeting, PA). ATP and phorbol 12-myristate 13-acetate (PMA) were from Sigma. [γ-^{32}P]ATP and [^{32}P]orthophosphate were purchased from Geneworks (Adelaide, South Australia), [*choline-methyl*-^{14}C]sphingomyelin from PerkinElmer Life Sciences, and TNFα from R&D Systems Inc. (Minneapolis, MN). Interleukin-1β (IL-1) was a gift from Synergin (Boulder, CO).

Cell Culture and Transfection—Human embryonic kidney cells (HEK293T, ATCC CRL-1573) were cultured on Dulbecco's modified Eagle's medium (CSL Biosciences, Parkville, Australia) containing 10% fetal calf serum, 2 mM glutamine, 0.2% (w/v) sodium bicarbonate, 1.2 mg/ml penicillin, and 1.6 mg/ml gentamycin. Transfections were performed using the calcium phosphate precipitation method (35). High transfection efficiency (>90%) of these cells was confirmed using immunofluorescence with the M2 anti-FLAG antibody (Sigma) and fluorescein-conjugated anti-mouse Ig (Selinus/Amrad, Melbourne, Australia). Cells were harvested and lysed by sonication (2 watts for 30 s at 4 °C) in lysis buffer containing 50 mM Tris/HCl (pH 7.4), 10% glycerol, 0.05% Triton X-100, 150 mM NaCl, 1 mM dithiothreitol, 2 mM Na_3VO_4, 10 mM NaF, 1 mM EDTA, and protease inhibitors (Complete™; Roche Molecular Biochemicals). Protein concentrations in cell homogenates were determined with either the Coomassie Brilliant Blue (Sigma) or bicinchoninic acid (Pierce) reagents using bovine serum albumin as standard.

Cloning of Human Sphingosine Kinase—Human SK1 (hSK) cDNA (GenBank™ accession number AF200328) was FLAG epitope-tagged at the 3′ end and subcloned into pcDNA3 vector, as described previously (23). For coexpression experiments hSK cDNA was hemagglutinin (HA) epitope-tagged at the 3′ end by *Pfu* DNA polymerase chain reaction with oligonucleotide primers 5′-TTGAACCATTATGCTGGCTATGA-3′ and 5′-TATCTAGAAGCTTAGGCGTAGTCTGGCACGTCGTATGGGT-ATAAGGGCTCTTCTGGCGGT-3′. The polymerase chain reaction product was digested with *Apa*I-*Xba*I, and the resultant HA-tagged hSK cassette was subcloned to generate pGEM4Z-hSK-HA. The HA-tagged hSK cDNA was then cloned into pcDNA3 by digestion with *Eco*RI-*Xba*I. Sequencing verified the integrity of the cDNA sequence.

Construction and Expression of hSK^{G82D}—The FLAG-tagged hSK cDNA (23) was cloned into pALTER (Promega Inc., Madison, WI) site-directed mutagenesis vector. Single-stranded DNA was prepared and used as a template for oligonucleotide-directed mutagenesis as detailed in the manufacturer's protocol. The mutagenic oligonucleotide (5′-CTGGAGACGATCTGATGCAC-3′) was designed to generate the hSK^{G82D} mutant by substitution of the Gly^{82} to Asp. The mutant was sequenced to verify incorporation of the desired modification. The hSK^{G82D} mutant cDNA was then subcloned into pcDNA3 (Invitrogen Corp., San Diego, CA) for transient transfection into HEK293T cells.

Enzyme Assays—SK activity was determined using D-*erythro*-sphingosine and [γ-^{32}P]ATP as substrates, as described previously (23). Neutral sphingomyelinase activity was determined using [*choline-methyl*-^{14}C]sphingomyelin as substrate, essentially as described previously (36). Briefly, whole cell lysates, prepared as described above, were added to an equal volume of 100 mM Tris/HCl buffer (pH 7.4) containing 0.2% Triton X-100, 10 mM $MgCl_2$, and [*choline-methyl*-^{14}C]sphingomyelin (50,000 cpm/assay) and incubated at 37 °C for 60 min. Radioactive phosphorylcholine produced was then extracted with chloroform/methanol (2:1, v/v) and quantified in the aqueous phase by scintillation counting. The measurement of PKC activity *in situ* was performed as described previously (37). Briefly, cells were seeded in 24-well plates and maintained in culture medium until 70–80% confluent. After the indicated treatments, the cells were washed with Dulbecco's modified Eagle's medium and placed in 60 μl of buffered salt solution (137 mM NaCl, 5.4 mM KCl, 0.3 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 5.5 mM glucose, and 20 mM HEPES) supplemented with 50 μg/ml digitonin, 10 mM $MgCl_2$, 25 mM β-glycerophosphate, and 10 μM [γ-^{32}P]ATP (5000 cpm/pmol). A PKC-specific peptide substrate (RKRTLRRL) was then added (to 200 μM) in the presence of 5 mM EGTA and 2.5 mM $CaCl_2$. After a 10-min incubation at 30 °C, the kinase reaction was terminated by the addition of 20 μl of 25% (w/v) trichloroacetic acid. Aliquots (65 μl) of the acidified reaction mixtures were spotted on phosphocellulose papers (Whatman P-81) and washed three times with 75 mM phosphoric acid and once with 75 mM sodium phosphate buffer, pH 7.5. The PKC-dependent phosphorylated peptide substrate bound to the filter was quantified by scintillation counting.

Measurement of Cellular S1P—Relative cellular S1P levels following TNFα treatment were determined after a 4-h preincubation of cells in serum and phosphate-free medium containing 100 μCi/ml [^{32}P]orthophosphate. Cells were washed with phosphate-buffered saline and harvested and lysed in 300 μl of 50 mM Tris/HCl buffer (pH 7.4) containing 1% Triton X-100, 2 mM Na_3VO_4, 10 mM NaF, and protease inhibitors (Complete™; Roche Molecular Biochemicals). Lipids were then extracted with 1.5 ml of chloroform/methanol,1 M NaCl (2:2:1, v/v) containing 50 μl of 3 M NaOH. Under these alkaline conditions S1P is water-soluble and partitions to the aqueous phase. The aqueous phase was then collected, acidified by the addition of 25 μl of concentrated HCl, and extracted with 1.2 ml of chloroform/methanol/concentrated HCl (100:100:1, v/v) to partition S1P to the organic phase. [^{32}P]S1P in the organic phase was then isolated by TLC on Silica Gel 60 with 1-butanol/ethanol/acetic acid/water (8:2:1:2, v/v) and quantitated by PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Western Blotting—SDS-polyacrylamide gel electrophoresis was performed on cell lysates according to the method of Laemmli (38) using 12% acrylamide gels. Proteins were blotted to nitrocellulose, and the membranes were blocked overnight at 4 °C in PBS containing 5% skim milk and 0.1% Triton X-100. SK expression levels were analyzed with either a monoclonal anti-HA antibody (12CA5) or M2 anti-FLAG antibody (Sigma). ERK activation in response to agonists was followed in cells serum-starved for 4 h using anti-ERK1,2 (Zymed Laboratories Inc., San Francisco, CA) and anti-phospho-ERK1,2 (Promega, Madison, WI) antibodies. Immunocomplexes were detected after conjugation to either horseradish peroxidase-conjugated anti-mouse (Pierce) or anti-rabbit (Selinus/Amrad, Melbourne, Australia) IgG using an enhanced chemiluminescence kit (Amersham Pharmacia Biotech).

RESULTS AND DISCUSSION

Production of a Catalytically Inactive Sphingosine Kinase by Site-directed Mutagenesis—We have recently cloned and characterized hSK (23). A search of the hSK amino acid sequence for signaling domains using the SMART search tool (39, 40) revealed similarity in residues 16–153 to the putative catalytic domain of diacylglycerol kinases (DGK). hSK showed an overall 36% identity to the consensus sequence of the DGK catalytic domain family and possessed 17 of the 24 very highly conserved amino acids of this domain (Fig. 1). One region of the hSK sequence showing highest similarity corresponded to the proposed ATP binding site of the DGK catalytic domain (47). This region in DGK displays some resemblance to the glycine-rich loop within the ATP binding site of many protein kinases (48,

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Drosophila DGK2  809 PVIVFINPKSCGNQGH-KLLGKFQHLINPRQI-FDLTQ-GGPKMGLDM-FRKAPNL---RVLACGGDGTV 871
Human DGKζ       295 PLLVFVNPKSCGNQGA-KIIQSFLWYINPRQI-FDLSQ-GGPKEALEM-YRKVHNL---RILACGGDGTV 357
Human DGKε       219 PLIILANSRSCGTNMGE-GLLGEFRILINPVQI-FDVTK-TPPIKALQL-CTLLPYYSA-RVLVCGGDGTV 282

Human SK1         16 RVLVLLNPRGCKGKALQLFRSHVQPLIAEAEISFTLMLTERRNHAREL-VRSEELGRWDALVVMSGDGLM  84
Mouse SK1         22 RVLVLLNPQGCKGKALQLFQSRVQPFIEEAEITFKLILTERKNHAREL-VCAEELGHWDALAVMSGDGLM  90
Human SK2        146 RLLLLVNPFGGRGLAWQWCKNHVLPMISEAGLSFNLIQTERQNHAREL-VQGLSLSEWDGIVTVSGDGLL 214
Mouse SK2        147 RLLILVNPFGGRGLAWQRCMDHVVPMISEAGLSFNLIQTERQNHAREL-VQGLSLSEWEGIVTVSGDGLL 215
Yeast LCB4       228 SILVIINPHGGKGTAKNLFLTKARPIIVESGCKIEIAYTKYARHAIDI-AKDLDISKYDTIACASGDGIP 296
Yeast LCB5       270 SIFVIINPFGGKGKAKKLFMTKAKPLILASRCSIEVVYTKYPGHAIEI-AREMDIDKYDTIACASGDGIP 338
S. pombe SK      107 RFIVFINPHGCKGKAKHIWESEAEPVFSSAHSICEVVLTRRKDHAKSI-AKNLDVGSYDGILSVGGDGLF 175
C. elegans SK     85 NLLVFINPNSGTGKSLETFANTVGPKIDKSLIRYEVVVTTGPNHARNVLMTKADLGKFNGVLILSGDGLV 154
Arabidopsis SK   279 RLLVFVNPFGGKKSAREIFVKEVKPLFEDADVQLEIQETKYQLHAKEF-VKSMDVSKYDGIVCVSGDGIL 347

Drosophila DGK2 872 GWVLSVLDQIQPPLQ--PAPAVGVLPLGTGNDLARALGWGGGYTD---EPIGKILREIG-MSQCVLMDRW 935
Human DGKζ       358 GWILSTLDQLRLKP----PPPVAILPLGTGNDLARTLNWGGGYTD---EPVSKILSHVE-EGNVVQLDRW 419
Human DGKε       283 GWVLDAVDDMKIKGQEKYIPQVAVLPLGTGNDLSNTLGWGTGYAG---EIPVAQVLRNVMEADGIKLDRW 350

Human SK1         85 HEVVNGLMERPDWETAI-QKPLCSLPAGSGNALAASVNHYAGYEQVTNEDLLTNCTLLLCRRLLSPMNLL 153
Mouse SK1         91 HEVVNGLMERPDWETAI-QKPLCSLPGGSGNALAASVNHYAGYEQVTNEDLLINCTLLLCRRRLSPMNLL 159
Human SK2        215 HEVLNGLLDRPDWEEAV-KMPVGILPCGSGNALAGAVNQHGGFEPALGLDLLLNCSLLLCRGGGHPLDLL 283
Mouse SK2        216 YEVLNGLLDRPDWEDAV-RMPIGVLPCGSGNALAGAVSHHGGFEQVVGVDLLLNCSLLLCRGGSHPLDLL 284
Yeast LCB4       297 YEVINGLYRRPDRVDAFNKLAVTQLPCGSGNAMSIS------CHWTNNPSYAALCLVKSIETRIDLMCCS 360
Yeast LCB5       339 HEVINGLYQRPDHVKAFNNIAITEIPCGSGNAMSVS------CHWTNNPSYSTLCLIKSIETRIDLMCCS 403
S. pombe SK      176 HEVINGLGERDDYLEAF-KLPVCMIPGGSGNAFSYN------ATGQLKPALTALEILKGRPTSFDLMTFE 238
C. elegans SK    154 FEALNGILCREDAFRIFPTLPIGIVPSGSGNGLLCSV--LSKYGTKMNEKSVMERALEIATSPTAKAESV 226
Arabidopsis SK   348 VEVVNGLLERADWRNAL-KLPIGMVPAGTGNGMIKSTLDTVGLRCCANSATISIIRGHKRSVDVATIAQG 515
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FIG. 1. **Sequence alignment of the putative catalytic domains of some diacylglycerol kinases with sphingosine kinases.** The putative DGK catalytic domains of *Drosophila* DGK2 (41) and human DGKζ (42) and DGKε (43), for which catalytically inactive mutants have been generated, are aligned with the partial amino acid sequences of the human SK1 (23) and SK2 (44), murine SK1 (45) and SK2 (44), *Saccharomyces cerevisiae* (LCB4 and LCB5) SKs (46), and EST sequences of putative SKs from *Schizosaccharomyces pombe*, *Caenorhabditis elegans*, and *Arabidopsis thaliana* (GenBank® accession numbers Z98762, Z66494, and AL022603, respectively). Highly conserved residues within the putative catalytic domain of diacylglycerol kinases are highlighted. The marked (●) residue indicates the site where mutagenesis (Gly → Asp) in the three DGKs ablates catalytic activity.

49) but is more divergent in hSK. A glycine residue in this region is known to be essential for DGK catalytic activity because mutation of this residue to aspartate ablates activity in all DGKs examined (41, 47, 50). Therefore, in an attempt to produce a catalytically inactive hSK, we also mutated the corresponding glycine (Gly^{82}) to aspartate in hSK. We then expressed this hSK^{G82D}, as well as the wild-type hSK (hSK^{WT}), in HEK293T cells and assayed the cell lysates for SK activity (Fig. 2). As previously reported (23), expression of hSK^{WT} produces a substantial (approximately 2000-fold) increase in SK activity over the endogenous levels. In contrast, similar expression of hSK^{G82D} produced no detectable increase in SK activity. This demonstrated that, as for DGKs, the Gly^{82} to Asp mutation ablated catalytic activity in hSK and due to its similarity to the glycine-rich loop of protein kinases, may suggest that this region of the enzyme is involved in ATP binding.

Expression of hSK^{G82D} Blocks Activation of Endogenous SK Activity by Various Cell Agonists—Numerous previous studies have shown that expression of catalytically inactive enzymes can result in dominant-negative function by preventing activation of the wild-type endogenous enzyme (*e.g.* Refs. 51–53). Therefore, we tested whether hSK^{G82D} could act in a similar manner to block endogenous SK activation by various cell agonists in HEK293T cells. Cells were transiently transfected with either pcDNA3-hSK^{G82D} or empty vector, and the SK activity was determined in response to treatment with TNFα, IL-1, and PMA. As observed previously (4, 9, 20), TNFα and PMA stimulated endogenous SK activity approximately 2-fold in the empty vector transfected HEK293T cells (Fig. 3). Similar stimulation of SK activity was also observed in these cells with IL-1 (Fig. 3). However, strikingly, in cells expressing hSK^{G82D} no activation of endogenous SK activity was observed in response to either TNFα or IL-1, whereas PMA treatment resulted in only a small increase in SK activity (Fig. 3). Basal levels of SK activity were maintained in both unstimulated and stimulated cells in the presence of hSK^{G82D}. Further examination of the time course of TNFα stimulation (Fig. 4) showed

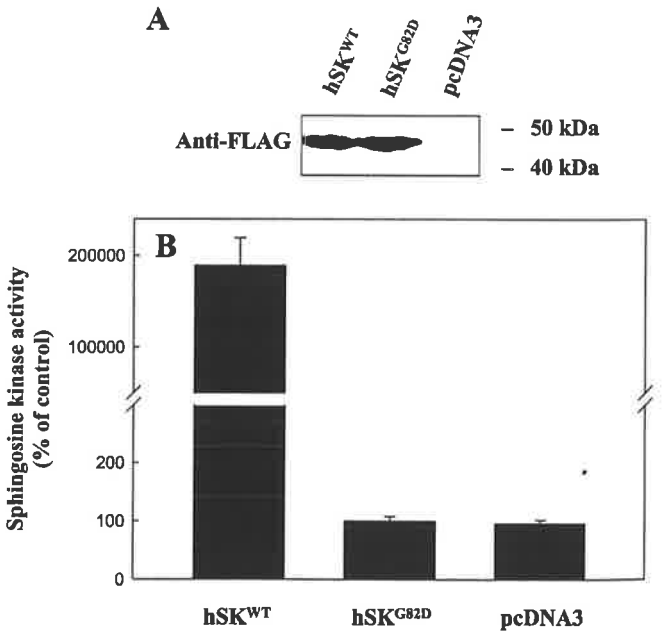

FIG. 2. **Site-directed mutagenesis of Gly^{82} to Asp in human sphingosine kinase ablates catalytic activity.** HEK293T cells transfected with either pcDNA3-hSK, pcDNA3-hSK^{G82D}, or empty pcDNA3 vector were harvested and analyzed for protein expression levels by Western blot using the anti-FLAG antibody (*A*) and sphingosine kinase activity (*B*).

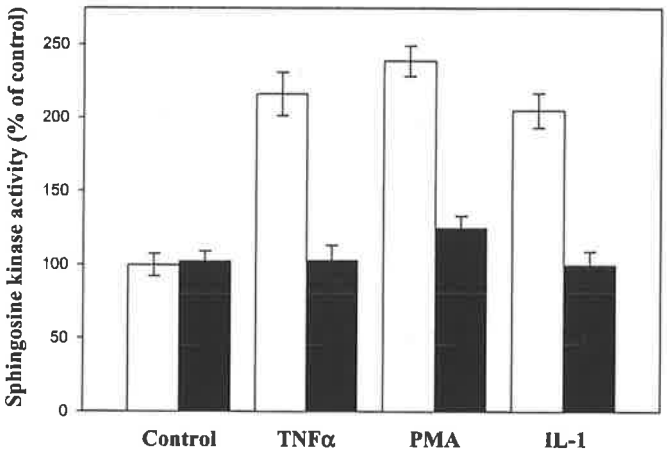

FIG. 3. **Expression of hSK^{G82D} in HEK293T cells blocks activation of endogenous sphingosine kinase activity by TNFα, PMA, and IL-1.** HEK293T cells transfected with either pcDNA3-hSK^{G82D} (*filled bars*) or empty pcDNA3 vector (*open bars*) were treated with 1 ng/ml TNFα and 100 units/ml IL-1 for 10 min and 100 ng/ml PMA for 30 min.

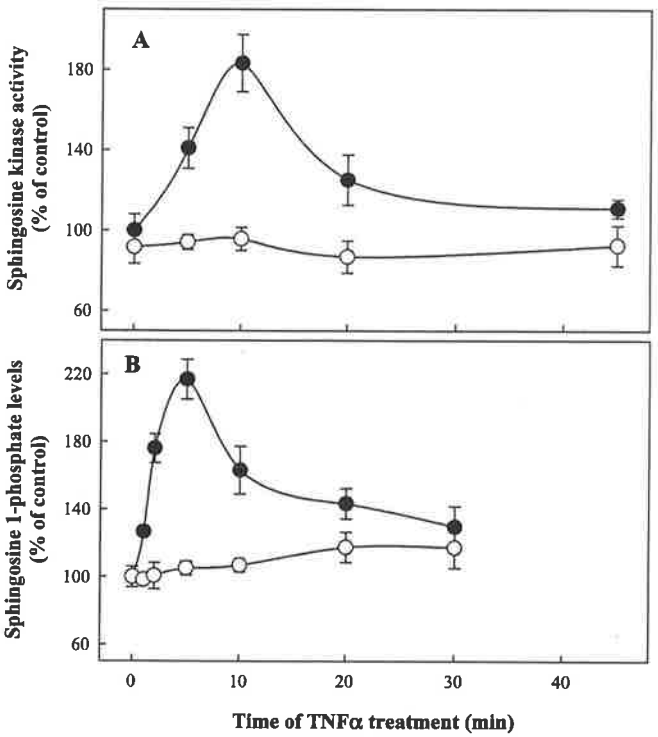

FIG. 4. **Time course of sphingosine kinase activation by TNFα in HEK293T cells expressing hSK^{G82D}.** HEK293T cells transfected with either pcDNA3-hSK^{G82D} (●) or empty pcDNA3 vector (○) were treated with 1 ng/ml TNFα. Cells were harvested at various times over 45 min of TNFα treatment (1 ng/ml), and the cell lysates were assayed for SK activity (*A*) and S1P levels (*B*). All values shown are relative to untreated HEK293T cells transfected with empty pcDNA3 vector.

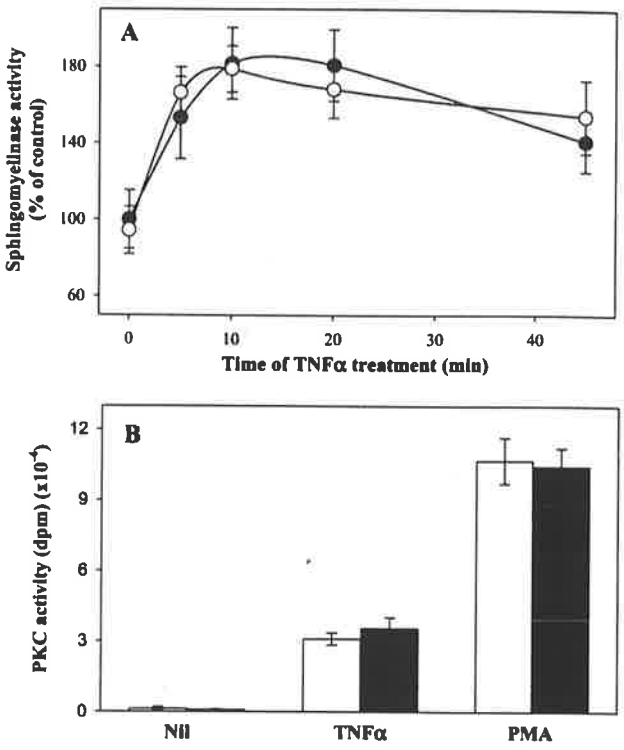

FIG. 5. **Expression of hSK^{G82D} in HEK293T cells does not affect activation of sphingomyelinase activity by TNFα or PKC activity by TNFα or PMA.** HEK293T cells were transfected with either pcDNA3-hSK^{G82D} (● and *filled bars*) or empty pcDNA3 vector (○ and *open bars*). *A*, cells were harvested at various times over 45 min of TNFα treatment (1 ng/ml), and the cell lysates were assayed for sphingomyelinase activity. SK activity from these lysates is shown in Fig. 4. All values shown are relative to untreated HEK293T cells transfected with empty pcDNA3 vector. *B*, cells were treated with 1 ng/ml TNFα for 10 min or 100 ng/ml PMA for 30 min and assayed for PKC activity.

that expression of hSK^{G82D} blocked activation of SK activity even after an extended period of stimulation. SK activity was maintained at unstimulated levels, demonstrating that hSK^{G82D} blocks activation but not basal SK activity. Cellular S1P levels were also measured in parallel with SK activity. As predicted, cells expressing hSK^{G82D} showed no transient increase in S1P levels in response to TNFα, while basal S1P levels were maintained. It is especially notable that hSK^{G82D} does not reduce S1P levels in either unstimulated or stimulated cells. This demonstrates that, in the intact cell, hSK^{G82D} does not block S1P formation by sequestering substrate, which would reduce both basal and stimulated S1P levels. This effect would be missed in *in vitro* SK assays where substrate is in excess but theoretically could be important in cells where substrate could be limiting. Also of note was that the time course for increased S1P levels occurs at a slightly earlier stage than that for SK activity. This may be due to increased S1P lyase (11) or S1P phosphatase (12) activity, clearing the cell of S1P, although currently nothing is known regarding the regulation of these enzymes.

Combined, these data indicate that hSK^{G82D} indeed acts as a dominant-negative SK (dnSK) in ablating activation of endogenous SK activity.

Specificity of hSK^{G82D} Dominant-Negative Function—To establish the specificity of hSK^{G82D} as a dominant-negative for SK activation, we examined other pathways stimulated by TNFα that should not be affected by hSK^{G82D}. Previous studies have shown that both sphingomyelinase (36, 54) and PKC (37, 55) activity are stimulated by TNFα. Therefore, HEK293T cells were transiently transfected with either pcDNA3-hSK^{G82D} or empty vector, and the sphingomyelinase and PKC activity was determined in response to treatment with TNFα. Activation of both enzymes by TNFα was unaffected by the presence of hSK^{G82D} (Fig. 5). This illustrates that hSK^{G82D} is a specific dominant-negative for SK activation by TNFα, blocking that activation and not other TNFα signaling pathways.

hSK^{G82D} Decreases TNFα-induced Activation of Overexpressed hSK^{WT}—We have previously shown that TNFα can rapidly induce an approximate 2-fold increase in the already high levels of SK activity resulting from the overexpression of hSK^{WT} in HEK293T cells in a parallel manner to the activation

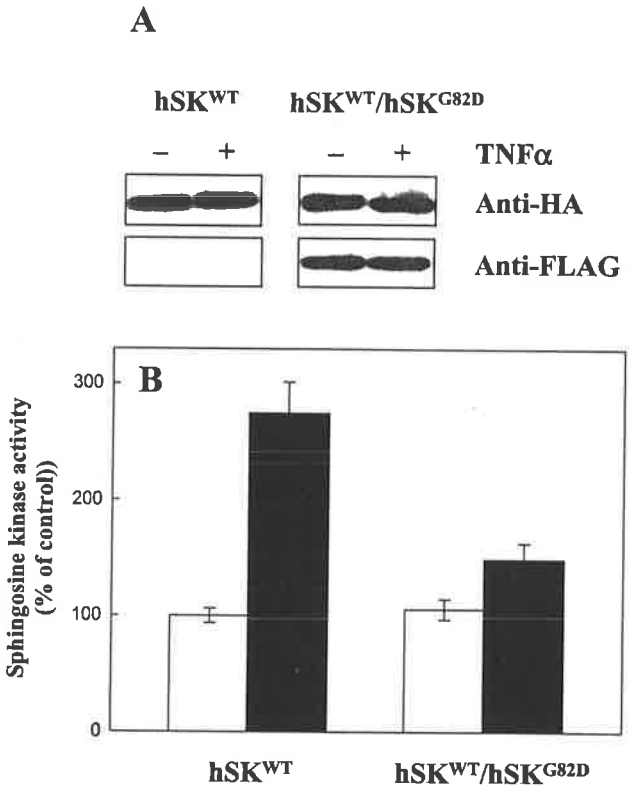

FIG. 6. **Expression of hSK^{G82D} in HEK293T cells decreases activation of overexpressed wild-type sphingosine kinase activity by TNFα.** HEK293T cells, either transfected with pcDNA3-hSK^{WT} or cotransfected with equal proportions of pcDNA3-hSK^{WT} and pcDNA3-hSK^{G82D} were harvested after being treated with 1 ng/ml TNFα for 10 min (*filled bars*) or without treatment (*open bars*). Recombinant protein expression (*A*) and SK activity (*B*) in the cell lysates were then determined.

of endogenous SK activity (23). Similar results were also recently reported with platelet-derived growth factor stimulation of NIH 3T3 fibroblasts expressing a murine SK (56). Therefore, we tested the ability of hSK^{G82D} to block the activation of these high levels of hSK^{WT} (2000-fold overexpression) by following TNFα-induced stimulation of SK activity in HEK293T cells coexpressing hSK^{WT} and hSK^{G82D}. To confirm similar levels of expression, the two SK forms were constructed with different epitope tags. The presence of equimolar levels of hSK^{G82D} blocked TNFα stimulation of hSK^{WT} activity by approximately 50% (Fig. 6). The high basal SK activity in unstimulated cells was unaffected by hSK^{G82D}. These data suggest that the mechanism whereby hSK^{G82D} acts as a dominant-negative is through direct competition with SK^{WT} for the, as yet unknown, upstream activator of SK. This proposed mechanism is further supported by the observation that, in untreated cells, hSK^{G82D} does not affect basal endogenous (Fig. 3) or overexpressed SK activity (Fig. 6). Similarly, cell extracts containing high levels of hSK^{G82D} did not inhibit the activity of purified human SK *in vitro* (data not shown), demonstrating that hSK^{G82D} blocks SK activation and not SK activity itself. By inference, these data also support our previous studies (23) suggesting that hSK is a constitutively active enzyme that does not require activation for basal activity.

Expression of hSK^{G82D} Prevents TNFα-induced ERK Activation—Having established that hSK^{G82D} acts as a specific dominant-negative to ablate activation of endogenous SK by a range of cell agonists, we examined its effect on a pathway proposed to be mediated by S1P and SK activation. Previous studies had shown that activation of extracellular signal-regulated kinase 1 and 2 (ERK1,2) activity by TNFα appears to be

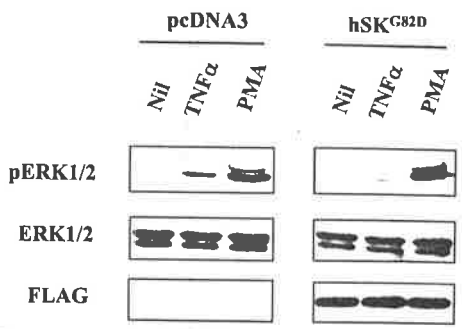

FIG. 7. **Expression of hSK^{G82D} in HEK293T prevents ERK activation by TNFα.** HEK293T cells transfected with either pcDNA3-hSK^{G82D} or empty pcDNA3 vector were treated with 1 ng/ml TNFα for 10 min or 100 ng/ml PMA for 30 min. Cells were harvested, and the cell lysates were analyzed by Western blotting with anti-FLAG, anti-ERK1,2, and anti-phospho-ERK1,2 antibodies.

mediated by SK activation through transient increases in cellular S1P levels (9). Thus, we examined the effect of hSK^{G82D} expression on ERK1,2 activation by TNFα. HEK293T cells were transiently transfected with either pcDNA3-hSK^{G82D} or empty vector, and the activation of ERK was determined in response to treatment with TNFα and PMA through Western blotting with an anti-phospho-ERK1,2 antibody. Treatment of HEK293T cells transfected with empty vector with TNFα rapidly stimulated activation of ERK1,2 (Fig. 7). In marked contrast, activation of ERK1,2 by TNFα is blocked in HEK293T cells expressing hSK^{G82D} (Fig. 7). PMA-induced activation of ERK1,2, however, remains unaffected demonstrating that expression of hSK^{G82D} does not inherently affect ERK1,2 activation. The activation of ERK1,2 by PMA in the presence of hSK^{G82D} is not surprising because it is known that ERK activation can be achieved by several pathways independent of SK and S1P (57, 58). Therefore, these data provide, for the first time, unequivocal evidence for the requirement of SK activation and raised cellular S1P levels in TNFα-induced ERK1,2 activation without resorting to the use of chemical inhibitors of questionable specificity. At present the mechanism of this S1P-mediated ERK1,2 activation is unclear. Previous studies have shown that exogenous S1P can activate the mitogen-activated protein kinase pathway via the EDG family of G protein-coupled S1P cell surface receptors (31, 32, 59, 60). Therefore, it is tempting to speculate that the increased intracellular S1P levels resulting from activation of SK activity lead to secretion of S1P and subsequent engagement of S1P cell surface receptors and activation of ERK1,2. However, previous studies have failed to detect S1P in media from HEK293T and NIH 3T3 cells producing high cellular S1P levels through overexpression of murine SK (56). This instead suggests that S1P may activate ERK1,2 via an intracellular mechanism, as has been suggested for other S1P-mediated pathways (61).

Conclusions and Implications of this Study—This study has described the construction and characterization of a dnSK that selectively blocks activation of SK by various cell agonists. Although the dnSK is based on human SK1, which we (23) and others (62, 63) have recently cloned, it should be noted that a second human SK isoform (hSK2) has also been identified recently (44). At present the relative abundance of hSK2 compared with hSK is not known, nor is it known if the activity of hSK2 is activated by cell agonists. However, the data from our current study suggest that hSK2 is either in relatively low abundance in HEK293T cells or is not activated, or its activation is also blocked by the dnSK.

The mechanism of action of the dnSK appears to be through competition for the, as yet unknown, SK activating molecule. The relatively high basal SK activity is unaffected by expres-

sion of the dnSK. This is an important observation supporting mounting evidence that SK has dual roles in the cell. Basal SK activity is likely to be involved in sphingomyelin turnover, assisting in clearing the cell of sphingosine and ceramide, two molecules with apparent pro-apoptotic functions (3, 25). In contrast, the activated SK activity seen upon cell stimulation with various cell agonists appears to confer a cell signaling role for this enzyme through the production of elevated S1P levels (1, 2). It is not yet clear whether the stimulated production of S1P confers a signaling role solely by raising S1P levels above a signaling threshold or whether the stimulated formation is localized to specialized sites active for signaling. The dnSK does not affect the housekeeping role of basal SK activity, thus overcoming any associated problems with accumulation of sphingosine and ceramide in the cell, as occurs with *N,N*-dimethylsphingosine treatment of cells. In contrast, by blocking SK activation the dnSK ablates SK-mediated cell signaling. Thus, the dnSK is a unique and powerful tool for the elucidation of the role of SK activation and the exact downstream targets of the resultant elevated cellular S1P levels.

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High Density Lipoproteins (HDL) Interrupt the Sphingosine Kinase Signaling Pathway

A POSSIBLE MECHANISM FOR PROTECTION AGAINST ATHEROSCLEROSIS BY HDL*

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The ability of high density lipoproteins (HDL) to inhibit cytokine-induced adhesion molecule expression has been demonstrated in their protective function against the development of atherosclerosis and associated coronary heart disease. A key event in atherogenesis is endothelial activation induced by a variety of stimuli such as tumor necrosis factor-α (TNF), resulting in the expression of various adhesion proteins. We have recently reported that sphingosine 1-phosphate, generated by sphingosine kinase activation, is a key molecule in mediating TNF-induced adhesion protein expression. We now show that HDL profoundly inhibit TNF-stimulated sphingosine kinase activity in endothelial cells resulting in a decrease in sphingosine 1-phosphate production and adhesion protein expression. HDL also reduced TNF-mediated activation of extracellular signal-regulated kinases and NF-κB signaling cascades. Furthermore, HDL enhanced the cellular levels of ceramide which in turn inhibits endothelial activation. Thus, the regulation of sphingolipid signaling in endothelial cells by HDL provides a novel insight into the mechanism of protection against atherosclerosis.

Numerous evidence from epidemiological, clinical, and genetic studies have clearly shown a potential protective role of high density lipoproteins (HDL)[1] against the development of atherosclerosis and associated coronary heart disease (1–4). Several mechanisms have been proposed for the cardioprotective function of HDL. These include the promotion of the efflux of cholesterol from atherosclerotic plaques and reducing the atherogenicity of LDL by inhibition of their oxidative modification (4–6). Recently, we and other groups have demonstrated an ability of HDL to inhibit endothelial adhesion protein expression, providing a new mechanistic explanation for its protective effect on atherosclerosis (7–11).

Atherosclerosis has been definitely characterized as an inflammatory disease (12). An important event in the initiation of atherosclerosis is adhesion of circulating monocytes to activated endothelial cells and their subsequent transendothelial migration to the subendothelium. This process is mediated by adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and E-selectin (13, 14). The inappropriate expression of these adhesion proteins in response to the "injury" are induced by various inflammatory stimuli, including cytokines and noncytokines such as interleukin-1, tumor necrosis factor-α (TNF), and oxidized or native LDL (14–16). Pathological studies have shown increased adhesion molecule expression in several components of the atherosclerotic plaque (17–20), and there is also evidence for a role of adhesion molecules in the acute atherothrombotic process (21). Furthermore, a direct association between an increased plasma concentration of soluble adhesion molecules and the increase in risk of future cardiovascular diseases has recently been reported (22, 23).

The ability of HDL to inhibit the cytokines-induced adhesion protein expression has been well documented. It has been reported that human HDL profoundly inhibit the expression of VCAM-1, ICAM-1, and E-selectin in human umbilical vein endothelial cells (HUVEC) activated by TNF or interleukin-1 (7). Total native HDL together with both HDL_2 and HDL_3 subfractions, or the reconstituted HDL particles showed the inhibitory effect in a concentration-dependent manner, although considerable variation existed among different experiments (7–11). The phenotype of inhibition on adhesion molecule expression by HDL differs from their well known function in promoting cholesterol efflux and protecting against lipid peroxidation, suggesting a distinct mechanism exists. We recently demonstrated a novel signaling pathway, sphingosine kinase (SphK) pathway, through the generation of sphingosine 1-phosphate (S1P), which is critically involved in mediating adhesion protein expression and endothelial cell activation after TNF stimulation (24). The SphK pathway has also emerged as a signaling pathway in mediating a variety of cellular functions such as cell growth, proliferation, and inflammatory reaction (24–29). In the present report we show that HDL profoundly inhibit the TNF-induced SphK activity and S1P generation, and subsequently reduce the activation of ERK and NF-κB signal cascades. We thus demonstrate that HDL interrupt a signaling cascade, the SphK pathway, which results in inhibition of endothelial activation. This could provide a new potential mechanism by which HDL protect against atherosclerosis, a cardiovascular inflammatory disease.

EXPERIMENTAL PROCEDURES

Materials—TNF was purchased from R&D Systems. C_2-Ceramide, S1P, sphingosine, *N,N*-dimethylsphingosine (DMS), and dihydrosphingosine were from Biomol (Plymouth Meeting, PA). [^{3}H]Serine and [*cho-*

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[1] The abbreviations used are: HDL, high density lipoproteins; apoA-I, apolipoprotein A-I; ERK, extracellular signal-regulated kinase; HUVEC, human umbilical vein endothelial cell; ICAM-1, intercellular adhesion molecule-1; LDL, low density lipoproteins; oxLDL, oxidized LDL; S1P, sphingosine 1-phosphate; SphK, sphingosine kinase; TNF, tumor necrosis factor-α; VCAM-1, vascular cell adhesion molecule-1; DMS, *N,N*-dimethylsphingosine; POPC, 1-palmityl-2-oleylphosphatidylcholine.

line-methyl-[^{14}C]sphingomyelin were from NEN Life Science Products. [γ-^{32}P]ATP was purchased from Bresatec (Adelaide, Australia), and ^{125}I-TNF was from Amersham Pharmacia Biotech (United Kingdom). *Escherichia coli* diacylglycerol kinase was from Calbiochem (La Jolla, CA). Anti-ERK1/2 antibodies were purchased from Zymed Laboratories Inc. (San Francisco, CA). Other chemicals were from Sigma.

Cell Culture and Flow Cytometry Analysis—HUVEC were isolated as described previously (30) and cultured on gelatin-coated culture flasks in Dulbecco's modified Eagle's medium containing 20% fetal calf serum, endothelial growth supplement (Collaborative Research) and heparin. In some experiments, cells were treated in Opti-MEM (Life Technologies, Inc.) containing 0.1% fatty acid-free bovine serum albumin as serum-free medium. The expression of cell-surface adhesion molecules was measured as described previously (24) by use of a Coulter Epics Profile XL flow cytometer.

Isolation and Preparation of Lipoproteins—As described previously (10), the lipoproteins were isolated from normal healthy adult donors by sequential ultracentrifugation in their appropriate density range: total HDL $1.07 < d < 1.21$, HDL_3 $1.13 < d < 1.21$, and LDL $1.019 < d < 1.063$ g/ml. The resulting preparations of lipoproteins were dialyzed against endotoxin-free PBS (pH 7.4) prior to use. Oxidized LDL was obtained by incubating LDL (500 μg/ml) with confluent cultures of HUVEC in Dulbecco's modified Eagle's medium containing 10 μM $CuSO_4$ for 24 h, and the oxidation was assessed by the increase of mobility on 1% agarose gel. Discoidal reconstituted HDL containing apoA-I and 1-palmityl-2-oleylphosphatidylcholine (POPC) were prepared by the cholate dialysis method described by Matz *et al.* (31).

^{125}I-TNF Binding Assay—The binding assay was performed in confluent HUVEC after preincubation with an increasing concentration of HDL_3 for 4 h. Cells were washed with M199 medium and incubated with 1 nM ^{125}I-TNF in the absence or presence of 500 nM unlabeled TNF in M199 medium containing 10% fetal calf serum. After 4 h of incubation at 4 °C, cells were washed three times with ice-cold M199 and then solubilized in 1 N NaOH, 1% SDS and radioactivity was determined in a γ-counter. Specific binding is defined as the difference between total binding and nonspecific binding with excess unlabeled TNF.

Metabolic Labeling and Sphingolipids Assay—HUVEC were labeled with [^{3}H]serine (10 μCi/ml) for 48 h as described previously (24). The cells were then washed three times and incubated for an additional 2 h in the presence or absence of HDL_3. After treatment with TNF for the indicated times, cellular lipids were extracted and resolved by thin layer chromatography (TLC) in two different solvent systems: (*a*) chloroform:methanol:acetic acid:water (50:30:8:5, v/v) and (*b*) 1-butanol: acetic acid:water (3:1:1, v/v). The samples were concomitantly run with standard sphingolipids including sphingomyelin, ceramide, sphingosine, and S1P. Sphingolipid spots were visualized by fluorography, quantitated by scintillation spectrometry, and normalized by radioactivity recovered in total cellular lipids.

Ceramide Measurement—In addition to metabolic labeling assay as described above, cellular ceramide was quantified with the diacylglycerol kinase reaction as described previously (32). Briefly, the cellular lipids were extracted with $CHCl_3/CH_3OH/HCl$ (1 N) (100:100:1) and resuspended into a sample buffer containing 7.5% *n*-octyl-β-D-glucopyranoside, 5 mM cardiolipin, and 1 mM diethylenetriamine-pentaacetic acid. The samples were reacted with diacylglycerol kinase and [γ-^{32}P]ATP in enzyme buffer containing 20 mM Tris/HCl (pH 7.4), 10 mM dithiothreitol, and 15% glycerol for 30 min at 22 °C. The product of the phosphorylation reaction, ceramide 1-phosphate, was extracted and resolved by TLC using $CHCl_3/CH_3OH/HAc$ (65:15:5) as solvent, detected and quantified by the Phosphoimage system. To exclude a possible error caused by some factors in the extracts affecting diacylglycerol kinase (33), synthetic C_2-ceramide was added in assays as an internal control. There were no changes in the phosphorylated C_2-ceramide in this assay system.

Measurement of SphK Activity—As described previously (24), cells were homogenized in 20 mM Tris buffer (pH 7.4) containing 20% glycerol, 1 mM dithiothreitol, 1 mM EDTA, 20 μM $ZnCl_2$, 1 mM Na_3VO_4, 15 mM NaF, 10 μg/ml leupeptin and aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 0.5 mM 4-deoxypyridoxine. After centrifugation at 13,000 × *g* for 30 min, SphK activity was measured in the supernatant by incubation with 10 μM sphingosine-bovine serum albumin complex and [γ-^{32}P]ATP for 20 min at 37 °C. The labeled lipids were extracted and resolved two times by TLC in the solvent of $CHCl_3/CH_3OH/NH_4OH$ (65:35:8, v/v) and 1-butanol:acetic acid:water (3:1:1, v/v), respectively. The radioactive spots corresponding to authentic S1P were visualized and quantified by the Phosphoimage system. For kinetic studies, cell extracts were prepared from HUVEC treated with TNF for 5 min after preincubation with an increasing concentration of HDL_3 for 4 h. The kinase assay was performed with various concentrations of sphingosine (0, 2.5, 5, and 10 μM).

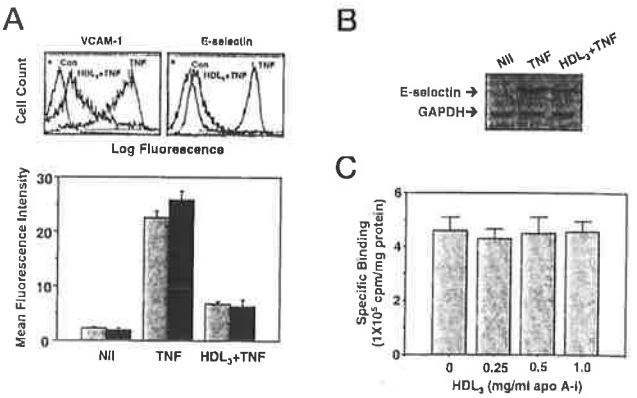

FIG. 1. **Effect of HDL_3 on TNF-induced adhesion protein expression in HUVEC.** *A*, confluent monolayers of HUVEC were preincubated with or without HDL_3 (1 mg/ml apo A-I). After 16-h incubation, the cells were treated with TNF (100 units/ml) for 4 h. The cell-surface expression of VCAM-1 and E-selectin was then measured and shown as the flow cytometric profiles. A negative control profile with the isotype-matched nonrelevant antibody (*Con.*) is also presented. The bar graph (*bottom*) shows the expression VCAM-1 (*gray bars*) and E-selectin (*dark bars*) after TNF stimulation in the absence or presence of HDL_3. Values represent mean ± S.D. from one experiment in triplicate, and the results are representative of four experiments conducted with four different HUVEC donors and four different HDL preparations. *B*, after the indicated treatment and TNF stimulation for 4 h, E-selectin mRNA levels were measured by Northern blotting assay with the α-^{32}P-labeled cDNA probes (24). Results are representative of three similar experiments. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase. *C*, binding of ^{125}I-TNF to HUVEC was performed as described under "Experimental Procedures" after preincubation with an increasing concentration of HDL_3. The data represented are mean values ± S.D. from three individual experiments.

To measure the SphK activity *in vivo*, the formation of S1P was measured in the permeabilized cells as described previously (25).

Electrophoretic Mobility Shift Assay—Nuclear extracts were prepared from HUVEC treated for 30 min with vehicle or the indicated agents after preincubation with or without HDL_3. The double-stranded oligonucleotides used as a probe in these experiments included 5′-GGATGCCATTGGGGATTTCCTCTTTACTGGATGT-3′, which contains a consensus NF-κB binding site in the E-selectin promoter that is underlined (34). Gel mobility shift of a consensus NF-κB oligonucleotide was performed by incubating a ^{32}P-labeled NF-κB probe with 4 μg of nuclear proteins. The specific DNA-protein complexes were completely abolished by addition of a 50-fold molar excess of unlabeled E-selectin NF-κB oligonucleotides.

RESULTS

HDL_3 Inhibits Adhesion Molecule Expression and Synthesis in Response to TNF—In order to minimize possible confounding effects of the variations between the distinct subfractions of total HDL, HDL_3 (d = 1.13~1.21 g/ml) was used in the present study. As shown in Fig. 1A, HDL_3 inhibited by ~70% the TNF-induced expression of VCAM-1 and E-selectin in HUVEC, which was consistent with our previous reports (7, 10). The inhibitory effect of HDL_3 was further identified by its reduction of E-selectin mRNA levels (Fig. 1B). To determine whether the inhibitory effect of HDL_3 on TNF-induced adhesion protein expression result from alterations of TNF access to its receptors, ^{125}I-TNF binding assay was performed. Fig. 1C shows that HDL_3 at concentrations of 0.25~1 mg/ml (apo A-I) did not significantly affect binding of TNF to HUVEC, suggesting that the effect of HDL_3 is secondary to a perturbation of subsequent signaling pathways at postreceptor sites.

HDL_3 Inhibits SphK Activation and S1P Formation—Since we have recently identified the SphK pathway as a potent signaling pathway in mediating TNF-induced endothelial activation, the effect of HDL_3 on this pathway was determined. Consistent with our previous report (24), TNF stimulation of

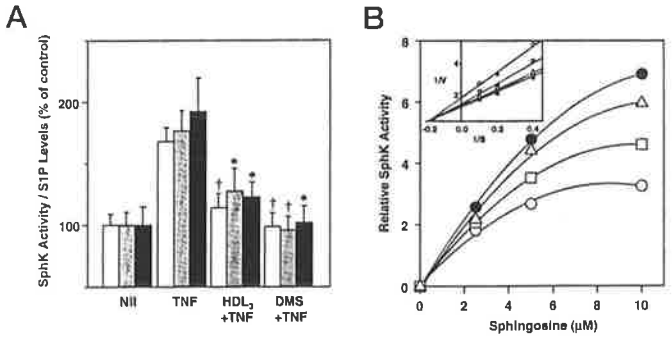

FIG. 2. **HDL_3 inhibits TNF-induced SphK activation.** *A*, after the preincubation with or without HDL_3 (1 mg/ml apoA-I) for 4 h or DMS (5 μM) for 30 min, cytosolic SphK activity (*white bars*), S1P formation *in vivo* (*gray bars*), and S1P levels in intact cells (*dark bars*) were measured, respectively, as described under "Experimental Procedures." The data represented are mean values ± S.D. from three individual experiments. *, $p < 0.02$; †, $p < 0.01$, *versus* TNF stimulation alone. *B*, for the kinetic study of HDL_3 inhibition on SphK, the cell extract was prepared from the cells treated with 100 units/ml TNF for 5 min after preincubation with 1 (○), 0.5 (□), and 0.25 mg/ml (△), or without HDL_3 (●) for 4 h. The kinase assay was performed with various concentration of sphingosine. The *inset* shows double-reciprocal plots following Lineweaver-Burk's method.

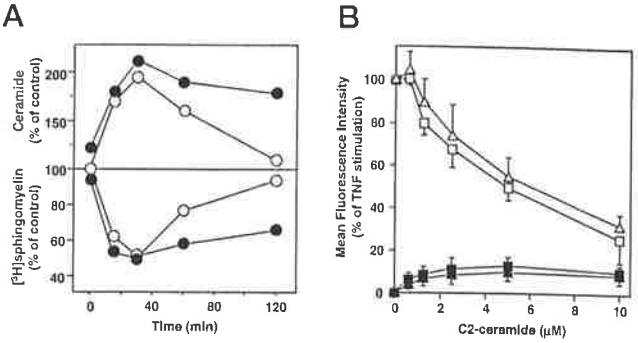

FIG. 3. **Effect of HDL_3 on TNF-induced sphingomyelin turnover.** *A*, HUVEC were preincubated with HDL_3 (1 mg/ml apoA-I) (●) or without HDL_3 (○), for 4 h, ceramide levels (*top*) and [^{3}H]sphingomyelin (*bottom*) were then measured, respectively, at the desired time point of TNF treatment. Results are representative of three similar experiments. *B*, HUVEC were treated with an increasing concentration of C_2-ceramide in the presence (*open symbols*) or absence (*closed symbols*) of TNF (100 units/ml) for 4 h. The cell-surface expression of E-selectin (△) or VCAM-1 (□) was measured by flow cytometry. The data are expressed as percentage of TNF stimulation in the mean fluorescence intensity (*M.F.I.*). Values represent mean ± S.D. from at least three independent experiments.

HUVEC caused a significant increase in cytosolic SphK activity. This activity was profoundly inhibited by HDL_3 preincubation at a physiological concentration ($p < 0.01$, Fig. 2A white bars). The inhibitory effect of HDL_3 was dose-dependent with half-maximal inhibition at about 0.41 mg/ml apoA-I (Fig. 2B). To characterize the inhibition of SphK by HDL_3, Lineweaver-Burk plots revealed that HDL_3 treatment altered the kinetics of SphK by decreasing its V_{max} without significant changes in the K_m (Fig. 2B, inset). Moreover, TNF-induced increases in S1P formation and its levels in intact cells were also markedly inhibited by HDL_3 to comparable levels as obtained by N, N-dimethylsphingosine (DMS), a competitive inhibitor of SphK (Fig. 2A, gray and dark bars). These data further confirm the inhibitory effect of HDL_3 on activation of SphK in endothelial cells.

HDL_3 Does Not Inhibit Sphingomyelinase Activation by TNF—Given the evidence that HDL_3 inhibited SphK activity and S1P production, we tested the possibility that this inhibition was due to a reduction in sphingomyelin-ceramide turnover, an essential upstream event in S1P metabolic pathway. We previously reported that TNF stimulation of HUVEC rapidly reduced sphingomyelin content and consistently increased cellular ceramide levels by approximately 2 fold peaking at 30 min with return to near basal levels by 2 h (24). Pretreatment with HDL_3 did not interrupt the TNF-promoted sphingomyelinase activation, but significantly delayed the reversion of post TNF sphingomyelin levels to base line and sustained the increased ceramide levels (Fig. 3A). There was a significant difference in the comparison of both sphingomyelin and ceramide levels between pretreatment with and without HDL_3 after 2 h of TNF stimulation ($p < 0.01$, three separate experiments). The parallel delay in sphingomyelin reversion and accumulation of ceramide strongly argued against the proposition that HDL_3 decreased S1P production by inhibiting the response of sphingomyelin-ceramide cycle to TNF. Interestingly, the addition of exogenous cell-permeable ceramide (C_2-ceramide) induced a dose-dependent inhibition of TNF-induced adhesion protein expression (Fig. 3B). Thus, the increased ceramide levels could be at least partly responsible for the inhibitory effect of HDL_3.

Intact HDL Particles Are Required for Their Inhibitory Effect—To gain an insight into which components of HDL_3 are responsible for the inhibitory activity, the effect of apoA-I and lipids isolated from HDL_3 was investigated. In marked contrast to intact HDL_3 particles, lipid-free apoA-I, at the same concentration as HDL_3, did not inhibit SphK activity. Similarly, HDL lipid constituents such as POPC in the form of small unilamellar vesicle also had no inhibitory effect (Fig. 4A). However, when apoA-I and POPC were reconstituted into discoidal HDL, the resulting complexes inhibited the TNF-induced SphK activation comparable to that seen with native HDL particles (Fig. 4A). In parallel, neither apoA-I nor POPC had any significant inhibitory activity on the TNF-induced expression of E-selectin (Fig. 4A, bottom panel), which was in agreement with our previous observations on VCAM-1 expression (10, 11).

As the cardioprotective ability of HDL *in vivo* appears to be dependent on the presence of LDL (3, 5), we tested whether the inhibitory effect of HDL on SphK is due to the interaction of LDL or other unknown factors in the serum. When cells were incubated in the serum-free conditions, the SphK activities in response to TNF in the presence or absence of HDL were to the same extent as that in the cells cultured with normal growth medium containing 20% fetal calf serum (compare Fig. 4B and 4A). The presence of LDL (250 μg/ml of apoB) in the cultures did not influence SphK activities either at the basal levels or post TNF stimulation. Further LDL had no effect on the inhibitory activity of HDL on SphK activation (Fig. 4B). Additionally, in the presence of oxLDL (250 μg/ml of apoB) HDL retained its ability to inhibit SphK activation (data not shown). Thus it is unlikely that the inhibitory effect of HDL resulted from the interaction of LDL, oxLDL or other unknown factors in the serum.

HDL_3-induced Reduction of Adhesion Protein Expression Is Related to the Inhibition of SphK Activity—Having shown that HDL inhibited SphK activity and the production of S1P, a novel identified inducer of adhesion protein expression, we further examined the linkage between the inhibition of SphK and reduction of endothelial activation. In the experiment illustrated in Fig. 5A, the HDL_3-induced dose-dependent inhibition of SphK activity was plotted against the reduction of E-selectin expression. There was a significant linear correlation between the inhibitory effects of HDL_3 on SphK activity and E-selectin expression ($r = 0.953$, Fig. 5A). Furthermore, when the formation of intracellular S1P was inhibited by DMS, a competitive inhibitor of SphK, the TNF-induced adhesion protein expression was also reduced (Fig. 5B). Conversely, both the HDL_3 and DMS inhibitory effects on TNF action were reversed by the

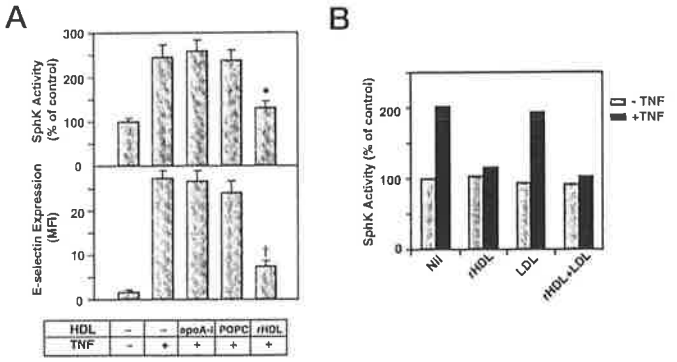

FIG. 4. **An intact conformation of HDL particle is required for the inhibition.** *A*, confluent monolayers of HUVEC were preincubated for 4 h with lipid-free apoA-I (1 mg/ml), POPC (5 mM), or discoidal reconstituted HDL (rHDL, 1 mg/ml apoA-I), followed by TNF (100 units/ml) stimulation. SphK activity (*top panel*) and the cell-surface expression of E-selectin (*bottom panel*) were measured, respectively. Values are mean ± S.D.; $n = 3$. *, $p < 0.01$; †, $p < 0.001$ *versus* TNF stimulation alone. *B*, HUVEC were preincubated with the serum-free medium in the absence (*Nil*) or presence of rHDL (1 mg/ml apoA-I), LDL (250 μg/ml apoB), or rHDL+LDL for 16 h, and treated with (*dark bars*) or without (*gray bars*) TNF (100 units/ml) for 10 min, SphK activity was then measured. Data are presented as mean of two separate experiments.

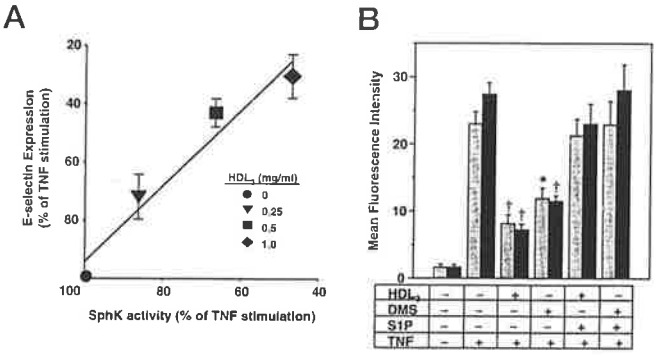

FIG. 5. **The reduction of adhesion protein expression is related to inhibition of SphK.** *A*, linear regression plot between the HDL_3-induced inhibition of SphK activity and the reduction of E-selectin expression. The cells were pretreated with an increasing concentration of HDL_3, and then SphK activity and E-selectin expression were measured after TNF stimulation, respectively. *B*, the HUVEC were pretreated with a vehicle, DMS (5 μM), S1P (5 μM), and/or HDL_3 (1 mg/ml), followed by TNF (100 units/ml) stimulation for 4 h. The expression of VCAM-1 (*gray bars*) or E-selectin (*dark bars*) was then measured. Values are mean ± S.D.; $n = 3$. *, $p < 0.01$; †, $p < 0.001$, *versus* TNF stimulation alone.

addition of S1P (Fig. 5B). This demonstrated that the inhibition of SphK is an important event in the HDL_3-mediated reduction of endothelial activation. As a control, neither DMS nor HDL_3 prevented S1P-induced adhesion protein expression (Fig. 5B), indicating a specific inhibitory effect on SphK. In addition, DMS did not change cellular ceramide levels (data not shown), suggesting the inhibition of SphK activity was not associated with altered ceramide levels.

HDL_3 Inhibits TNF-promoted ERK and NF-κB Activation—The MAP kinase, ERK, has been proposed to be a downstream target in the SphK pathway mediating a variety of cellular functions including adhesion protein expression (24, 26, 29). Fig. 6A showed that both TNF and S1P were approximately equipotent in stimulating ERK activities. Treatment with DMS significantly inhibited TNF-activated ERK, indicating the involvement of SphK in the TNF-activated ERK signal cascade. Preincubation of HUVEC with HDL_3 also reduced TNF-stimulated ERK activation by 49 ± 6.2% ($p < 0.01$), consistent with its effect on reducing cellular levels of S1P.

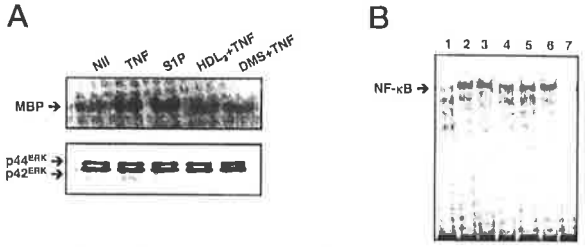

FIG. 6. **Effect of HDL_3 on ERK and NF-κB activation.** *A*, HUVEC were preincubated with or without HDL_3 (1 mg/ml) for 4 h and treated with the indicated agents for 30 min. ERK activities were then assayed with myelin basic protein (*MBP*) as substrate after immunoprecipitation with antibodies against $p42/p44^{ERK}$. The kinase reaction products were separated on 10% SDS-PAGE. In parallel, an aliquot of the same cell lysates was blotted with anti-$p42/p44^{ERK}$ antibodies to ensure equal ERK expression. *B*, NF-κB binding activity was measured by electrophoretic mobility shift assay after treatment with a vehicle (*lane 1*), TNF (100 units/ml, *lane 2*), S1P (5 μM, *lane 3*), DMS (5 μM) + TNF (*lane 4*), HDL_3 (1 mg/ml) + TNF or + S1P (*lanes 5* and *6*) for 30 min. The specific NF-κB binding complexes were identified by competition analyses with the addition of a 50-fold molar excess of unlabeled NF-κB oligonucleotides (*lane 7*). Results in *A* and *B* are representative of at least three similar experiments.

We have previously demonstrated a role of SphK pathway for TNF-promoted NF-κB activation (24) that is essential for the transcription of adhesion molecule genes (35). Fig. 6B shows that HDL_3 significantly inhibited the TNF-induced activation of NF-κB by 51 ± 17% ($p < 0.01$), but did not inhibit that induced by S1P. This inhibition was comparable to that induced by DMS, the SphK inhibitor, suggesting that HDL_3 inhibited the SphK pathway resulting in an inhibition of NF-κB which could account for the reduction of adhesion protein expression. Furthermore, HDL_3 treatment did not inhibit the phorbol ester-promoted NF-κB activation (data not shown), indicating a specificity of HDL_3 effect in this pathway.

DISCUSSION

In this report, we show a novel mechanism of atheroprotection by HDL. In this model, HDL interrupt a signal transduction pathway, the SphK pathway, which is critically involved in endothelial cell activation and adhesion protein expression. The expression of adhesion proteins on activated endothelial cells plays an essential role for the inflammatory processes in the pathogenesis of atherosclerosis (12). The importance of adhesion molecules in atherogenesis is strongly supported by several lines of evidence: (i) adhesion molecules are present in atherosclerotic plaques (17–21); (ii) increased plasma levels of adhesion molecules are associated with the risks of atherosclerosis (22, 23); and (iii) a deficiency of adhesion molecules significantly reduces the formation of atherosclerotic fatty streaks in knockout mice lacking the genes of ICAM-1, P-selectin, or both ICAM-1 and P-selectin (36, 37).

The nature of the inflammatory signals and associated molecular mechanisms that activate adhesion molecule expression in endothelial cells in the atherogenic lesion are unknown. Factors such as TNF and interleukin-1 that are commonly found in inflammatory atherogenic lesions induce the expression of adhesion molecules in cultured endothelial cells. Thus, TNF-stimulated adhesion molecule expression on HUVEC provides a useful model to investigate the signaling pathways involved in the regulation of endothelial cell activation. In this model we have recently identified a novel signaling pathway, the SphK pathway, in mediating TNF-induced adhesion protein expression and endothelial cell activation (24). We found that TNF consistently stimulated SphK activity and the generation of S1P, and blockage of SphK by its inhibitor, DMS, inhibited NF-κB activation and adhesion protein expression. An inhibitory effect of HDL_3 was clearly seen in this pathway:

HDL_3 inhibited (i) SphK activity, (ii) S1P generation, (iii) S1P levels, (iv) ERK activation and (v) nuclear translocation of NF-κB. Moreover, HDL_3-induced inhibition of SphK activity is linear correlating with the reduction of adhesion protein expression, and the inhibitory effects of HDL_3 were reversed by the addition of S1P (Fig. 5). Taken together, these results strongly indicated that the inhibition of SphK activation by HDL_3 could account for its inhibitory effect on adhesion protein expression and endothelial activation.

The finding that HDL_3 not only inhibited the activity and the V_{max} of SphK in a dose dependent manner but also the generation of S1P and its levels in intact cells (Fig. 2) indicated a primary inhibitory effect of HDL_3 on the SphK pathway. On the other hand, it is possible that HDL_3 may affect endothelial phenotype by an effect on the sphingomyelin-ceramide turnover since HDL_3 increased the TNF-dependent ceramide generation and inhibited the reaccumulation of sphingomyelin (Fig. 3A). It is uncertain whether the ceramide accumulation is primarily due to prolonged hydrolysis by sphingomyelinase or to inefficient metabolism by downstream catalysis. The inhibition of adhesion molecule expression by exogenous ceramide (Fig. 3B) indicated a two-pronged inhibition on endothelial activation by HDL: reduction of S1P formation and increase in ceramide levels. Thus, it is assumed that HDL may reset the 'biostat' of ceramide and/or S1P to modulate cellular responses to TNF stimulation and to inhibit endothelial cell activation. This sphingolipid biostat has been proposed in regulating a variety of cellular functions such as cell growth, proliferation and cell death (38, 39).

HDL may exert the protective effect against atherosclerosis by several mechanisms including (i) promoting cholesterol efflux from the peripheral tissues, (ii) reducing LDL oxidation, or (iii) protecting the vasculature against the cytotoxic effect of oxLDL (5, 6, 40). In addition to these effects of HDL, we now demonstrate a novel mechanism whereby HDL interrupt intracellular signaling involved in the pathogenesis of atherosclerosis. The inhibitory effect of HDL on the SphK pathway is very likely independent of the above-mentioned known antiatherogenic ability of HDL, since (i) an intact conformation of HDL particle is required for the inhibition; (ii) the delipidated apoA-I is unable to mimic HDL effect; (iii) the inhibition is serum-independent; and (iv) the interaction of LDL or oxLDL is unlikely to be involved in the inhibition of SphK activation (Fig. 4).

HDL may function on cells in either a receptor-dependent or -independent manner (40). Our preliminary data that HDL particles have no direct inhibitory effect on SphK activity *in vitro* (results not shown) suggested that the access of HDL to cell membrane and putative HDL-binding proteins could be necessary. However, the fact that lipid-free apoA-I is able to access putative HDL receptors such as SR-B1 (41, 42) suggests that binding *per se* may not completely explain the inhibition of SphK pathway. It is possible that the apolipoprotein is critical to the effect but only when it is in an intact conformation that results from its association with phospholipids. Alternately, HDL induced inhibition of SphK pathway may act through other putative HDL-binding proteins in endothelial cells.

In conclusion, this is the first demonstration that HDL interrupt a signaling cascade—the SphK pathway that is involved in regulation of endothelial cell activation, a key event in atherogenesis. This provides a mechanistic explanation for the well-documented ability of HDL to protect against atherosclerosis and might ultimately lead to the development of novel strategies for the prevention and treatment of this disease.

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Chemotactic desensitization of neutrophils demonstrates interleukin-8 (IL-8)-dependent and IL-8-independent mechanisms of transmigration through cytokine-activated endothelium

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SUMMARY

We have recently shown that an exogenous gradient of interleukin-8 (IL-8) induces the transendothelial migration of neutrophils. Treatment of endothelium with the cytokines IL-1 or tumour necrosis factor (TNF) also causes neutrophil transmigration, and recent evidence suggests that this may be due to endogenous IL-8 produced by the endothelium. We have used specific chemotactic desensitization of neutrophils to investigate the role of IL-8 in transmigration through cytokine-activated endothelium. Preincubation of neutrophils with IL-8 reduced their chemotactic transmigration response to an IL-8 gradient by 81%, demonstrating desensitization. Transmigration in response to cytokine-activated endothelium was inhibited by 104% after IL-8 preincubation, thus tending to support the role of IL-8. However, preincubation with another neutrophil chemotactic factor *N*-formyl-methionyl-leucyl-phenylalanine (FMLP), which did not affect the IL-8 response, also inhibited transmigration, by 74%. This suggests that FMLP preincubation acts to inhibit a non-IL-8-dependent mechanism of transmigration through cytokine-activated endothelium. Chemotactic factor pretreatment of neutrophils did not reduce their adhesion to activated endothelium, but specifically blocked the transmigration step. We have therefore shown that chemotactic transmigration can be subjected to factor-specific desensitization, and have used this to provide evidence supporting a role for IL-8 in transmigration through cytokine-activated endothelium, as well as suggesting a further IL-8-independent mechanism. These data also provide a mechanism for the observed defect in accumulation of neutrophils at inflammatory sites when chemotactic factors are infused intravenously.

INTRODUCTION

Extravasation of neutrophils is essential to the acute inflammatory process. In order that this occurs in a site-directed manner, local factors at the inflammatory lesion determine the capture of circulating neutrophils. We and others have shown that there are at least two mechanisms by which neutrophil transendothelial migration may be accomplished; a transendothelial gradient of neutrophil chemotactic factors,[1] and secondly, the activation of endothelium by the 'proinflammatory' cytokines tumour necrosis factor (TNF) and interleukin-1 (IL-1),[1-3] which cause the endothelium itself to induce neutrophil transmigration in the absence of an exogenous chemotactic gradient.

We have investigated the mechanism by which cytokine-activated endothelium induces neutrophil transmigration. We have previously shown that an exogenous gradient of the potent neutrophil chemoattractant IL-8 induces neutrophil transmigration across non-activated endothelium.[1] Endothelial cells activated by IL-1 and TNF produce IL-8.[4] We have therefore hypothesized that cytokine-induced (endogenous) IL-8 secreted by endothelial cells is responsible for neutrophil transmigration, by chemotaxis, across cytokine-activated endothelial monolayers. Huber *et al.* recently showed that neutralizing anti-IL-8 antibodies partially inhibited transmigration through cytokine-activated endothelium,[5] offering direct evidence in support of this hypothesis.

To test this hypothesis, we have used the approach of neutrophil desensitization to IL-8 [and the control chemotactic factor *N*-formyl-methionyl-leucyl-phenylalanine (FMLP)], and observed their response to activated endothelium. Our results are consistent with a role for IL-8 in neutrophil transmigration across cytokine-activated endothelium, but indicate that other non-IL-8-dependent mechanisms are also involved.

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MATERIALS AND METHODS

Reagents

The 72 and 77 amino acid forms of IL-8 were chemically synthesized as described previously.[1] The 72 amino acid form had equivalent potency to recombinant 72-IL-8 in assays for induction of neutrophil chemotaxis, elastase release, cytosolic-free calcium and superoxide production.[6] Endotoxin levels in the stock preparation were <10 pg/ml, as determined by limulus amoebocyte assay.

IL-1 was kindly supplied by Immunex (Seattle, WA) (10^8 thymocyte mitogenic units/mg). Endotoxin levels were <40 pg/ml. Human purified fibronectin (Boehringer Mannheim, Mannheim, Germany) was diluted in phosphate-buffered saline pH 7·3 (PBS) to 50 μg/ml for use in coating plastic surfaces. FMLP and heparin were obtained from Sigma (St Louis, MO). Endothelial cell growth supplement (ECGS) was obtained from Collaborative Research (Bedford, MA). Recombinant human TNF-α (batch no. 3056–55, at 0·5 mg/ml, equivalent to 2×10^7 U/ml) was kindly supplied by Genentech (South San Francisco, CA).

BSA (Commonwealth Serum Laboratories, Melbourne, Australia) in PBS was labelled with ^{125}I (Amersham International, Amersham, U.K.) by the Chloramine-T method.[7] Activity of each preparation was approximately 5×10^5 c.p.m./ml, and the trichloracetic acid (TCA) precipitable activity was $>97\%$.

Neutrophil preparation

Neutrophils were prepared from fresh blood from healthy volunteers. Citrated blood was dextran sedimented, and the buffy coat separated by Ficoll–Hypaque (Nycomed, Oslo, Norway) gradient centrifugation followed by hypotonic lysis of remaining red cells. Purity of preparations was $>93\%$ neutrophils as judged by morphological examination of Wright's stained cytocentrifuge preparations.

Endothelial cells

Human umbilical vein endothelial cells (HUVEC) were isolated by collagenase treatment of umbilical veins,[8] and maintained in endotoxin-free M199 with Earles salts (Cytosystems, Sydney, Australia) with 20% foetal calf serum (FCS) (Flow, North Ryde, Australia), supplemented with 20 mM HEPES, non-essential amino acids, sodium pyruvate, ECGS 25 μg/ml and heparin 25 μg/ml (HUVEC medium). Cells were used between passages 2 and 6. Endotoxin levels in the media used were <50 pg/ml.

Preparation of transmigration apparatus

Transwells (Costar, Cambridge, MA) (6·5 mm diameter, polycarbonate membrane with 3 μm pores) were coated with fibronectin (50 μg/ml for 30 min at room temperature) and seeded with endothelial cells (EC) at 5×10^4 cells/well in 150 μl of HUVEC medium. These were placed in 24-well cluster trays (Costar), the lower well containing 700 μl of HUVEC medium, and cultured for 4 days at 37°, 5% CO_2 (adaptation of methods in refs 2, 9, 10). Confluence was determined both prior to and following treatment with cytokines by measuring permeability to radiolabelled bovine serum albumin (BSA).[11] Briefly, endothelial monolayers were washed and 150 μl of medium containing [^{125}I]BSA (1:20 dilution of stock solution) were pipetted into the well. The transwells were then placed into 24-well trays, the lower compartment containing 700 μl of medium. After 60 min of incubation at 37°, 5% CO_2, the transwells were removed, 200 μl was sampled from the lower well and the radioactivity counted. Transwells without endothelium (filter alone) were used for comparison. Diffusion of BSA was expressed as a percentage of equilibrium, which was simulated by mixing 150 μl of the medium containing [^{125}I]BSA with 700 μl of medium. Transwells were used after 4 days of culture, at which time BSA diffusion was generally $<10\%$ of equilibrium at 60 min. Two wells from each batch were sampled, and experiments where HUVEC monolayers showed permeability $>10\%$ were discarded.

Transmigration experiments (transwells)

In some experiments, monolayers were preincubated with IL-1 or TNF-α by adding either of these in equal concentrations to the upper and lower wells 4 hr prior to the assay. Neither IL-1 nor TNF-α treatment increased the permeability of the monolayers (BSA diffusion—unstimulated HUVE monolayer $7\cdot0\pm1\cdot0\%$, IL-1-treated HUVE monolayer $5\cdot5\pm0\cdot8\%$, $P<0\cdot257$ by independent Student's t-test, mean of five experiments performed in duplicate). Monolayers were washed with assay medium (M199, 2·5% FCS, 10 mM HEPES) prior to each assay, and 10^6 neutrophils in 100 μl of assay medium were then added and the transwells placed in 24-well culture trays which had been precoated with gelatin. The lower well contained 600 μl of assay medium, with or without IL-8 or FMLP. This assembly was incubated for 60 min, after which the wells were shaken to dislodge neutrophils from the lower surface of transwells and the transwells removed. The medium in the lower wells was thoroughly mixed, and aliquots taken for counting of migrated neutrophils in a Coulter Counter (model ZF; Harpenden, U.K.). Counts are expressed as a percentage of the cells added.

Desensitization of neutrophils

Neutrophils (10^7/ml) were preincubated in assay medium at 37° for 30 min with frequent manual agitation, with chemotactic factors in the concentrations indicated in the text. At the end of the incubation the cell suspension was placed on ice, and washed twice in PBS at 4° before resuspending in assay medium for transmigration experiments. These cells remained viable by criteria of trypan blue exclusion ($<99\%$) and showed no increase in release of lactate dehydrogenase at IL-8 concentrations of up to 10^{-6} M. Control cells were subjected to the same treatment except the initial incubation was in medium alone. The purpose of cooling to 4° was to preserve the desensitized state, since washing at room temperature resulted in less effective specific desensitization. The transmigration of neutrophils was not affected by washing at 4°, since transmigration of control cells was comparable with unwashed cells to all stimuli tested, as was the response of desensitized cells to the heterologous chemotactic stimulus (data not shown).

Transmigration experiments (slides)

Methods were adapted from those previously described.[12,13] Endothelial cells (10^5 cells/well, in 300 μl HUVEC medium) were plated into wells on 8-well Labtek chamber slides (Nunc, Naperville, IL) precoated with fibronectin, and allowed to reach confluence over 3–4 days. Four hours prior to experiments, 100 U/ml IL-1 was added to some wells. All wells were washed twice with assay medium immediately prior to the addition of

neutrophils. Neutrophil suspensions, preincubated either in chemotactic factors or medium and washed as described above, were diluted to 10^6 cells/ml and 200 μl was added to the endothelial monolayers. The slides were then incubated at 37°, 5% CO_2 for 20 min. After this, the medium and non-adherent cells were tipped off, and the upper chamber structures were removed. The slides were then viewed under phase contrast microscopy within the next 5 min, and five high power fields (40 × objective) were randomly selected from each well and photographed. Adherent cells were clearly recognized by their rounded or polarized phase bright appearance, while transmigrated neutrophils were easily distinguished since they were spread out on the glass, phase dark but with a bright halo. Counts are given as cells/high power field (photograph) and are means of the five fields. Pooled figures refer to pooling of the means from different wells.

Statistical methods

The variations are expressed as the standard error of the mean (SEM). *P*-values were calculated by Student's paired or independent *t*-tests, or analysis of variance (ANOVA), as indicated.

RESULTS

IL-1 preincubation of endothelium induces neutrophil transmigration

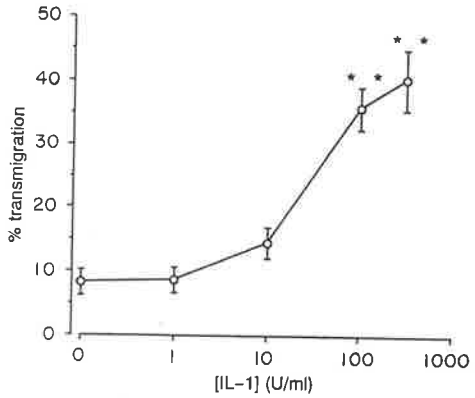
Endothelial monolayers cultured in transwells were incubated in IL-1 at varying concentrations for 4 hr and then washed in assay medium before the addition of 10^6 neutrophils for 60 min. IL-1 increased transmigration in a dose-dependent manner (Fig. 1), with a plateau occurring at 100 U/ml. Similar results were obtained with TNF (data not shown). To activate endothelium in subsequent experiments, 100 U/ml IL-1 was used. The level of transmigration induced by this dose of IL-1 was variable with different endothelial cultures and neutrophil donors, but was usually three- to fivefold greater than baseline. The permeability of the monolayers to radiolabelled albumin was not increased by treatment with 100 U/ml IL-1 (see Materials and Methods).

IL-1 preincubation of endothelial monolayers cultured on glass slides also resulted in significant increases in both adhesion and transmigration of neutrophils (see Figs 5, 6).

Chemotactic transmigration and desensitization to IL-8 and FMLP

In order to investigate whether transendothelial migration of neutrophils in response to chemotactic gradients could be desensitized specifically, transmigration in response to gradients of (72 amino acid) IL-8 and FMLP was tested after preincubation with these agents. Gradients of 1 nM were chosen for testing desensitization because these are in the middle of the dose–response curve for these factors, so a positive or negative effect should be easily observed; also, the level of transmigration at these doses is similar to that seen with cytokine-activated endothelium. Dose–response experiments indicated that for both factors, 100 nM preincubation produced greater homologous desensitization than 1 or 10 nM without affecting the heterologous response; higher concentrations (1000 nM) reduced transmigration to the heterologous factor (data not shown). Preincubation of neutrophils in 100 nM IL-8 reduced their transmigration to a chemotactic gradient of 1 nM IL-8 by 81% (Fig. 2). The transmigration response of IL-8-preincubated neutrophils to a 1 nM FMLP gradient was not different to control neutrophils. Neutrophils preincubated in 100 nM FMLP were inhibited by 69% from transmigration to a 1 nM FMLP chemotactic gradient, whereas such neutrophils were not inhibited from migrating towards 1 nM IL-8 (Fig. 2), or IL-8 at a range of concentrations (Fig. 3).

Since it was intended to test the response of desensitized neutrophils to activated endothelium which produces predominantly the 77 amino acid form of IL-8, desensitized neutrophils were also tested for response to gradients of 77-IL-8. FMLP preincubation did not inhibit neutrophil transmigration in response to 77-IL-8 over a range of concentrations (Fig. 3). Preincubation with 72 amino acid IL-8 desensitized effectively to the 77 amino acid form (data not shown).



gure 1. Neutrophil transmigration in response to IL-1-activated ıdothelium, showing dose of IL-1 preincubation. Endothelial monoyers in transwells were incubated for 4 hr in IL-1 at the indicated ıncentrations, and then washed prior to the addition of 10^6 neutroıils. Transmigrating neutrophils were quantified as described in aterials and Methods. Each point is the mean ± SEM of six experiental values (three experiments in duplicate). $^{**}P < 0{\cdot}005$ in compari-n with 0 IL-1 by independent Student's *t*-test.

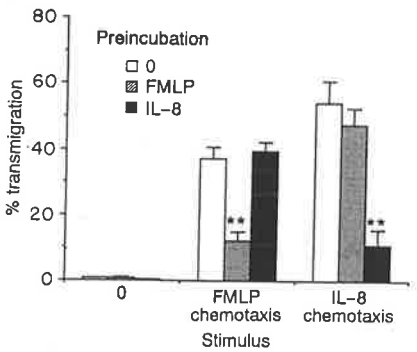


Figure 2. Specific chemotactic desensitization by preincubation with chemotactic factors. Neutrophils were preincubated with 100 nM IL-8, 100 nM FMLP or medium (0) as indicated, for 30 min, at 37°. After washing they were placed on endothelial monolayers in transwells and subjected to a chemotactic gradient (stimulus) of either 0, 1 nM FMLP or 1 nM IL-8. Columns represent the mean ± SEM results of four experimental values (two experiments in duplicate). $^{**}P < 0{\cdot}005$ in comparison with preincubation in medium alone (0), by independent Student's *t*-test.

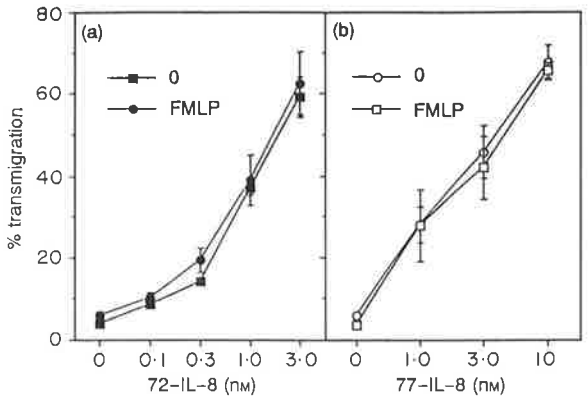


Figure 3. Effect of FMLP preincubation on neutrophil transmigration in response to a chemotactic gradient of 72 amino acid IL-8 [72-IL-8 (a)] or 77 amino acid IL-8 [77-IL-8 (b)], over a range of IL-8 concentrations. Neutrophils were preincubated in 100 nM FMLP or assay medium alone (0) at 37° for 30 min and washed. They were then placed on endothelial monolayers in transwells, with the indicated concentration of IL-8 in the lower compartment. Results are the mean ± SEM of four experimental values (two experiments in duplicate). There was no significant difference between FMLP-preincubated and control cell transmigration at any IL-8 concentration.

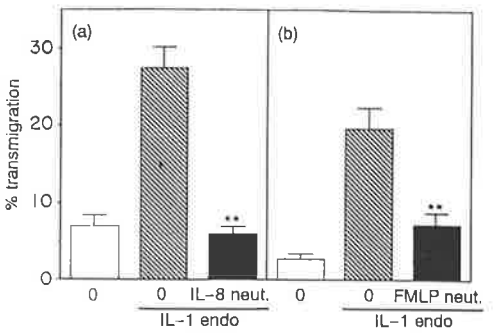


Figure 4. Inhibition of transmigration response to IL-1-activated endothelium by preincubation of neutrophils with IL-8 (a) or FMLP (b). Transmigration response of control neutrophils (preincubated in medium alone) to activated endothelium in each series of experiments (▨); preincubation of neutrophils for 30 min at 37° in 100 nM IL-8 or FMLP resulted in inhibition (■) [compare with baseline unstimulated transmigration (□)]. Pooled data from seven experiments in duplicate (a); five experiments in duplicate (b); columns represent mean ± SEM. $^{**}P < 0.005$ in comparison to control-incubated neutrophils by independent Student's *t*-test.

Preincubation of neutrophils with either FMLP or IL-8 inhibits their response to IL-1-activated endothelium

Transwell assay

Neutrophils preincubated in (desensitized to) 72-IL-8 or FMLP were assayed for transmigration response to IL-1 (100 U/ml) activated endothelium (no exogenous chemotactic gradients present). Both chemotactic stimulants inhibited the response of the neutrophils significantly (Fig. 4), although the mean inhibition by FMLP (74%) was less than that by IL-8 (104%) ($P < 0.05$).

Inhibitory effects of preincubation with either chemotactic factor were maximal at 30 min of preincubation, and a clear dose–response of preincubation was seen, with 100 nM producing maximal inhibition (data not shown).

Slide assay

Experiments using the slide transmigration assay illustrate and confirm the inhibition of transmigration after neutrophil preincubation in chemotactic agents.

Transmigration through cytokine-activated endothelium of 72-IL-8 preincubated neutrophils was virtually abolished and FMLP-preincubated neutrophils substantially inhibited (Figs 5, 6). Inhibition increased progressively with preincubation con-

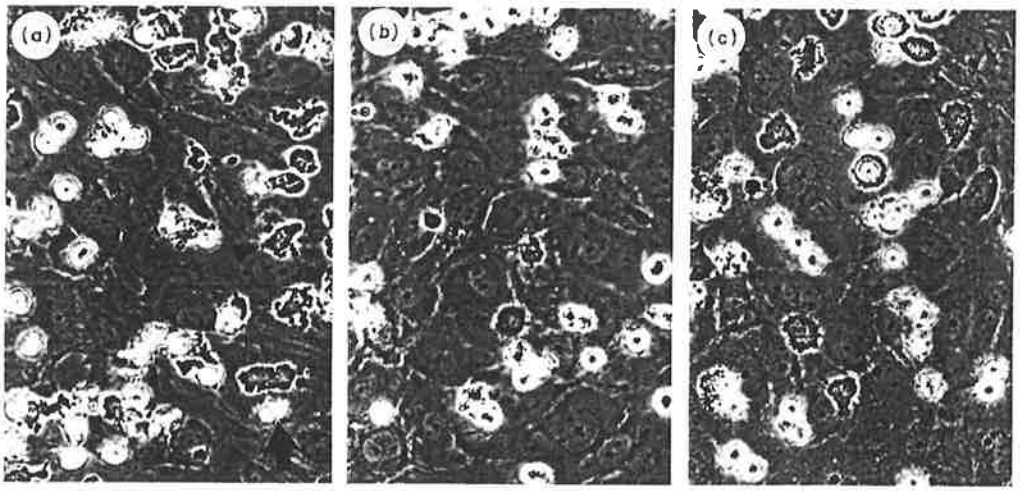


Figure 5. Photomicrographs illustrating the adhesion and transmigration of neutrophils on IL-1-activated endothelium. Endothelial monolayers were cultured to confluence on glass chamber slides (Labtek, Naperville, IL) and preincubated in 100 U/ml IL-1 for 4 hr, then washed. Neutrophils were preincubated in medium alone [controls (a)], IL-8 (b) or FMLP (c) and then co-incubated with the endothelial monolayers for 20 min. Panel (a) shows both adherent (arrow) and transmigrated (arrowheads) neutrophils. Panel (b) shows that after IL-8 preincubation, there are still numerous adherent cells, but none have transmigrated. In (c), FMLP preincubation has reduced the transmigration but not eliminated it completely. Representative fields, original magnification × 400. For quantitation of multiple fields, see Fig. 6.

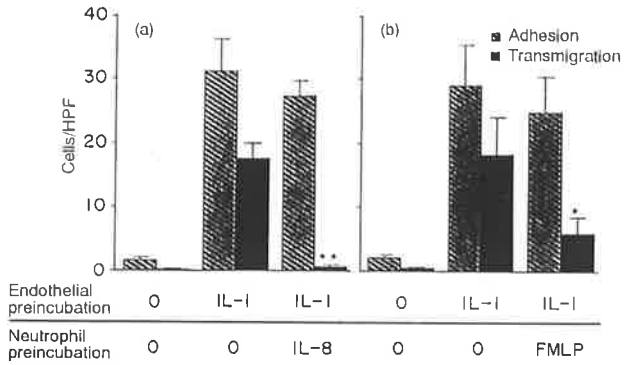


Figure 6. Effect of IL-8 (a) and FMLP (b) preincubation of neutrophils on their adhesion and transmigration response to endothelial monolayers cultured on glass slides. Endothelial monolayers were preincubated with 100 U/ml IL-1 or medium alone (0) for 4 hr and washed. Neutrophils preincubated in medium alone (0) or in 100 nM IL-8 or 100 nM FMLP were then added to the endothelial monolayer, and after a 20-min co-incubation, adhesion and transmigration were quantified by microscopy as described in Materials and Methods. Mean counts ± SEM of eight slide assays (IL-8), five slide assays (FMLP). Comparisons were made between factor preincubated and control preincubated neutrophils for both adhesion and transmigration: $^{**}P < 0{\cdot}005$, $^{*}P < 0{\cdot}05$, by paired Student's *t*-test.

centration of IL-8 and FMLP (data not shown). The slide assay also demonstrates that chemotactic preincubation did not alter the number of adherent neutrophils.

DISCUSSION

Neutrophils can be desensitized to the effects of various agonists by prior exposure to these agonists. This may occur by receptor down-modulation, in which case the desensitization will be specific to ligands which utilize the particular receptor that has been modulated. Other mechanisms for desensitization have also been proposed, such as exhaustion of post-receptor intracellular mediators; however this often results in non-factor-specific inhibition.[14]

We have desensitized neutrophils to the chemotactic effects of IL-8 and a control chemotactic factor, FMLP. These chemoattractants operate via distinct but related receptors of the G-protein family.[15,16] Despite the similarity of these receptors, others have found that they are not cross-modulated, i.e. that homologous desensitization is specific for each of these agents.[17] By incubating neutrophils in suspension with IL-8 or FMLP at concentrations supramaximal for chemotactic responses, we were able to desensitize selectively neutrophils for chemotactic transmigration to each agent (Fig. 2). They were able to transmigrate normally in response to the heterologous chemotactic stimulus, indicating, importantly, intact motility apparatus and capacity for transendothelial migration. Inhibition of the homologous response was significant but not total in the case of either factor.

Cytokine-activated endothelium induces the transendothelial migration of neutrophils,[2,3] and IL-8 production by activated endothelial cells[4] has been implicated in this.[5] We have used an ELISA to confirm that IL-8 is produced by IL-1-activated HUVEC cultured in transwells (data not shown). We therefore tested the transmigration response of desensitized neutrophils to IL-1-activated endothelium. Neutrophils which have been preincubated with IL-8 were totally inhibited from transmigrating across IL-1-activated endothelium (by a mean of 104%, Fig. 4). Surprisingly, FMLP preincubation also significantly inhibited transmigration (by 74%), although FMLP-preincubated neutrophils respond normally to gradients of both 72 and 77 amino acid forms of IL-8 over a range of concentrations (Fig. 3). This implies that there are IL-8-independent mechanisms of transmigration, which are inhibited by FMLP preincubation of neutrophils. IL-8 preincubation may inhibit the IL-8-independent mechanisms as well as desensitizing to the IL-8-dependent mechanism, accounting for the total inhibition. This conclusion is consistent with recent work by Huber *et al.*[5] which showed that neutralizing antibodies against IL-8 only partially inhibit transmigration of neutrophils through cytokine-activated endothelium.

The role of IL-8 as a mediator of transmigration for cytokine-activated endothelium may seem inconsistent with reports of its inhibition of neutrophil adhesion to activated endothelium.[18] We have used direct observation methods to demonstrate that preincubation with IL-8 reduces the number of endothelium-associated neutrophils, but this is due to an inhibition of transmigration only, and not of adhesion (Figs 5, 6). IL-8 injected intravenously, i.e. acting on neutrophils on the lumenal side of the endothelium, has been reported to have anti-inflammatory effects.[19] This may correspond to our finding of inhibition of transmigration by preincubation of neutrophils in IL-8; indeed, intravenous FMLP had similar effects.[19] Therefore, IL-8 stimulation of neutrophils before their encounter with endothelium will have different effects to IL-8 released locally by activated endothelial cells.

The nature of the IL-8-independent mechanism of transmigration that we propose is unknown, but there are several possibilities. Another chemotactic factor in addition to IL-8 may be secreted from the endothelial cells which induces neutrophil transmigration in an equivalent manner to IL-8; the neutrophil receptors for this factor may be 'cross down-modulated' by incubation of the neutrophils in either IL-8 or FMLP. However, the majority of the chemotactic activity in the supernatants of activated endothelial cells is accounted for by IL-8[4,20] in 72 or 77 amino acid form, or its close relative MGSA (melanoma growth stimulatory activity)[21] which shares the same receptor.[22]

A further possible explanation for the IL-8-independent mechanism of transmigration is that the migration response to activated endothelium does not involve chemotaxis in the classical sense. It may be mediated by cell-surface receptor–ligand interactions between neutrophils and activated endothelial cells, and the neutrophil surface structures could be altered by stimulation with IL-8 or FMLP. A candidate molecule is L-selectin (LAM-1), which is shed from the neutrophil surface after stimulation by IL-8.[23,24] However, L-selectin is currently thought to be involved in the rolling of leucocytes along the endothelial wall, but not in their transmigration.[25] A second candidate is sialylated Lewis-X, the neutrophil ligand for the TNF-induced endothelial adhesion molecule E-selectin. However, this molecule is not altered in expression after IL-8 treatment of neutrophils.[26]

The neutrophil integrins of the CD18 group are clearly essential for transendothelial migration. Neutrophils from patients who congenitally lack this molecule are not able to transmigrate *in vitro*,[13] or localize at inflammatory sites *in vivo*,[27]

and in addition, antibodies to this molecule block transmigration.[13] It seems paradoxical that chemotactic stimulation, which increases expression and activation of the CD18 complexes LFA-1 and MAC-1,[23] decreases transmigration. Recently it has been found that contact of neutrophils with E-selectin induces the adhesive conformation of MAC-1.[28] It has been postulated that E-selectin displayed by activated endothelium could act as a solid phase chemoattractant.[28] Thus for transmigration to occur, the localized and sequential activation of MAC-1 may be important. Generalized activation of CD18 complexes by preincubation of neutrophils with chemotactic factors in suspension may antagonize this process.

Current evidence favours a three-step model for the extravasation of neutrophils at inflammatory sites.[25] The first step is the rolling of neutrophils along the endothelial surface, mediated by the selectins; the second is firm adhesion, mediated by the integrin–ICAM interaction; and the third is the transendothelial migration of cells into the tissues. The production of IL-8 by activated endothelial cells suggests that an IL-8 gradient may be one of the mechanisms of the transmigration step. Our finding that desensitization of neutrophils to IL-8 reduces their transmigration in response to activated endothelium supports this. However, our finding that FMLP desensitization, which does not reduce the IL-8 response, also reduces transmigration, points to other, non-IL-8-dependent mechanisms of transmigration through activated endothelium.

In vivo, neutrophils which are activated by cytokines intravascularly are shown to be defective in migration into inflammatory sites.[19,29,30] There are now several mechanisms to account for this. Shedding of neutrophil surface L-selectin after chemotactic factor stimulation may reduce the ability of endothelial adhesion molecules to capture the neutrophil from the circulation.[24] As shown here, factor-specific desensitization of neutrophils may inhibit transmigration in response to transendothelial chemotactic gradients. In addition, preactivation of neutrophils may also inhibit their transmigration through cytokine-activated endothelium.

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Transendothelial migration of neutrophils involves integrin-associated protein (CD47)

(inflammation/extravasation/chemokines/β_2 integrins)

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ABSTRACT **Inflammation is a primary pathological process. The development of an inflammatory reaction involves the movement of white blood cells through the endothelial lining of blood vessels into tissues. This process of transendothelial cell migration of neutrophils has been shown to involve neutrophil β_2 integrins (CD18) and endothelial cell platelet-endothelium cell adhesion molecules (PECAM-1; CD31). We now show that F(ab')$_2$ fragments of the monoclonal antibody B6H12 against integrin-associated protein (IAP) blocks the transendothelial migration of neutrophils stimulated by an exogenous gradient of the chemokine interleukin 8 (IL-8; 60% inhibition), by the chemotactic peptide *N*-formylmethionylleucylphenylalanine (FMLP; 76% inhibition), or by the activation of the endothelium by the cytokine tumor necrosis factor α (98% inhibition). The antibody has two mechanisms of action: on neutrophils it prevents the chemotactic response to IL-8 and FMLP, and on endothelium it prevents an unknown but IL-8-independent process. Blocking antibodies to IAP do not alter the expression of adhesion proteins or production of IL-8 by endothelial cells, and thus the inhibition of neutrophil transendothelial migration is selective. These data implicate IAP as the third molecule essential for neutrophil migration through endothelium into sites of inflammation.**

The movement of leukocytes from blood into tissues or transendothelial migration (TM) is a complex process. Initial events leading to the adhesion of leukocytes to endothelial cells have been carefully studied. Leukocyte capture by selectins from the axial stream, tethering by chemokines, and subsequent firm adhesion to the endothelial cell by means of leukocyte integrins are all required for movement of cells into tissues (1, 2). Chemokines and integrins also play a role in the process of leukocyte diapedesis across the endothelial barrier (3–5). Chemokines can be elaborated in the tissues by various cell types or by invading microorganisms, and the cellular specificity of TM is likely to be determined by the type of chemokine elaborated. However, after activation with tumor necrosis factor α (TNF-α) or interleukin 1β (IL-1β) endothelial cells are also capable of secreting chemokines, and, as luminal material is removed by the circulation, a chemokine gradient is generated (3–5). This has led to the hypothesis that TM after the activation of endothelium is mediated by an endogenous chemokine gradient. However, desensitization (6), as well as neutralization, experiments (3) have suggested that mechanisms other than those involving chemokines also can mediate TM.

Two molecules have been implicated in neutrophil TM so far. β_2 integrins on the neutrophil are essential, as evidenced by the lack of neutrophil chemotaxis in patients with a genetic defect in β_2 integrin or by neutrophils treated with anti-β_2 integrin blocking antibodies (7–10). β_2 integrins can exist in different affinity states, and chemotaxins readily induce the appearance of a high-affinity state (11). It has thus been assumed that TM not only involves β_2 integrin but also a conformational change in these molecules. Antibodies to platelet/endothelium cell adhesion molecule 1, an antigen that is present both on the neutrophil and on endothelium at interendothelial cell junctions, also blocks TM (12). However, the effect of this antibody appears to be primarily on the endothelium rather than the neutrophil (ref. 13 and C. Bernard, J.R.G., S. Albelda, and M.A.V., unpublished data). Since cell movement is not associated with leakage of plasma components or a loss of electrical resistance (4), there is also likely to be a strong adhesion between the transmigrating cell and the endothelium. Evidence for the local interaction between leukocytes and endothelium is the localized calcium flux induced in endothelial cells surrounding the intruding leukocyte (14).

Since TM requires integrins we hypothesized that a protein or proteins that regulate the function of integrins may have important regulatory roles in TM. Integrin-associated protein (IAP) is a 50-kDa membrane protein with an amino-terminal immunoglobulin domain and carboxyl-terminal membrane spanning region that is probably identical with the rhesus factor-related antigen CD47 (15, 16). IAP is present on both neutrophils and endothelial cells and has been shown to amplify the response of neutrophils to adhesive and chemotactic processes involving Arg-Gly-Asp (RGD)-containing proteins that appear to be recognized primarily by β_3 integrins (17, 18). On endothelial cells IAP allows the development of calcium flux after contact with extracellular matrix proteins (19). Because IAP is involved in Ca^{2+} regulation in endothelium and this Ca^{2+} rise is required for neutrophil TM, we hypothesized that IAP could have a role in TM. The current experiments were designed to test this hypothesis.

MATERIALS AND METHODS

Purification of Human Neutrophils. Neutrophils were purified from normal donors by dextran sedimentation and gradient centrifugation, followed by hypotonic lysis of erythrocytes. Neutrophils were resuspended in RPMI medium 1640 supplemented with 10 mM Hepes and 2.5% fetal calf serum (FCS) (Flow Laboratories). The purity of neutrophils prepared in this way was >95%, as judged by morphology of stained cytocentrifuged preparations, and the viability was >98%, as judged by trypan blue exclusion.

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Abbreviations: TM, transendothelial migration; TNF, tumor necrosis factor; IAP, integrin-associated protein; IL-*n*, interleukin *n*; FMLP, *N*-formylmethionylleucylphenylalanine; HUVEC, human umbilical vein endothelial cell; FCS, fetal calf serum; RGD, Arg-Gly-Asp.

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Endothelial Cell Culture. Human umbilical vein endothelial cells (HUVECs) were isolated by collagenase treatment of umbilical veins (20). HUVECs were resuspended and maintained in medium 199 supplemented with Earle's salts (Cytosystems, Sydney, Australia), 20% FCS, 20 mM Hepes, 2 mM glutamine, 1% nonessential amino acids, 1 mM sodium pyruvate (Cytosystems), 0.225% sodium bicarbonate, and antibiotics. HUVECs were cultured in gelatin-coated tissue culture flasks and passaged when they reached confluence by detaching them with trypsin/EDTA (Cytosystems). Cells were used between passages 2 and 6 for all experiments. Endotoxin levels in medium were <50 pg/ml as determined by a Limulus amoebocyte assay.

Cell Culture Reagents. Purified human fibronectin (Boehringer Mannheim) was diluted in phosphate-buffered saline (PBS), pH 7.3, to 50 μg/ml for use in coating transwells. HUVECs were cultured in heparin (Sigma) and endothelial cell growth supplement (Collaborative Research), both at a final concentration of 20 μg/ml.

Stimuli. Recombinant human TNF-α, (batch number 3056-55 at 0.5 μg/ml, 2×10^7 units/ml) produced in *Escherichia coli* (Genentech), was used at 100 units/ml in all experiments. *N*-formylmethionylleucylphenylalanine (FMLP); Sigma) was prepared as a stock solution at 1 mM in absolute alcohol, stored at −20°C, and diluted prior to use to 0.1 mM. Chemically synthesized IL-8 was a gift from Ian Clark-Lewis (Centre for Biomedical Research, Vancouver) (21). IL-8 was used at 0.05 mM for all chemotaxis and TM experiments.

Antibodies. Polyclonal rabbit AB24 antiserum to IL-8 was a gift from S. L. Kunkel, University of Michigan. Anti-IL-8 serum was used at a 1:500 dilution in all TM assays. For the IL-8 ELISA, immunoglobulin was purified from this serum by sequential ammonium sulfate precipitation and protein A column chromatography. $F(ab')_2$ fragments of monoclonal antibodies directed against IAP (B6H12 functional and 2D3 nonfunctional control) were used at 5 μg/ml unless indicated (18).

B6H12 and 2D3 are well characterized antibodies recognizing IAP (CD47) (16, 18). Both antibodies recognize IAP by immunoprecipitation and Western blot, and both antibodies react with CHO cells only when they have been transfected with human IAP cDNA (15). While B6H12 inhibits IAP function in neutrophils, endothelial cells, and transfected CHO cells, 2D3 is not inhibitory in any assay to date (15, 19, 22). Nonetheless, 2D3 recognizes IAP with an affinity that is approximately equal to that of B6H12 (23).

2D3 and B6H12 recognize distinct epitopes on IAP, as shown in both competition assays (18) and by binding to human–mouse hybrid IAP proteins (F.P.L. and E.J.B., unpublished data). The antibody 49-1B11 is directed against E-selectin and was raised in house. Its specificity was shown by reactivity to cells transfected with E-selectin cDNA.

Chemotaxis Assay. Chemotaxis assays were performed by using transwells (Costar). A 24-well culture tray was precoated with gelatin for 10 min at room temperature to prevent neutrophil adhesion. The gelatin was aspirated off, and 600 μl of assay medium (RPMI medium 1640 supplemented with 2.5% FCS) was added to the compartment in the presence or absence of chemokine. The transwells (6.5 mm diameter, 3 mm pore size) were added to this 24-well tray and 10^6 neutrophils in 100 μl of assay medium were placed into the transwells. Neutrophils which had migrated through the filters after a 1-h incubation at 37°C in an atmosphere of 95% air/5% CO_2 were retrieved from the lower compartment and counted by using a Coulter counter (model ZF). Cell counts were expressed as a percentage of the total number of cells added.

TM Assay. Transwells were precoated with fibronectin at 50 μg/ml for 30 min at room temperature and then seeded with HUVECs at 5×10^4 cells per well in 150 μl of HUVEC medium. Transwells were then placed into 24-well trays containing 700 μl of HUVEC medium and cultured for 3 or 4 days at 37°C in an atmosphere of 95% air/5% CO_2. Prior to the experiment some HUVEC monolayers cultured in transwells were incubated with TNF-α for 4 h or incubated with antibody for 30 min at 37°C and 95% air/5% CO_2. The transwells were then washed in assay medium and transferred to a 24-well tray which had been precoated with gelatin and which contained 600 μl of assay medium with or without chemokine or antibody. Medium was then aspirated from the transwell and replaced by 10^6 neutrophils in 100 μl of assay medium. In some experiments the neutrophils were preincubated for 30 min at room temperature with anti-IAP $F(ab')_2$ antibody, which was either washed out or left present throughout the assay, depending upon the experiment. After 1 h, migrated neutrophils were counted by using a Coulter counter, and the counts were expressed as a percentage of the total number of neutrophils added.

IL-8 ELISA. Supernatants were taken from cultures of HUVECs grown in 96-well trays, which had been treated exactly as for transmigration assays. IL-8 in these supernatants was quantitated by ELISA (24). Protein A-purified anti-IL-8 at a concentration of 1 μg/ml in bicarbonate buffer, pH 9.6, was used to coat 96-well polyvinyl chloride plates (Costar) at 4°C overnight. Plates were washed three times in PBS/0.05% Tween 20 (wash medium) between each step. Samples and standards (synthetic IL-8 in HUVEC medium), 100 μl/well, were incubated for 1 h, at 37°C in 95% air/5% CO_2. Purified anti-IL-8 was biotinylated using Sulfo-NHS-Biotin (Pierce), according to the manufacturer's directions. Biotinylated anti-IL-8 was diluted 1:250 in wash medium supplemented with 1% FCS, and after washing, 100 μl was added to each well for 30 min at 37°C and in 95% air/5% CO_2. A conjugate of streptavidin/biotinylated horseradish peroxidase (Amersham) was diluted 1:1000 in wash medium supplemented with 1% FCS, and after being washed, 100 μl was added to each well for 30 min at 37°C and 95% air/5% CO_2. Finally, 100 μl of a 1 mg/ml solution of *o*-phenylenediamine (Sigma) in citrate buffer containing 0.03% H_2O_2 was added to each well for 30 min at room temperature. The reaction was stopped by the addition of 50 μl of 1 M H_2SO_4 to each well, and the absorbance determined at 490 nm by using a microplate reader (Bio-Rad). Sample values were calculated by using an asymmetric sigmoidal curve fit of the standard values in ng/ml of IL-8. Samples from each separate supernatant were assayed in triplicate, and the mean value ± SEM was represented.

Flow Cytometry. Flow cytometry was used to determine the effect of anti-IAP antibodies on the expression of E-selectin on HUVECs. HUVECs were cultured to confluence in 24-well trays. Cells were then either left unstimulated or stimulated with TNF-α for 4 h, washed, and then incubated with anti-IAP at 5 μg/ml for 30 min at 37°C. After being washed, cells were incubated at 4°C for 30 min with an anti-E-selectin antibody directly conjugated to fluorescein isothiocyanate. After being washed, HUVECs were released from the 24-well plate by trypsinization and fixed for flow cytometric analysis. The mean fluorescence intensity of these cells was determined by using a Coulter Epics Profile II flow cytometer, counting 10,000 cells for each experimental point.

Statistics. Means were calculated and compared by Student's two-tailed *t* test.

RESULTS

Anti-IAP Antibody B6H12 Inhibits Neutrophil TM. $F(ab')_2$ fragments of two antibodies against IAP were added to two systems in which neutrophil TM through confluent endothelial monolayers was induced. Anti-IAP monoclonal antibody B6H12 blocks stimulation of phagocytosis by RGD peptide (18), integrin-mediated increase in endothelial $[Ca^{2+}]$ (19), and neutrophil adhesion to entactin (25). Anti-IAP monoclo-

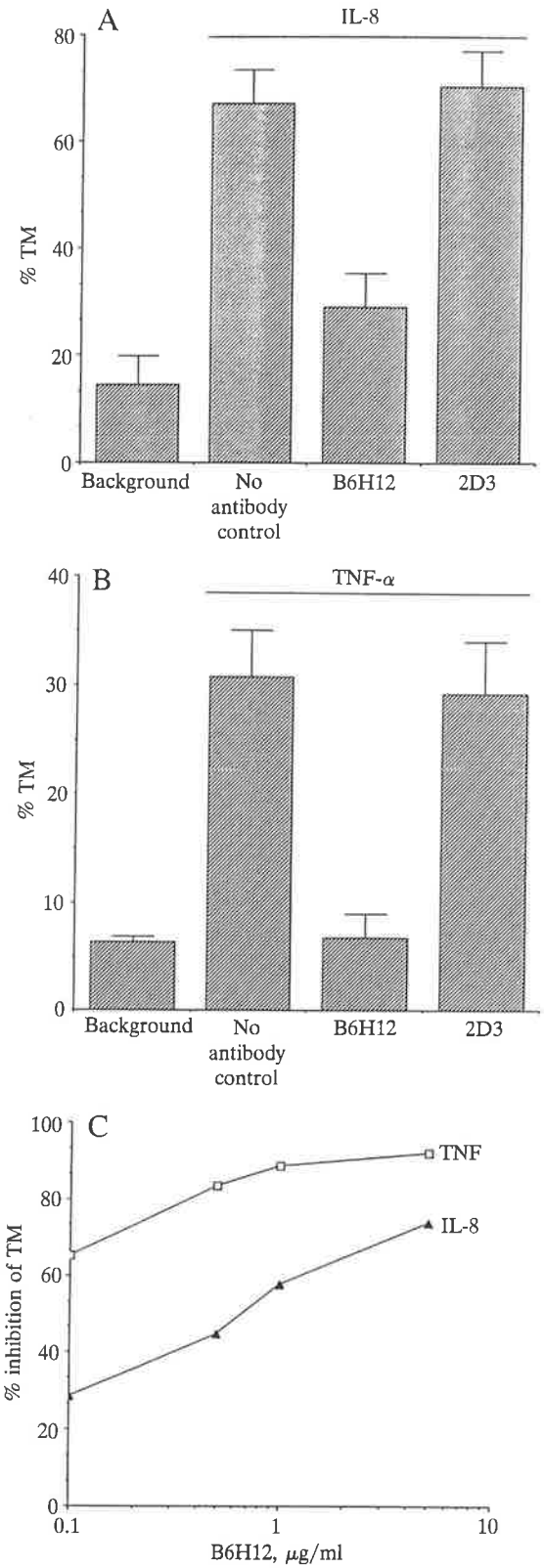

FIG. 1. Effect of anti-IAP on IL-8-stimulated (*A*) and TNF-α-stimulated (*B*) TM of neutrophils. HUVEC monolayers were prepared on polycarbonate filters in the transwell system. These were either stimulated with IL-8 at 5×10^{-9} M in the lower compartment (*A*) or by TNF-α at 100 units/ml for 4 h at 37°C (*B*). Neutrophils in the presence or absence of 5 μg of anti-IAP per ml were added to the upper compartment and incubated at 37°C for 1 h. The percent TM was determined by counting cells in the lower compartment and comparing that number with the total number of cells added. The data represent mean ± SE from three different experiments, each consisting of a duplicate determination. The antibody B6H12 significantly reduced TM in both IL-8-stimulated (60% inhibition; $P = 0.002$) and TNF-α-stimulated (98% inhibition; $P = 0.0002$) cultures. A representative dose-response effect of B6H12 inhibition of TM in both TNF-α- and IL-8-stimulated cultures is shown in *C*. The data represent the mean percent inhibition of TM of one experiment with duplicate determinations that is representative of two similar experiments. Background, unstimulated control culture.

Table 1. Expression of IAP by HUVECs

	Exp. 1		Exp. 2		Exp. 3	
Antibody	−TNF	+TNF	−TNF	+TNF	−TNF	+TNF
None	0.24	—	0.22	0.31	—	0.21
B6H12	7.89	8.72	8.00	9.44	4.53	5.66
2D3	8.61	9.10	10.4	10.5	4.87	6.00

Confluent endothelial cells in multiwell plates were either untreated or treated with 100 units of TNF-α per ml for 4 h at 37°C before being processed for flow cytometry. IAP expression was determined by incubating cells with 5 μg of anti-IAP per ml for 30 min at 4°C, washing them, and then incubating them with fluorescein isothiocyanate-conjugated anti-mouse antibody. A unimodal distribution was seen in all cases. Values shown are the mean peak fluorescence.

nal antibody 2D3, which binds to IAP with affinity equal to that of B6H12, has no inhibitory effects in these systems (22).

In the first system, TM was induced by the addition of the chemokine IL-8 placed into the lower compartment. $F(ab')_2$ fragments of B6H12 blocking but not 2D3 nonblocking anti-IAP antibodies strongly inhibited this process by 60% ± 6.4% (mean ± SEM; $n = 3$; Fig. 1*A*). TM induced by FMLP was also inhibited by 78% ± 3.1%; $n = 3$; data not shown). In the second system of TM, endothelial cell monolayers were pretreated for 4 h with 100 units of TNF-α per ml. The cells were then washed, and neutrophils were added. TM in this system was totally inhibited (98% ± 2%; $n = 4$) by B6H12 (Fig. 1*B*). Dose-response experiments (Fig. 1*C*) showed that the inhibition was dose dependent and that TNF-α-induced TM was more sensitive to B6H12 than IL-8-induced TM. Endothelial cell IAP was recognized equally well by B6H12 and 2D3, and there was no significant change in the expression of these molecules after 4 h of treatment with TNF-α (Table 1).

Anti-IAP Inhibits Chemotactic TM by an Effect on the Neutrophil. Preincubation experiments were performed to test whether the effect of anti-IAP in the chemokine-induced TM was mediated on the neutrophil or the endothelium. As seen in Fig. 2*A*, pretreatment of the neutrophils but not the endothelium resulted in the inhibition of TM in response to an exogenous gradient of IL-8. This suggested that anti-IAP may be a general inhibitor of neutrophil mobility. To test this, cell movement was measured in a transwell system without endothelium. Indeed, anti-IAP inhibited neutrophil chemotaxis to IL-8, as well as to the chemotactic agent FMLP (Fig. 3). The possibility that anti-IAP inhibits β_2 integrin expression was tested by flow cytometry. Neither B6H12 blocking nor 2D3 nonblocking antibody induced a significant change in CD18 expression (data not shown).

Anti-IAP Inhibits TM Through Activated Endothelium by an Effect on the Endothelium. Preincubation experiments revealed a different pattern of inhibition where TM through TNF-α-activated endothelium was measured (Fig. 2*B*). While preincubation of neutrophils with anti-IAP had a partial effect, preincubation of endothelial cells was also effective. Since preincubation of endothelial cells with B6H12 had little or no effect on chemotactic movement, we suggest that this inhibition reveals a mechanism that acts directly on the endothelium. The possibility that anti-IAP is a broad spectrum inhibitor of endothelial responses to TNF-α was tested by measuring TNF-α-induced expression of E-selectin and IL-8. Anti-IAP antibodies reduced neither of these responses (Table 2), suggesting that the effect on TM was specific.

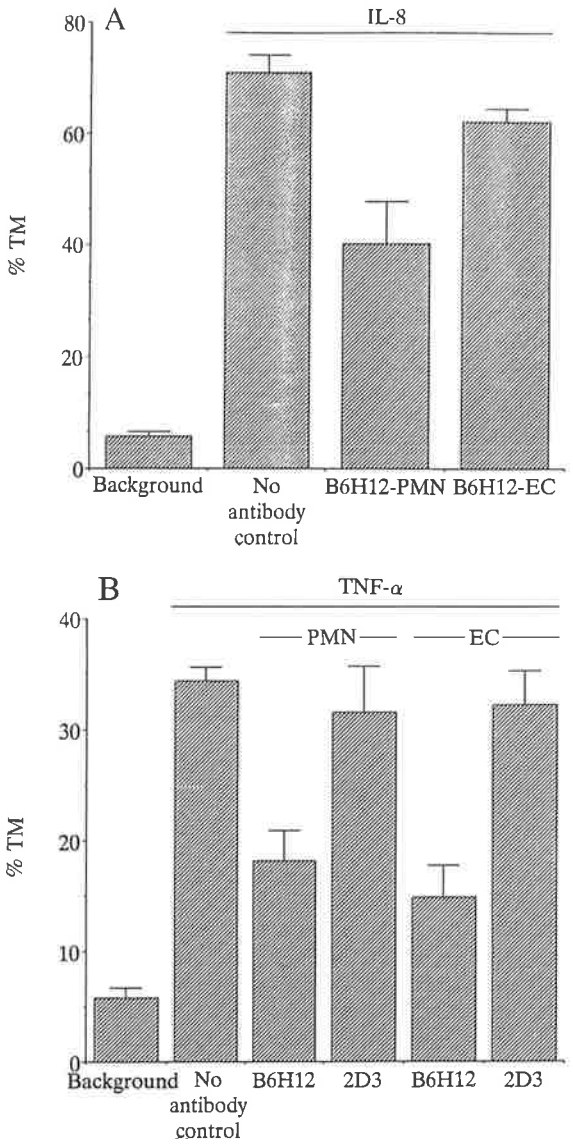

FIG. 2. Effect of anti-IAP on IL-8-stimulated (*A*) and TNF-α-stimulated (*B*) TM of neutrophils. Neutrophils (PMN) or endothelial cells (EC) were selectively pretreated with anti-IAP at 5 μg/ml for 30 min at 37°C, and the antibody was washed out prior to the TM assay. After 1 h at 37°C migrating cells were enumerated, and the percent TM was calculated. Data represent mean ± SE of three separate experiments, each consisting of duplicate determinations. In the IL-8-stimulated cultures (5×10^{-9} M for 1 h at 37°C; *A*), pretreatment of PMNs produces a significant reduction in TM ($P = 0.002$), whereas pretreatment of ECs produces a nonsignificant effect ($P = 0.1$). In TNF-α-stimulated cultures (100 units/ml for 4 h at 37°C; *B*), pretreatment of either PMNs or ECs produces a significant reduction in number of migrating cells ($P = 0.003$ and $P = 0.003$, respectively). Background, unstimulated control culture.

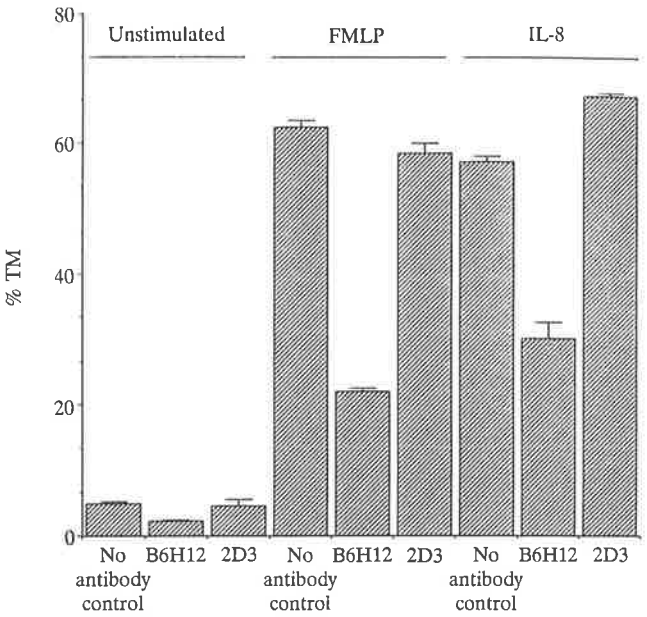

FIG. 3. Effect of anti-IAP on FMLP- and IL-8-stimulated migration of neutrophils through a polycarbonate filter. FMLP at 10^{-8} M or IL-8 at 5×10^{-9} M was placed in the lower compartment of a transwell. Neutrophils in the presence or absence of 5 μg of anti-IAP per ml were added to the upper compartment. After 1 h at 37°C, migrating cells in the lower compartment were enumerated in the lower compartment and the percent of migrating cells was determined by comparison with the total number of neutrophils added. The data represent mean ± SE of three experiments, each consisting of duplicate determinations. The effect of B6H12 on migration is significant in both FMLP ($P = 0.0006$) and IL-8 ($P = 0.001$) stimulated chemotaxis.

DISCUSSION

The chief finding in this paper is that antibodies against IAP strongly inhibit neutrophil TM. The use of $F(ab')_2$ antibodies eliminates the possibility that binding through the Fc receptor confounded our results, and the use of antibodies that bound to the same structure to similar extents but had different capacities to block the response makes nonspecific steric effects unlikely. Thus, we believe that IAP has an essential role involved in the process of neutrophil TM.

Two complementary mechanisms appear to operate. IAP is involved in neutrophil responses to chemoattractants, whether exogenously applied (Figs. 1*A*, 2*A*, and 3) or generated endogenously by activated endothelial cells (Figs. 1*B* and 2*B*). Previous publications have shown that anti-IAP inhibits chemotactic responses to RGD-containing peptides or proteins by a mechanism that involves the β_3-like integrin, leukocyte response integrin (LRI) (18, 25). However, TM is exquisitely β_2 integrin dependent, and it has previously been shown that IAP does inhibit β_2 integrin function (23, 24). A pathway involving LRI and IAP has been shown in phorbol-ester-stimulated Mac-1 activation (26) and in monocyte binding of the filamentous hemagglutinin of *Bordatella pertussis* (27). Since anti-IAP does not change β_2 integrin expression, the possibility exists that anti-IAP functions by inhibiting the

Table 2. Effect of anti-IAP on IL-8 production and E-selectin expression

Antibody	IL-8, ng/ml*	E-selectin, MIF†	
		−TNF-α	+TNF-α
None	1.124 ± 0.145	0.252	2.049
B6H12 (5 μg/ml)	1.136 ± 0.115	0.248	2.287
2D3 (5 μg/ml)	1.408 ± 0.194	0.254	2.295

*Endothelial cells in 96-well plates were treated with 100 units of TNF-α per ml for 4 h, washed, and incubated for a further hour in the presence or absence of anti-IAP antibodies. Supernatant was collected, and IL-8 concentration was determined. Similar conclusions were reached when IL-8 production was determined over the 4-h TNF-α incubation period. Values shown are the mean ± SEM of three determinations.

†E-selectin expression was determined after incubating endothelial cells in the presence or absence of 100 units of TNF-α per ml for 4 h at 37°C. Cells were then washed and incubated in the presence or absence of anti-IAP antibodies for 30 min. After being washed, endothelial cells were incubated for 30 min at 4°C with fluorescein isothiocyanate-conjugated anti-E-selectin. Similar results were obtained in a duplicate experiment. Values shown are the mean intensity of fluorescence (MIF; linear values).

expression of an epitope such as the "24" epitope (28) on β_2 integrins that is associated with its activation (3). In preliminary experiments, a variable inhibition (5–100%) of FMLP-stimulated expression of the 24 activation epitope was observed (data not shown). Nevertheless it is possible that β_3 integrins recognizing extracellular matrix or basement membrane proteins are also involved in TM and that engagement of β_3 integrins results in β_2 integrin activation.

The second mechanism of action of IAP is revealed by preincubation experiments of the endothelium. In this instance there is no inhibition of responses to exogenous chemotactic gradients, yet responses to activated endothelium are strongly suppressed. Indeed, IAP proteins have been demonstrated to be present on endothelial cells and to be involved in the generation of transient calcium fluxes in response to plating on fibronectin but not when stimulated with histamine (19). As neutrophil TM has also been shown to be associated with the generation of calcium fluxes (14), it is tempting to hypothesize that IAP exerts its effect through this process. It is clear that the mechanism of IAP is not global, since other TNF-α-mediated responses (Table 2) are not inhibited.

Our results also demonstrate that TM through activated endothelium involves at least two different mechanisms. Although there has been indirect evidence for an IL-8-independent process in neutrophil TM (6), our data show a direct effect on endothelium that does not appear to involve IL-8, either at the level of responsiveness or production (Fig. 2*A* and Table 2). Indeed, the effect of anti-IL-8 antibodies and anti-IAP appear to be additive (data not shown). It is interesting to note that in the literature anti-IL-8 has inhibited TM through activated endothelium by between 30% and 70% (3, 6). It is possible that TM has two phases: initially it is driven by IL-8, but once the first neutrophils have transmigrated, the digestion of basement membrane proteins results in the release of other chemotaxins like entactin (25) that propagate the process. IAP inhibits responses to IL-8 and entactin (Fig. 1 and ref. 22) and entactin-mediated TM may be one of the as yet uncharacterized IL-8-independent processes.

Finally, our findings have considerable clinical relevance. Other agents that inhibit TM—e.g., anti-β_2 integrin antibodies—have been noted to inhibit reperfusion injuries, frost bite, and adult respiratory distress-like syndromes (28). While our findings only address one cell type in an *in vitro* system, they suggest a central role for IAP in the regulation of leukocyte–endothelial interaction and raise the possibility that antagonists of IAP may also have a therapeutic function.

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A NEW METHOD FOR THE PURIFICATION OF HUMAN EOSINOPHILS AND NEUTROPHILS, AND A COMPARISON OF THE ABILITY OF THESE CELLS TO DAMAGE SCHISTOSOMULA OF *SCHISTOSOMA MANSONI*

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Centrifugation of human white blood cells over either Ficoll-Hypaque or slightly hypertonic Metrizamide discontinuous gradients reliably produces separate fractions that are enriched for either neutrophilic or eosinophilic granulocytes. This single step purification routinely yields 90 to 100% pure neutrophils and 85 to 100% pure eosinophils. Metrizamide gradients, in particular, reproducibly provide high yields of 90 to 100% pure eosinophils from normal subjects with 2 to 3% eosinophils in their peripheral blood. The method does not damage cells as judged by morphologic or functional criteria.

The purified cell populations were tested for their ability to damage antibody-coated schistosomula either by the measurement of ^{51}Cr release from labeled organisms, or by direct morphologic assessment. Neutrophils were superior in their ability to release ^{51}Cr from labeled organisms, but eosinophils adhered to the organisms to a greater extent and induced microscopically detectable damage.

During experimental infection with *Schistosoma mansoni*, it has been found that animals bearing a primary infection acquire the capacity to resist superinfection with a secondary challenge of cercariae (1, 2). A similar resistance to superinfection, in the presence of a continued primary infection, may occur during natural infections of man (3). In mice, this acquired resistance appears to be a result of an antibody-dependent cell-mediated immune response, in that immunity can be transferred with antibody, but depends on the presence in the recipient of a radiosensitive cell (4, 5). In this context, Butterworth *et al.* (6, 7) have previously reported that human granulocytes, but not mononuclear cells, can induce an antibody-dependent release of ^{51}chromium from labeled larvae (schistosomula) of *Schistosoma mansoni in vitro*. Experiments with anti-eosinophil and anti-neutrophil sera suggested that the eosinophil was a major effector cell. Preparations, containing up to 98% eosinophils and usually devoid of detectable neutrophils, would both induce release of ^{51}chromium and cause marked damage detectable by phase contrast and electron microscopy (8–10). Comparable observations have since been reported with rat eosinophils (11). These findings led to experiments by Mahmoud *et al.* (12) to test directly for the role of eosinophils *in vivo*, in which it was found that ablation of circulating eosinophils by treatment with anti-eosinophil serum resulted in a loss of both actively acquired and passive immunity in the mouse.

In our early studies, granulocyte preparations that had been depleted of eosinophils by centrifugation over two separate density gradients had no detectable effect in the ^{51}chromium release assay, whereas eosinophil-enriched fractions from the same gradients had a marked effect. In these experiments, however, the yield of neutrophils in the "eosinophil-depleted" fractions was low, amounting to 5 to 10% of the total neutrophils applied, and it was possible that they contained a small subpopulation of damaged or otherwise inactive cells. In addition, since the cells tested in these fractionation experiments were usually obtained from patients with eosinophilia, it was possible that the eosinophil-enriched fractions contained functionally abnormal cells.

In pursuing this problem further we have now modified a technique originally devised by Ross and his colleagues (13, 14) for the separation of bone marrow cells, based on the centrifugation of cells over multiple discontinuous gradients, in order to obtain both neutrophils and eosinophils in *high* yield from *normal* human peripheral blood. On comparing the functional properties of these cells, we have found that neutrophils as well as eosinophils can mediate damage to antibody-coated schistosomula sufficient to allow release of ^{51}chromium from labeled organisms. However, only in the case of the eosinophil, which adheres to schistosomula much more extensively than does the neutrophil, does this initial lesion progress to gross damage detectable microscopically after 18 to 24 hr of incubation. This suggests that the initial lesion is repairable in the case of the neutrophil, but is irreversible in the case of the eosinophil.

MATERIALS AND METHODS

Media. Minimal essential medium (MEM; GIBCO, Grand Island, N. Y.) and MEM supplemented with 10% fetal calf serum inactivated at 56°C for 1 hr (MEM/FCS) were made to

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contain 100 units/ml penicillin G, 100 μg/ml streptomycin (Microbiological Associates, Bethesda, Md.), 1% L-glutamine, and 25 mM HEPES[4] buffer. Other media were MEM/FCS to which 30 mg/liter DNase (Worthington Biochemical Corp., Freehold, N. J.: 2045 units/mg) were added (MEM/FCS/DNase); Earle's balanced salt solution with 0.5% lactalbumin hydrolysate, 100 units/ml penicillin G, 100 μg/ml streptomycin (E/lac); Tyrode's solution, each liter of which contained 1 g of dextrose, 1 g $NaHCO_3$, 0.2 g of KCl, 8 g of NaCl, and 0.05 g of Na_2HPO_4 (anhydrous); and Tyrode's solution containing 0.1% gelatin and 30 mg/liter of DNase (Tyrode's gel/DNase).

Parasite cycle and preparation of schistosomula. The methods involved are described in detail elsewhere (15, 16). Briefly, *S. mansoni* was maintained by passage in laboratory-bred *Biomphalaria glabrata* snails and CF mice (Charles River Labs, Wilmington, Mass.). Schistosomula were prepared by allowing cercariae to penetrate an isolated preparation of rat skin *in vitro* (17). The organisms were stored overnight in E/Lac containing 10% heat-inactivated fetal calf serum. After overnight storage at 4°C, the schistosomula were labeled with ^{51}Cr sodium chromate (New England Nuclear, Boston, Mass.) at 1000 μCi/10^4 organisms for 3 to 4 hr at 37°C, and were washed four times in MEM and twice with MEM/FCS, or six times in MEM/FCS.

Human sera. Serum samples were obtained from patients with *S. mansoni* infection and were heat inactivated at 56°C for 1 hr before use. Serial dilutions in MEM/FCS were tested for their ability to induce chromium release from labeled schistosomula in the presence of unpurified normal peripheral blood leukocytes. The antisera had no direct toxicity to schistosomula, and dilutions were chosen that showed high levels of cell-dependent cytotoxicity.

Preparation of effector cells. Peripheral blood leukocytes were obtained from normal or *S. mansoni*-infected individuals by sedimenting 5 volumes of freshly drawn heparinized blood (10 units heparin/ml of blood) with either 1 volume of 4.5% dextran (Dextran 150,000, Sigma Chemicals, St. Louis, Mo.) or 1 volume of 6% Macrodex in normal saline (Macrodex-dextran 70, Pharmacia Laboratories, Piscataway, N. J.) for either 30 min at 37°C or 60 min at 37°C, respectively. The leukocyte-rich supernatant (buffy coat) was collected and washed twice in MEM/FCS/DNase by centrifugation at 1000 rpm for 10 min. This procedure also served to remove platelets from the buffy coat.

Purification of effector cells. Buffy coat cells were purified by centrifugation over Ficoll-Hypaque (F-H) or Metrizamide (M) gradients.

a) Ficoll-Hypaque. Discontinuous gradients were prepared according to the method originally described by Ross *et al.* (13). Various densities of F-H were prepared by diluting with water a stock solution of 15% Ficoll (Ficoll 400, Pharmacia Fine Chemicals, Piscataway, N. J.) and 20% Hypaque (Winthrop Labs, Division of Sterling Drugs, Inc., N. Y.), which had a density of approximately 1.17 g/liter. This stock solution could be stored for up to 2 months at 4°C.

Gradients were prepared by placing 5×10^7 buffy coat cells in 2 ml MEM/FCS/DNase in a 25-ml plastic Universal tube (Sterilin, Ltd., England) and underlayering this solution with a series of 4-ml volumes of F-H solutions of increasing densities by using 18 G stainless steel cannulas fixed onto plastic syringes. The densities used were between 1.07 and 1.12 g/liter. The tube was then spun in a centrifuge with a nonflexible shaft at 1200 × G for 45 min at 20°C, after which cells were collected from each density interface and washed three times in MEM/FCS/DNase. The cell count was estimated in a Coulter counter and a differential count was made on each cell fraction by using cytocentrifuge-prepared slides stained with Giemsa. In each fraction the viability of cells was estimated by trypan blue exclusion.

In certain instances, in order to perform studies on mononuclear cell fractions, buffy coat cells were centrifuged over lymphocyte-separating medium (LSM; Bionetics, Rockville, Md.). The pellet obtained was then used as a source of granulocytes.

b) *Metrizamide.* In order to eliminate the slight variations in tonicity that are present between various densities of F-H gradients, we set up discontinuous gradients with freshly prepared M (Nyegaard & Co., A/S, Oslo). An isotonic stock solution of 39% M in water, which has a density of approximately 1.22 g/liter, was diluted with isotonic Tyrode's gel/DNase to various densities. The dilutions were made on a percentage basis and the density was calculated. Centrifugation of buffy coat cells over discontinuous gradients containing this isotonic M was found to give very poor separation. To test whether a slightly hypertonic gradient would allow better cell separation, we made a stock solution of 30% M in Tyrode's gel/DNase (density 1.16 to 1.17 g/liter, about 540 mOsmoles/liter) and diluted it with Tyrode's gel/DNase to solutions of 18 to 25% (w/v), which had densities ranging approximately from 1.10 to 1.13 g/liter. Gradients were prepared by carefully layering 2-ml volumes of decreasing densities of M solution into a 15-ml conical centrifuge tube (Falcon Plastics, Oxnard, Calif.), on top of which were placed 5 to 10×10^7 buffy coat cells in MEM/FCS/DNase in a 2-ml volume. Each tube contained five to six gradient steps. The tube was then spun in a centrifuge with a nonflexible shaft at 1200 × G for 45 min at 20°C, after which the cells were collected from each interface, and counted. A differential count was estimated by using cytocentrifuge-prepared slides stained with Giemsa. Viability was estimated by exclusion of trypan blue.

Cytotoxicity assay. The cytotoxicity assay has been described in detail elsewhere (16). Briefly, aliquots of 0.1 ml of schistosomula (500/ml) were dispensed into 38 x 7-mm round-bottomed plastic tubes (LP/2, Luckham, Ltd.). Dilutions of antiserum (0.2 ml) and effector cells (0.1 ml) were added to each of four replicate tubes, and the tubes were incubated in humidified airtight plastic boxes at 37°C. Control tubes received an appropriate volume of medium alone, cells and medium, or antiserum and medium.

At the end of the incubation period (7 hr), the contents of each tube were resuspended and centrifuged at 200 × G for 5 min. One-half of the supernatant was then withdrawn into a second tube, and both tubes were counted for ^{51}Cr in a Packard well-type gamma counter (Packard Instrument Co., Inc., Downers Grove, Ill.). The percentage of isotope release was calculated from the corrected count rates in both tubes, thereby avoiding the errors attributable to variation in total count rate that occur if calculations are based on the count rate in the supernatant alone.

Adherence assay. Aliquots containing 100 organisms were dispensed into 38 x 7 mm round-bottomed plastic tubes (LP/2, Luckham, Ltd.) to which dilutions of effector cells (0.1 ml) and antibody (0.2 ml) were added. Tubes were then incubated in humidified airtight boxes at 37°C for various periods of time. At the end of the incubation period, usually 3 to 4 hr, the contents of each tube were gently resuspended and allowed to

[4] Abbreviations used in this paper: F-H, Ficoll-Hypaque; M, Metrizamide; HEPES, *N*-2-hydroxy-ethylpiperazine-*N'*-2-ethanesulfonic acid.

sit for 3 min (which is sufficient time for the schistosomula, but not cells, to settle to the bottom). Most of the supernatant was then removed. The residual 50 μl were gently resuspended with a Pasteur pipette and placed on a slide previously coated with 0.1% toluidine blue in methanol. The number of adherent cells on each organism was then counted in a wet mount. Organisms were counted as dead if they were immotile, had lost their translucency, and had become granular and opaque in their appearance (17). In addition, these organisms took up the toluidine blue dye in an intense and granular fashion, in contrast to live and actively motile organisms which failed to take up toluidine blue. Finally, many of the dead organisms had lost their shape and showed gross physical disruption of their tegument, and were sometimes surrounded by multiple layers of cells.

In order to facilitate the expression of adherence, the results of the first five experiments were grouped into negative controls (no antibody added) and groups that had positive adherence, and the distribution of organisms with various numbers of adherent cells was plotted. In the negative control groups, out of 657 schistosomula counted, only 3.3% had three or more adherent cells, whereas in the positive groups, 57.7% of 405 organisms had three or more adherent cells. The results of the adherence assay, therefore, will be expressed in most instances as the percent of schistosomula in each preparation with three or more cells attached. In one experiment (Table III) in which adherence was scored after 7 and 20 hr, estimates were made of the organisms bearing more than 5 or more than 20 cells, because of the increase in background adherence at these later times.

Each experiment presented was scored by two to four different observers who were not aware of the experiment being tested. The correlation in one experiment between the scoring of the two observers was better than $r = 0.8$, with $p < 0.01$ with Spearman's rank correlation coefficient.

Statistics. For the ^{51}Cr release data, comparisons of individual pairs of mean values (usually cells with antibody *vs.* cells without antibody) were made by the Student *t*-test with the logarithms of the individual values. Similar comparisons were made for the adherence and morphologic damage data, except that in this case the arithmetic values were used.

RESULTS

Comparison of cell separation on various discontinuous gradients. Buffy coat cells from a single individual were separated by using either F-H or hypertonic or isotonic M gradients. F-H and hypertonic M gradients separated buffy coat cells into fractions that contained approximately 90% pure monocytes, neutrophils or eosinophils. The isotonic gradient was not effective (Table I). The viability of cells after separation, as judged by trypan blue exclusion, was 100%, and by both light and electron microscopy they appeared morphologically intact and not degranulated. The efficiency of separation of buffy coat cells into purified components was approximately the same with F-H or hypertonic M. It can be seen from Figure 1, in which the purity of eosinophil-rich fractions is plotted against the starting percentage in whole blood for a large number of samples, that F-H and M yielded, respectively, 85 to 95% and 90 to 100% eosinophils, provided that the starting blood preparation contained at least 2 to 3%.

The recovery of cells from F-H gradients was of the order of 40 to 70%, whereas from M it was regularly greater than 90%. The increased recovery from M gradients was largely attributable to the small volumes of M steps, allowing their full collection in each fraction spanned. In the case of F-H gradients, part of each density step was discarded in order to reduce the number of washes necessary to remove the gradient material. When all the F-H gradient was collected, the cell recovery was better than 80%.

TABLE I

Comparison of cell purification on various kinds of discontinuous gradients

F-H density[a]	Differential Cell Count and % Yield	M Concentration[a,b]	Differential Cell Count and % Yield[c]	
			Hypertonic	Isotonic
g/liter		%		
1.07	0/6/94[d] (50%)[e]	18	0/2/98 (25%)	1/81/18 (90%)
1.08	2/59/39 (6%)	20	0/90/10 (3%)	15/82/3 (9%)
1.09	0.5/91/8 (8%)	22	0/100/0 (70%)	33/65/2 (1%)
1.10	2/90/8 (27%)	23	90/10/0 (3%)[f]	LY[g]
1.11	25/71/4 (3%)	24	82/15/3 (0.2%)	LY
1.115	73/26/0 (3%)			
1.12	88/12/0 (2%)			

[a] Refers to the density or concentration of the gradient steps from the top of which cells were collected.

[b] Ficoll-Hypaque gradients are made up to exact densities, Metrizamide ones on the basis of percentage concentration. The approximate densities of Metrizamide solutions are as follows: 18%, 1.09; 20%, 1.11; 22%, 1.12; 23%, 1.125; and 24%, 1.13 g per liter.

[c] Buffy coat cells from the same person were used for these experiments. The two Metrizamide gradients were done simultaneously, the Ficoll-Hypaque on a different day. The yield of cells from the Ficoll-Hypaque gradient was 75% and from Metrizamide ones, 95%.

[d] The three numbers represent the per cent of eosinophils, neutrophils, and mononuclear cells, respectively. Buffy coat cells contained 7% eosinophils, 61% neutrophils, and 32% mononuclear cells. Two hundred cells were counted for each differential.

[e] This number indicates the percentage of cells in this fraction compared to the total yield from the separation.

[f] In seven separate experiments using blood from the same individual, the best purity of eosinophils obtained was 98, 100, 99, 98, 97, 93, and 97%.

[g] LY, low yield of cells (less than 0.1% of total yield), no satisfactory slide could be made for differential.

The reproducibility of purification with these gradients can be judged by the purity of eosinophils obtained from one individual at different times. For example, with M gradients, the purity of eosinophils obtained from the blood of one individual on six different occasions was 89, 98, 99, 98, 97, and 93%; from another individual tested on four different occasions, it was 92, 91, 95, and 97%. In two instances we separated paired samples of the same person's blood at the same time by the two methods. In these two experiments, F-H gradients produced 83% and 80% pure eosinophils, whereas M gradients yielded 91 and 90% pure eosinophils. In both cases, the fractions contained approximately 50% of the recovered eosinophils.

Comparison of the ability of various cell fractions to cause antibody-dependent ^{51}Cr release from labeled schistosomula. Equal numbers of buffy coat cells or various fractions thereof, obtained either from F-H or M gradients, were mixed with ^{51}Cr labeled schistosomula and culture medium that did or did not contain antiserum. The per cent of ^{51}Cr release after 7 hr of incubation in three separate experiments is shown in Figure 2. The percentage of eosinophils and neutrophils in each fraction is also indicated. Similar patterns of ^{51}Cr release were obtained with cell fractions from F-H (Fig. 2*A*) or M (Fig. 2*B, C*) separations. In both cases, practically pure populations of eosinophils (fraction 1.12 in Fig. 2*A*, fraction 25 in Fig. 2*B*, and

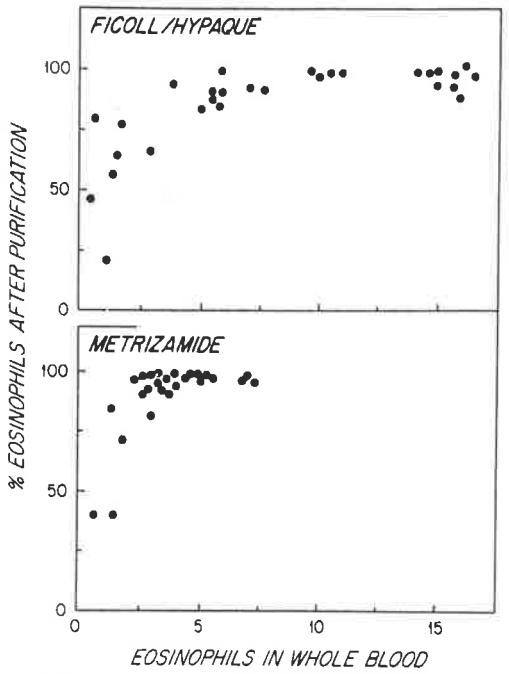

Figure 1. Per cent eosinophils after purification of buffy coat cells on either Ficoll-Hypaque (*top*) or hypertonic Metrizamide (*bottom*) discontinuous gradients. The per cent of eosinophils in the blood from which the cells were purified is indicated along the abscissa.

fraction 24 in Fig. 2*C*) and neutrophils (fraction 1.08 in Fig. 1*A*, and fractions 21 and 22 in Fig. 2*B*, *C*) caused significant antibody-dependent ^{51}Cr release. The highest release, however, was seen in the neutrophil-rich fractions. This observation is contrary to our previous reports that purified neutrophil populations were not effective in this system (8). Part of the explanation for this discrepancy is suggested by Figure 2*B*, where it is shown that different fractions containing almost 100% neutrophils cause differing amounts of chromium release. To test whether the method we used to purify neutrophils in our earlier reports (8) indeed produced only a small population of inactive neutrophils, we compared neutrophils obtained by M gradients with those from the Hypaque gradient method used in our earlier papers. The recovery of neutrophils (78% pure, contaminated only with monocytes) from the interface of the Hypaque gradient was only 5%. These cells, described in our previous paper as eosinophil-depleted neutrophils, caused only a 1 to 2% antibody-dependent ^{51}chromium release, as opposed to the pellet, containing 80% neutrophils and 16% eosinophils, which caused a 10% release. By contrast, fraction 20 of the M gradient, which contained 70% of the neutrophils recovered from the gradient, was 92% pure neutrophils and caused a 17% antibody-dependent ^{51}chromium release. This ^{51}chromium release was equal to that caused by an equal number of unseparated buffy coat cells in this experiment. Thus, it seems likely that the separation method used in our earlier experiments yielded an eosinophil-depleted, but unrepresentative fraction of neutrophils that was inactive in the chromium release assay.

The total amount of ^{51}Cr release that can be obtained from any cell population can be estimated by multiplying the antibody-dependent ^{51}Cr release for a given number of cells by the total number of cells in the fraction. If this calculation is applied to the data in Figure 2, most of the activity is seen to reside in the neutrophil fractions.

The fractionation procedures are seen to reveal not only a morphologic but also a functional heterogeneity. Evidence that the cells were separated into fractions with different properties is the enrichment or depletion of activity on a cell-for-cell basis observed in purified fractions. Two types of experiments, reported in Table II, suggest that the separation procedure does not cause the selective loss of a major functional component present in the buffy coat cells. First, cell populations obtained after mixing all the separated fractions were compared with unseparated buffy coat cells for their ability to mediate anti-

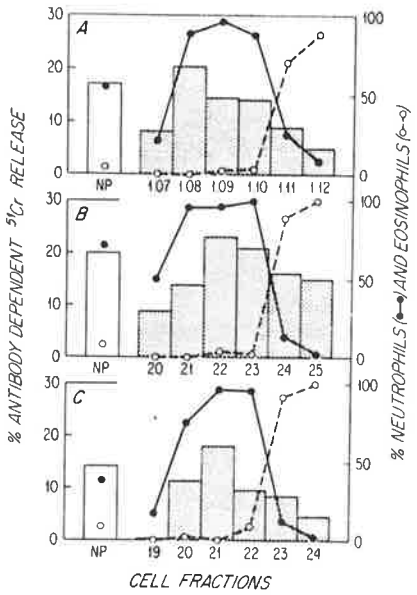

Figure 2. Antibody-dependent ^{51}Cr release (release in the presence of cells and antibody − release in the presence of cells alone) induced by nonpurified (NP) buffy coat cells (*open bar*) and by Ficoll-Hypaque (*A*) or Metrizamide (*B*, *C*) discontinuous gradient purified fractions (*stippled bars*) from three normal individuals. The per cent of neutrophils (●) and of eosinophils (○) in each fraction is also indicated. The difference between the sum of these percentages and 100 gives the per cent mononuclear cells in each fraction. The total number of cells ($\times 10^6$) in the fractions shown are: *A*, 1.07:6.6; 1.08:2.8; 1.09:13.9; 1.10:11.2; 1.11:2.7; 1.12:1.9. *B*, 20%:6.0; 21%:7.2; 22%:18.0; 23%:10.6; 24%:5.2; 25%:4.6. *C*, 19%:15.4; 20%:27.9; 21%:13.6; 22%:6.0; 23%:8.8; 25%:4.5. Effector:target ratio, 2×10^3:1. The following ^{51}Cr release values differ by $p < 0.05$. In Fig. 2*A*, 1.08 from 1.07, 1.09, 1.11, and 1.12; in Fig. 2*B*, 22 from 20, 21, and 24; and in Fig. 2*C*, 21 from 20, 22, 23, and 25.

TABLE II

Effect of the separation procedures on the ability of cells to release ^{51}Cr from labeled schistosomula

Expt.	Effector Cell	% ^{51}Cr Release at an Effector:Target Ratio of 3×10^4:1	
		Ab^+	Ab^-
1	Buffy coat	24[a]	16
	Reconstituted[b] F-H	25	14
2	Buffy coat	32	13
	Reconstituted[b] M	30	13
3	Buffy coat	33	20
	Buffy coat cells spun on F-H cushion (d = 1.12 g/liter)	40	20

[a] In all instances ^{51}Cr release in Ab^+ differs from that in Ab^- by $p < 0.01$.

[b] This population of cells was obtained by mixing all the fractions obtained after centrifugation of buffy coat cells over discontinuous gradients.

body-dependent ^{51}Cr release: no difference was observed. Secondly, buffy coat cells were spun on a F-H cushion of a density (1.12 g/liter) that does not allow cell separation, yet allows the cells to experience the stress of prolonged high speed centrifugation. No difference was observed between this cell preparation and unmanipulated buffy coat cells in the ^{51}Cr release assay.

Comparison of the ability of eosinophil- and neutrophil-rich fractions from schistosome-infected patients to cause ^{51}Cr release from labeled schistosomula. Neutrophil- and eosinophil-rich fractions from patients with *S. mansoni* infection were examined for their ability to release ^{51}Cr from labeled schistosomula. In these experiments, mononuclear cells were first separated from buffy coat cells by centrifugation over lymphocyte-separating medium. The granulocyte pellet obtained was then passed over F-H gradients. Representative results from three patients (A, B, and D) and one normal individual (C) are shown in Figure 3.

The same pattern of ^{51}chromium release was seen in normal and in infected individuals, in that neutrophil-rich fractions caused the highest amount of release. In these experiments, purified eosinophils from the patients, but not from the normal individual, produced significant amounts of antibody-dependent chromium release. A more extensive comparison of eosinophils from eosinophilic and normal individuals will be published elsewhere (see 27).

Dose-response curves comparing eosinophil- and neutrophil-mediated ^{51}Cr release from labeled schistosomula. In order to test whether the difference in the relative activity of eosinophil- or neutrophil-rich fractions depends on the effector: target ratio, we carried out titrations of the effector cell number against a constant number of target organisms. The results in Figure 4 show that both cell populations cause high levels of ^{51}Cr release, and that at higher effector:target cell ratios neutrophils caused significantly more release than eosinophils.

Time course of eosinophil- or neutrophil-mediated antibody-dependent ^{51}Cr release from labeled schistosomula. The amount of ^{51}Cr release at various times after mixing labeled schistosomula with either eosinophil- or neutrophil-rich cells in the presence or absence of antibody is shown in Figure 5. Neutrophil-mediated ^{51}Cr release was greater than that mediated by eosinophils at each time point examined. At 2 hr, neutrophil-mediated release of ^{51}Cr was evident, whereas eosinophil-mediated release was not. This experiment was repeated three more times with similar results.

Time course of adherence of buffy coat cells to schistosomula. The time course of adherence of cells to schistosomula in the presence or absence of antibody is shown in Figure 6. Increased adherence in the groups with antibody present was seen at all time points tested. After 4 hr of incubation, a number of dead schistosomula appeared, and after 7 hr the background adherence, i.e., that with no antibody present, increased.

Comparison of the ability of eosinophil- or neutrophil-rich fractions to adhere to antibody-coated schistosomula. Equal numbers (usually 10^5) of unseparated buffy coat cells or purified fractions were mixed with 100 schistosomula in the presence or absence of antibody, and were incubated for 2 to 3 hr before testing for adherence. The details of four such experiments are shown in Figure 7. Antibody-dependent adherence was seen in each cell fraction examined, but was greater with eosinophil-rich than with neutrophil-rich fractions. Compared to buffy coat cells, neutrophils were equal to or slightly inferior and eosinophils were superior in their capacity to adhere. Cells from uninfected individuals (Fig. 7*C*) and those from patients with

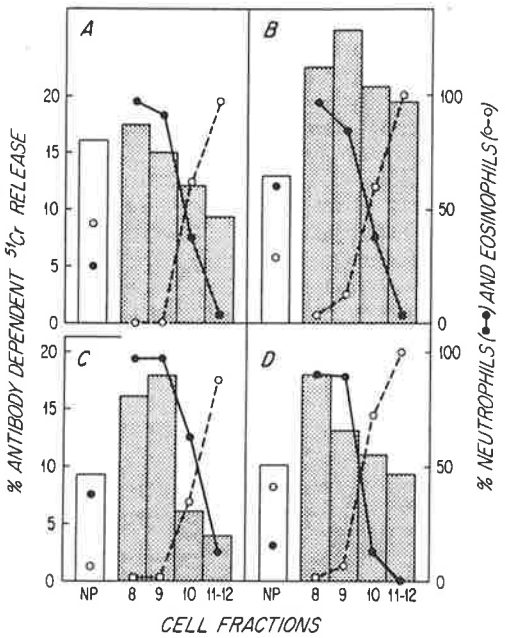

Figure 3. Antibody-dependent ^{51}Cr release by nonpurified (NP) buffy coat cells (*open bar*) and by Ficoll-Hypaque purified fractions (*stippled bars*) from three patients with eosinophilia and schistosomiasis (*A*, *B*, *D*) and from one normal individual (*C*). The per cent of neutrophils (●) and eosinophils (○) in each fraction is also shown. Effector:target ratio, 2×10^5:1. Numbers under the columns refer to the gradient density of the fraction: 8 = 1.08, 9 = 1.09, etc. The following data are available on the patients used: *A*, female, 10 yr, 400 eggs/g of feces, 10% peripheral eosinophilia; *B*, female, 16 yr, 4910 eggs/g of feces, 7% eosinophilia; *D*, male, 13 yr, 1220 eggs/g of feces and 7% eosinophilia.

The chromium release values in fractions 8 and 9 differ from their control values at $p < 0.001$. The values in fraction 10 differ from control values at $p < 0.005$ in *A*, <0.001 in *B*, <0.05 in *C*, and <0.001 in *D*. The values in fractions 11 and 12 differ from control values at $p < 0.025$ in *A*, <0.001 in *B*, <0.2 in *C*, and <0.025 in *D*.

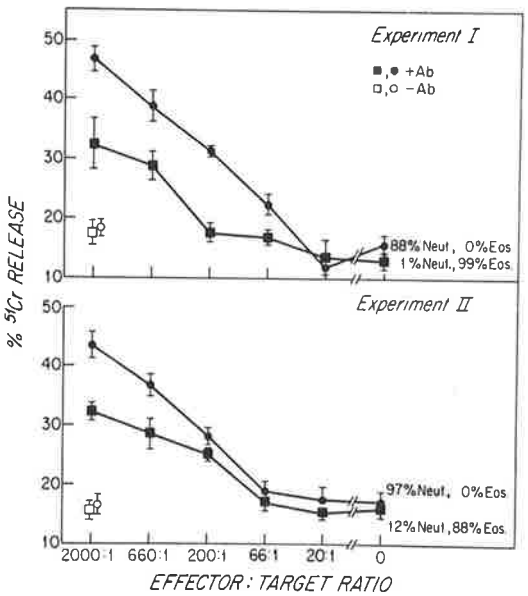

Figure 4. Dose-response curve comparing the ability of eosinophil-rich (■) and neutrophil-rich (●) cell fractions to release ^{51}Cr from labeled schistosomula in the presence of antibody. Open symbols indicate the amount of released ^{51}Cr in the absence of antibody at the highest cell concentration. The fractions were obtained by purifying on Ficoll-Hypaque discontinuous gradients buffy coat cells from two patients with schistosomiasis. Vertical bars span 2 S.E.M.

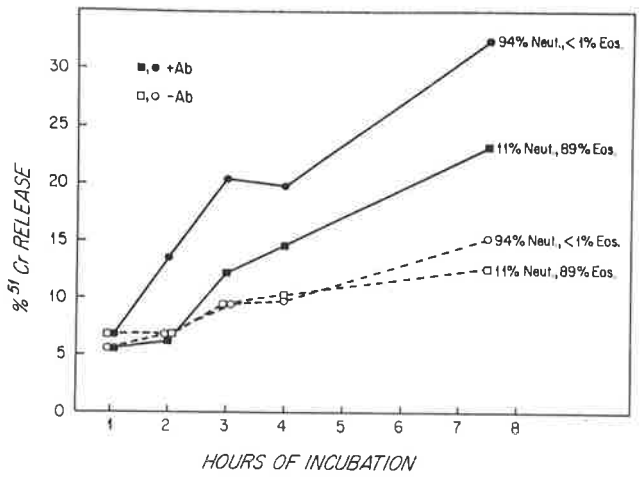

Figure 5. Time course of ^{51}Cr release, in the presence of (+Ab) (■, ●) or in the absence of (−Ab) (□, ○) antibody, induced by neutrophil- (●, ○) or eosinophil-rich (■, □) cell populations. Effector:target ratio, 2×10^5:1. Neutrophils caused significant ($p < 0.01$) ^{51}Cr release at the 2, 3, 4, and 7½ hr time point, whereas eosinophils caused significant release only at the 7½ hr time point.

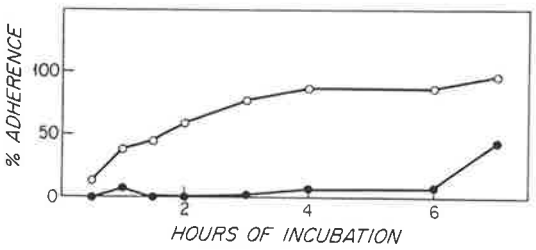

Figure 6. Time course of adherence of buffy coat cells to schistosomula in the presence (○) or absence (●) of anti-schistosomular antibody. Effector:target ratio, 2×10^6:1. Schistosomula which bore three or more adherent cells were counted as positive.

schistosomiasis (Fig. 7*A*, *B*, *D*) behaved in a comparable fashion. In the experiments shown in Figure 7*B* and *D*, we were especially concerned with the pure cell fractions, and the testing of fraction 10, containing a mixture of eosinophils and neutrophils, was deliberately omitted.

Comparison of the ability of eosinophil- and neutrophil-rich fractions to cause microscopically detectable damage to schistosomula in vitro. In order to determine the damage induced *in vitro* after attachment of either eosinophils or neutrophils, mixtures of enriched cells, antibody, and schistosomula were examined by microscopy. One such experiment, showing death after 24 and 48 hr of incubation, is shown in Figure 8. Eosinophil-rich but not neutrophil- or monocyte-rich fractions killed the parasite, and the percentage of dead parasites increased over the course of the incubation. The results of a further experiment (Table III) show that although the adherence pattern was similar, albeit with a higher background, to that observed earlier, death of schistosomula occurred to a much greater extent with the eosinophil-rich fractions. This experiment was repeated five times, with comparable results.

DISCUSSION

Success with the technique described here for separating eosinophils and neutrophils from normal peripheral blood depends on three factors. These are the material chosen for gradients, the speed of centrifugation, and the use of slightly hypertonic reagents. Although the F-H mixture and M both

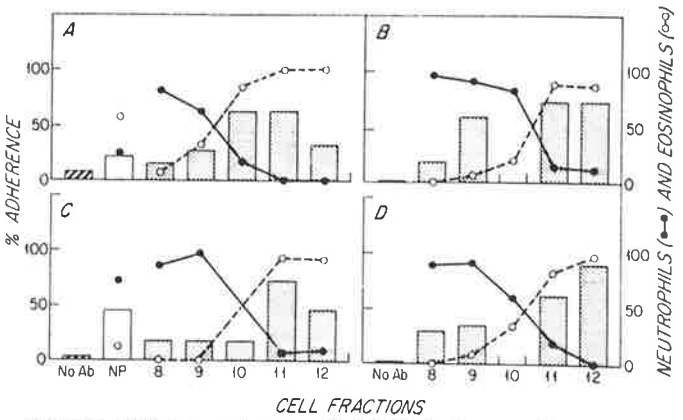

Figure 7. Adherence of nonpurified (NP) buffy coat cells (*open bar*) and Ficoll-Hypaque purified fractions (*stippled bars*) from three patients with schistosomiasis (*A*, *B*, *D*) and one normal individual (*C*) to antibody-coated schistosomula. The adherence by nonpurified buffy coat cells in absence of antibody is shown (*striped bar*). Adherence of purified fractions to schistosomula in the absence of antibody was similar to that of NP cells. The percentage of neutrophils (●) and eosinophils (○) in each cell preparation is also shown. Effector:target ratio, 10^5:1. Numbers under the columns refer to the density of the gradient fraction: 8 = 1.08, 9 = 1.09, etc. The following data are available on the patients used: *A*, male, 13 yr, 160 eggs/g feces, 33% peripheral eosinophilia; *B*, male, 11 yr, 140 eggs/g feces, 29% eosinophilia; *D*, male, 12 yr, 1090 eggs/g feces and 25% eosinophilia.

gave good separation, cell recovery and degree of purification were better with M. With both materials, viability and functional integrity of cells in all fractions were good. Centrifugation at 1200 × G for 45 min yielded more clear-cut interface layers than is commonly observed when cells are centrifuged on density gradients at 400 × G. This reduced the chance of contamination between fractions and had no deleterious effect on viability or function. Finally, the deliberate use, in the case of the M gradients, of slightly hypertonic reagents improved the separation between eosinophils and neutrophils. The reason for this is not clear. It is likely, though, that the reduction in cell volume attributable to hypertonicity led to a relatively greater increase in the density of the eosinophil-rich fraction, by virtue of its higher content of dense granular material. Such gradients now allow the routine preparation of eosinophils and neutrophils from normal human peripheral blood, in high yield and at a purity of greater than 90% in the case of neutrophils, and 90 to 100% in the case of eosinophils.

Eosinophils and neutrophils recovered from such gradients appear functionally intact as judged by the following criteria. The cells were 100% viable after separation. Buffy coat cells that had been centrifuged at 1200 × G for 45 min on a dense cushion of F-H or M that did not allow separation, or cells that were mixed again after separation (Table II) induced the same ^{51}Cr release from schistosomula as unseparated buffy coat cells. In addition, the inferior ability of eosinophils to cause ^{51}Cr release was unlikely to have resulted from damage during the separation procedure, because these cells were clearly better than neutrophils in their adherence to and killing of antibody-coated schistosomula (Figs. 7, 8).

When tested for their ability to induce antibody-dependent release of ^{51}chromium from labeled schistosomula, it was found that both eosinophil-rich and neutrophil-rich fractions were active (Figs. 2, 3). This finding conflicts with the results of our previous studies (8), in which we were unable to detect an effect of eosinophil-depleted, neutrophil-rich preparations in the

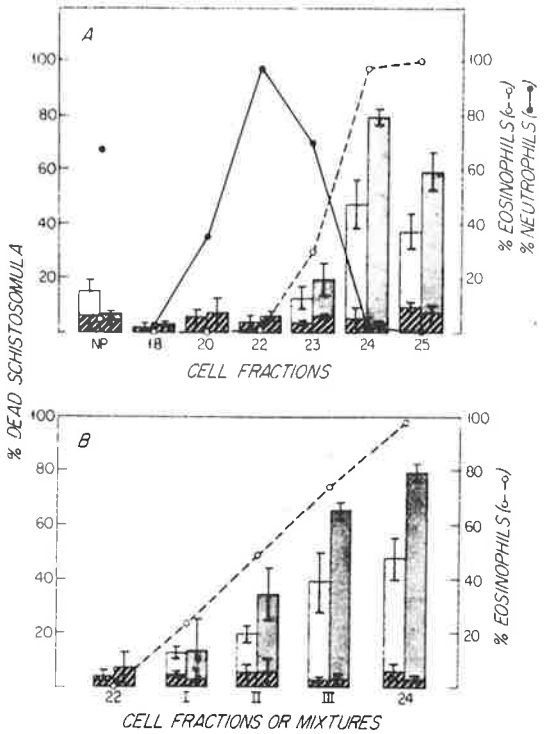

Figure 8. In vitro death of schistosomula cultured for 18 hr (*light bars*) and 42 hr (*dark bars*) in the presence of antibody and human nonpurified (NP) buffy coat cells or various fractions produced by Metrizamide gradients (indicated by numbers underneath the columns). Death in the absence of antibody is shown by the cross hatched bars. In *A* the per cent of eosinophils (○) and neutrophils (●) in each cell preparation tested is also shown. The difference between the sum of these two numbers and 100 gives the per cent of mononuclear cells in each fraction. In *B*, the per cent eosinophils (○) is shown in each fraction. The difference between this number and 100 gives the per cent of neutrophils in each fraction. I, II, and III are cell mixtures from fractions 22 and 24. An effector:target ratio of 4000:1 was used. Vertical bars span 2 s.d. At the 18 hr time point four replicates, and at the 42 hr time point two replicates were scored.

^{51}chromium release assay. The reason for this discrepancy is probably that the small yield of neutrophils with our earlier separation technique was nonrepresentative, and we were selecting out a population of neutrophils that was functionally inactive. Neutrophils were found to be more active than eosinophils in inducing release of ^{51}Cr in terms of the speed of the reaction and the number of cells required to produce an equivalent effect (Figs. 4, 5). In a separate paper (27), we report that buffy coat cells from patients with schistosomiasis and eosinophilia show no consistent differences from cells from normal individuals and that eosinophils from such patients are better, as a group, than those from normal individuals in the induction of ^{51}chromium release.

Our original suggestion (8) that eosinophils from patients with a schistosome-induced eosinophilia were less active than normal eosinophils was based on the *lack* of increase in the cytotoxic activity of unpurified leukocytes from eosinophilic blood, in comparison with normal blood, that would be expected if all eosinophils were equally effective and were the only effector cell. In light of the data presented herein, the observation that unpurified cells from eosinophilic and from normal individuals behave in a comparable fashion in the ^{51}chromium release assay now can be explained on the basis of the relative ability of neutrophils and eosinophils in these unpurified cell preparations to induce release of the label. Thus, eosinophil-rich leukocyte preparations contained fewer neutrophils, and therefore showed a lower overall neutrophil-dependent activity; but this decrease was probably balanced by a slightly increased activity of the eosinophils in these preparations. We would therefore no longer argue that eosinophils from *most* patients with a schistosome-induced eosinophilia are blocked by immune complexes, even though in some individual patients a block can be observed that can be reversed by mild trypsinization of the cells (8). In spite of these unexpected findings in the ^{51}Cr release assay, we find that both antibody-dependent adherence to schistosomula and microscopically-detectable damage is much *more* marked with the eosinophil-rich than with the neutrophil-rich fractions.

The degree of eosinophil adherence was somewhat surprising, in view of the well-documented paucity of eosinophil Fc receptors in comparison with those of neutrophils (18–20). Four alternative explanations can be offered. First, in contrast to a classical rosetting technique, in which an excess of antibody-coated particles are allowed to interact with a small number of rosette-forming cells, the adherence reaction described here involved an excess of effector cells offered to a small number of antibody-coated schistosomula. Therefore it is possible that the reaction conditions allowed the selection of a very small proportion of eosinophils with Fc receptors qualitatively different from or of much greater affinity than those of most neutrophils. This is not likely, however, because in other experiments (27), the adherence of a population of eosinophils was unaltered by the partial depletion of Fc rosette-forming cells. Secondly, it is possible that the antischistosomular sera contain antibodies of relatively restricted subclasses, with which eosinophils react

TABLE III

Comparison of the ability of eosinophil-rich and of neutrophil-rich preparations to adhere to or kill schistosomula

Source of Cells	Composition of Cell Preparations N/E[a]	Antibody	Duration of Incubation: 7 hr % Adherence >5	7 hr % Adherence >20	7 hr % Dead	24 hr % Adherence >5	24 hr % Adherence >20	24 hr % Dead
M 20[b]	80/1	+	**46**[c]	4	4	27	4	4
		−	6	0	1	9	6	6
M22	99/1	+	**54**	**7**	5	**36**	8	10
		−	7	0	5	11	2	11
BC	63/4	+	**77**	**12**	6	**62**	**21**	**19**
		−	10	0	4	10	6	7
Mix[d]	80/20	+	**91**	**52**	13	**89**	**36**	23
M 22/24		−	18	7	9	19	6	6
M 23	30/70	+	**100**	**90**	**26**			
		−	14	3	3			
M 24	7/93	+	**99**	**96**	**30**	**97**	**83**	**52**
		−	27	6	6	67	18	**7**

[a] N/E denotes the per cent of neutrophils (N) and of eosinophils (E) in the fractions. The difference the sum of these two numbers and 100 represents the number of mononuclear cells in the fraction.

[b] Cell preparations were either obtained from buffy coat cells of one individual, or from Metrizamide gradients on which buffy coat cells were purified. The number following M denotes the per cent Metrizamide contained in the step from the top of which the cells were recovered.

[c] Adherence of cells (>5 or >20 cells adhering) and death of schistosomula were scored in duplicate preparations containing 50–100 organisms by two observers, each of whom was unaware of the nature of the preparations being scored. Values in bold face differ from the controls without antibody at $p < 0.05$ by Student's t test.

[d] Cells from M 22 and M 24 were mixed at a ratio of 4:1.

[e] Not done.

better than neutrophils. This possibility, although not excluded, is unlikely, since in our hands (Bout, Butterworth, David, and Vadas, unpublished data) several subclasses of human IgG behave in similar fashions. Thirdly, it might be argued that the neutrophil dies more quickly than the eosinophil after interacting with the schistosomulum, and becomes more quickly detached. This could result either from rapid spontaneous death or by an active process on the part of the schistosomulum in ridding itself of bound effector cells. Evidence against this possibility is the excellent neutrophil adherence to dead schistosomula seen after prolonged incubation. In addition, in some preliminary experiments (Dessein, Butterworth, and Vadas, unpublished observations), we have found that cultured neutrophils, although somewhat less effective than fresh ones, are still able to adhere to a fresh preparation of antibody-coated schistosomula. Finally, a functional change may occur in the eosinophils, but not in the neutrophils, after interaction with antibody-coated schistosomula, such that the eosinophil remains adherent for longer periods of time. This functional change may be associated with degranulation. This last possibility is the one we favor. In preliminary experiments (Vadas, Butterworth, Sherry, and Hogan, unpublished data), we have found that after the initial Fc-dependent binding of eosinophils to schistosomula occurs, a second event takes place that renders this union irreversible. In this context, it is worth noting that the pattern of adherence differs in the two cell types. Schistosomula incubated with an excess of neutrophils for long periods usually show an "all-or-none" effect, originally described by Dean *et al.* (21, 22). Organisms are either completely ensheathed with neutrophils and are dead, or else they are free of more than one or two cells and are alive. This suggests some functional heterogeneity within the schistosomulum preparation. In contrast, schistosomula incubated with a comparable excess of eosinophils show an intermediate gradation, ranging from organisms that are free of cells, which occur rarely, to those with many cells attached.

In addition to demonstrating the strongest adherence to schistosomula, of the cell types examined, the eosinophil was the only cell capable of killing the parasite *in vitro* (Fig. 8, Table III). Recent observations (see 29) indicate that the capacity of the eosinophil to induce microscopically detectable damage (and the release of ^{51}chromium) may in part be attributable to the release of the "major basic protein" that is characteristic of the eosinophil granules (24, 25). Thus it may be postulated that eosinophils and neutrophils both induce ^{51}chromium release, perhaps by different mechanisms, but that since the major basic protein is limited to the eosinophil, it is only this cell that can induce gross, microscopically detectable damage.

The role of complement (C) in promoting eosinophil- or neutrophil-mediated damage was not studied in these experiments, but is currently under investigation. Although previous workers have reported that both neutrophils (21, 22) and eosinophils (26) can mediate damage to schistosomula which is dependent on the fixation of C by either the classical or the alternative pathway, these results must be viewed with a certain amount of caution. It has been recognized for several years that the fixation of C alone is sufficient to damage schistosomula over a 2- to 4-day culture period (17); therefore, it is possible that the action of the added effector cell is simply to enhance damage that has already been initiated by C but is not detectable under the particular conditions used for the assay. To exclude this possibility, it will be necessary to expose the organisms to purified C components, serially interacted up to but not beyond C3. Until this is done, no definite statement can be made, although it would be reasonable to assume that C fixation would enhance the damaging effect of cells bearing C3 receptors.

A point that stems from the results presented here concerns the relevance and relative merits of ^{51}chromium release and microscopy as estimates of cytotoxicity to schistosomula. Preliminary attempts have been made to reintroduce *in vivo* organisms that have been subjected to eosinophil- or neutrophil-mediated damage *in vitro*, to test whether they can subsequently develop into adult worms. These experiments have been technically unsatisfactory so far, because of poor survival of control organisms. Comparable difficulties have been reported by Mahmoud *et al.* (28). Although it has been found previously that in the case of buffy coat cells and of eosinophils there is a good correlation between chromium release and microscopically detectable damage, this is clearly not the case for purified neutrophils. It may now be suggested that the ^{51}chromium release assay, which is easy to carry out with large numbers of samples and which is entirely objective, represents a useful measure of early changes in the parasite that may or may not progress to gross damage. In contrast, the microscopical assay is subjective, insensitive, and excessively time-consuming, but it does afford a more clear-cut indication of death of the organism. Ideally, some intermediate assay should be sought that is both objective and incontrovertibly associated with irreversible damage to the organism.

Finally, the distinguishing features of the eosinophil as an effector cell active against schistosomula now appear to be: first, the ability to mediate gross, morphologic damage; secondly, an enhanced ability to bind to antibody-coated schistosomula, by comparison with neutrophils; and thirdly, an enhanced recruitment into the sites of schistosomulum migration in immune animals (see 23).

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ENHANCEMENT OF HUMAN BLOOD EOSINOPHIL CYTOTOXICITY BY SEMI-PURIFIED EOSINOPHIL COLONY-STIMULATING FACTOR(S)*

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Eosinophils are a major component of the host-immune response to helminth infections (1, 2), and factors modulating their helminthotoxic capacity are now receiving considerable attention. Lymphokines secreted by cells form *Schistosoma mansoni* egg granulomas (3) and eosinophil chemotactic factor of anaphylaxis tetrapeptides released by mast cells (4–6) potentiate the ability of eosinophils to destroy helminths in vitro; the release of these factors in vivo probably results in the local activation of tissue eosinophils.

Recent studies suggest that circulating eosinophils isolated from the blood of eosinophilic patients are activated: they have a low surface charge, high levels of certain lysosomal and membrane enzymatic activities (7), and enhanced helminthotoxicity in vitro (8). Also, blood eosinophils in eosinophilic patients are often vacuolated and degranulated (9–11), and certain substances normally stored in their granules have been found in abnormal concentrations in the blood of some of these patients (12, 13). It has been suggested that these cells, unlike eosinophils in normal individuals, release their granule content in the blood in response to unknown stimuli. Some of these eosinophil-derived substances are toxic for mammalian cells (14, 15), and they could be responsible for the tissue damage observed in some patients with hypereosinophilic syndrome (12, 16–18).

The mechanisms causing these changes in the properties of blood eosinophils are not known. Because they occur in association with eosinophilia, it is possible that some eosinopoietic factors induce them. Colony-stimulating factors (CSF)[1] could probably have this dual function because they have been reported to stimulate progenitor cells (19) as well as mature cells (20–22).

We tested this hypothesis using a human placental conditioned medium that is a source of human eosinophil CSF (23, 24). The data show that a material contained in the human placental conditioned medium markedly enhances eosinophil cytotoxicity.

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[1] *Abbreviations used in this paper:* Ab, antibody; Con A, concanavalin A; CSA, colony-stimulating activity; CSF, colony-stimulating factors; D, deoxyribonuclease; HPCM, human placental conditioned medium; MEM, minimum essential medium.

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This material copurifies with eosinophil CSF on phenylsepharose and Sephadex G-100 columns, suggesting that eosinophil CSF might be the molecule(s) responsible for the enhancement of eosinophil cytotoxicity. Studies on the mechanism of this enhancement suggest that normal blood eosinophils develop, after a short time incubation with this factor(s), properties that resemble some of the properties presented by circulating eosinophils in hypereosinophilic patients.

Materials and Methods

Life Cycle of S. mansoni. A Puerto Rican strain of *S. mansoni* was routinely maintained by passage through outbred mice and *Biomphalaria glabrata* snails. Schistosomula were prepared by allowing cercariae to penetrate an isolated preparation of rat skin in vitro (25, 26).

Antisera. Sera from patients with *S. mansoni* infection, either single or in pools, were used as a source of antischistosomular antibodies. All sera were heat-inactivated at 56°C for 1 h and had previously been tested for their ability to mediate microscopically detectable eosinophil-dependent damage to schistosomula in vitro.

Effector Cells. Neutrophils and eosinophils were recovered from the blood of normal individuals by fractionation on metrizamide gradients as previously described (27). Cytocentrifuge smears of different cell fractions were stained with Wrights Giemsa for immediate examination, and fractions were pooled as appropriate. Purity of cell preparation is indicated in figure legends; in the case of eosinophils, the contaminating cells were neutrophils; in the case of neutrophils, the contaminating cells were mononuclear cells with occasional eosinophils. Cells and schistosomula were washed and resuspended in minimal essential Eagle's medium supplemented with 25 mM of Hepes, 100 U/ml penicillin G, 100 μg/ml streptomycin, 1% glutamine, 10% fetal calf serum (FCS), and 30 mg/liter deoxyribonuclease, as previously described (27) (MEM/FCS/D). Cell concentrations were adjusted to 2 or 8 × 10^6 cells/ml, yielding effector cell-to-target schistosomulum ratios of 1,000:1 (adherence assay) or 4,000:1 (killing assay). Because neutrophils also adhere to antibody (AB)-coated schistosomula, the eosinophil adherence assays were performed with eosinophils that were >98% pure.

Adherence Assay. Aliquots containing 100 schistosomula (50 μl), eosinophils (50 μl), and appropriate dilutions of antischistosomular antiserum (50 μl) and a fraction of placental conditioned medium (50 μl) were incubated for 1–6 h in humidified airtight boxes at 37°C. At the end of the incubation period, schistosomula and cells that had sedimented at the bottom of the tubes were gently resuspended in 50 μl of assay medium and placed on a slide previously coated with 2 drops of 0.1% toluidine blue in methanol. The number of adherent cells on each organism was then counted at a magnification of 100×. In most experiments, results are recorded as the percentage of schistosomula bearing >20 cells. This threshold of 20 cells per schistosomula was chosen because it corresponds usually to the degree of eosinophil adherence required to kill the schistosomula when incubation is prolonged up to 24 h for the killing assay. Concanavalin A (Con A)-dependent eosinophil adherence is weaker than antibody-dependent eosinophil adherence at 37°C (28). Therefore, Con A-dependent eosinophil adherence was recorded as the percentage of parasites bearing >10 cells.

Killing Assay. 100 schistosomula, 4 × 10^5 eosinophils, and appropriate dilutions of antischistosomular antiserum and placental conditioned medium were incubated in plastic tubes as indicated for the adherence assay. Damage was determined after 24 h of culture. Larvae were scored as dead if they were immotile and had taken up toluidine blue in an intense and granular fashion (27). Separate experiments have shown that schistosomula considered dead by these criteria are unable to mature into adult worms when reinjected into mice.[2]

Inhibition of Eosinophil Protein Synthesis by Puromycin. Eosinophils (93% pure) were resuspended (8 × 10^6 cells/ml) in methionine-free Dulbecco's medium supplemented with 20 mM Hepes, 20 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.01 mM [^{35}S]methionine (sp act, 1,000 Ci/mmole) with or without 5 μg/ml puromycin (63178; Sigma Chemical Co., St. Louis, MO). After 3 h incubation, 10^6 cells were deposited on a filter paper (Whatman, 3 mM)

[2] Dessein, A. J., A. E. Butterworth, M. A. Vadas, and J. R. David. Maturation of *Schistosoma mansoni* after culture in vitro with granulocytes and antibody. Manuscript submitted for publication.

that was immediately immersed in 10% boiling trichloroacetic acid; the cells on the filters were then washed three times in 10% trichloracetic acid and twice in 90% ethanol, dried, and counted. ^{35}S incorporation (10^6 cells) was as follows: cells incubated without puromycin, 63,000 ± 5,000 cpm; cells incubated with puromycin, 22,000 ± 3,000 cpm; and cells kept at 4°C, 25,000 ± 4,000 cpm.

Preparation of Conditioned Media. Human placental conditioned medium was prepared as described previously (24). Briefly, pieces of fresh human placenta were incubated for 7 d in RPMI 1640 medium containing 5% FCS. The supernatant was then collected, pooled, and tested for CSF activity.

Purification of CSF from Human Placental Conditioned Medium (HPCM)

GEL FILTRATION ON SEPHADEX G-100. HPCM was concentrated 10-fold using an Amicon DC-2A apparatus (Amicon Corp., Scientific Sys. Div., Lexington, MA) with a H1P10 hollow filter cartridge and dialyzed against distilled water. It was then absorbed to calcium phosphate gel and eluted with 0.05 M sodium phosphate buffer as described previously (29). This concentrated material (25 ml) was then applied to a column of Sephadex G-100 (29) (LKB-Produkter, Bromme, Sweden), 2.6 × 100 cm, and eluted at a flow rate of 15 ml/h with phosphate-buffered (0.02 M, pH 7.3) saline (0.15 M) containing polyethylene glycol 6,000 (0.005% wt/vol). Fractions of 5 ml were collected and assayed separately before pooling.

FRACTIONATION ON PHENYL SEPHAROSE CL-4B. Samples of HPCM (either calcium phosphate eluates or active fractions from gel filtration) were applied to a column of phenyl sepharose CL-4B, 2.6 × 20 cm, (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated in phosphate-buffered saline (29). The column was eluted with the same buffer until eluate absorbance reached background level, and then the eluate was changed to 60% (vol/vol) ethylene glycol in distilled water. CSF failing to bind to the resin in phosphate-buffered saline was designated fraction-α, and that eluting with ethylene glycol was designated fraction-β.

Results

Enhancement by HPCM of the Antibody-dependent Eosinophil-mediated Killing of Schistosomula. Schistosomula were incubated with purified human blood eosinophils with or without HPCM and human antischistosomular serum. In the presence of HPCM there was a 4- to 10-fold increase in parasite death scored after 20 h of culture. This enhancement of eosinophil cytotoxicity was observed with eosinophils from the blood of all 15 volunteers tested (eosinophil count between 1 and 15%) and with all 10 human antischistosomular sera assayed. Table I shows the details of such experiments performed with eosinophils from the blood of five different individuals. HPCM without eosinophils was not toxic to the larvae; it should be noted, however, that it allowed a modest antibody-independent eosinophil-mediated killing of schistosomula.

Neutrophils adhered to antibody-coated larvae but failed to damage them in our assay (27). HPCM did not stimulate neutrophils to kill the larvae (Table I).

Enhancement of eosinophil cytotoxicity was maximum at antibody concentrations that allowed a marginal killing of schistosomula by control eosinophils (Fig. 1). Moreover, in the presence of HPCM, eosinophils required 5 to 10 times less antibodies than control eosinophils to demonstrate a similar killing ability (Fig. 2).

Enhancement of eosinophil cytotoxicity was proportionate to the dilution of HPCM (Fig. 2). This effect was observed at dilutions of up to 1/500. Small but significant enhancement of the antibody-independent eosinophil killing of schistosomula is observed in most experiments at dilutions up to 1/100.

Enhancement by HPCM of the Complement-dependent Eosinophil-mediated Killing of Schistosomula. Purified human eosinophils kill schistosomula coated with human complement (30, 31). This antibody-independent damage is maximum with mechanically

TABLE I

Enhancement by HPCM of the Ab-dependent Eosinophil-mediated Damage to Schistosomula

Patient	HPCM	Percent dead schistosomula				
		Ab	Eosinophils + Ab			Neutrophils + Ab
		1/40*	1/40	1/120	0‡	1/5
1	−	4 ± 2 (A)§	14 ± 2	ND‖	3 ± 1	ND
	+	3 ± 3	62 ± 5¶	ND	14 ± 2¶	ND
2	−	2 ± 2 (B)	70 ± 12	30 ± 2	3 ± 2	ND
	+	3 ± 4	98 ± 2¶	85 ± 2¶	20 ± 2¶	ND
3	−	7 ± 4 (C)	20 ± 3	5 ± 2	7 ± 3	8 ± 4
	+	7 ± 3	85 ± 10¶	48 ± 4¶	13 ± 5	10 ± 5
4	−	5 ± 3 (C)	12 ± 5	4 ± 2	5 ± 2	9 ± 3
	+	6 ± 4	90 ± 2¶	38 ± 10¶	7 ± 4	8 ± 4
5	−	3 ± 3 (A)	21 ± 4	6 ± 3	1 ± 1	6 ± 4
	+	4 ± 1	79 ± 4¶	49 ± 5¶	10 ± 4	9 ± 3

Eosinophils and neutrophils were purified (>90% pure) from the blood of five different patients (blood eosinophil count 1–8%), and their ability to kill schistosomula in the presence of antischistosomular Ab was tested as described in Materials and Methods. HPCM (1/100) was added at the beginning of the culture. Numbers represent arithmetic means of duplicate determinations ± SD obtained in five separate experiments (one experiment for each patient).

* Dilutions of human antischistosomular antiserum.

‡ No antibody.

§ Letters in parentheses refer to the human antischistosomular serum used in the corresponding experiment.

‖ Not determined.

¶ Values that differ significantly from their controls (incubations without HPCM) $P < 0.01$.

prepared schistosomula; skin-prepared schistosomula are much less susceptible (32). HPCM caused a 4- to 10-fold enhancement of the complement-dependent eosinophil-mediated killing of skin-prepared schistosomula. Eosinophils incubated with HPCM killed schistosomula at fresh normal human serum concentrations that were 5 to 10 times lower than those required by control eosinophils (Fig. 3).

Copurification of Eosinophil Cytotoxicity Enhancing Activity with Eosinophil Colony-stimulating Activity (CSA). Chromatography on phenyl-Sepharose columns resolves the HPCM into two major fractions, α and β (29). Both fractions have granulocyte-macrophage-CSA, but only fraction-α has eosinophil CSA; when tested in the killing assay, only fraction-α enhanced the antibody-dependent eosinophil-mediated killing of schistosomula (Table II). As was found with unfractionated HPCM, fractions α and β were unable to convert antibody-dependent neutrophil adherence to schistosomula into a killing reaction.

It was also found that eosinophil cytotoxicity-enhancing activity and eosinophil colony-stimulating activity are associated with molecule(s) having a similar apparent molecular weight (~30,000). The HPCM was filtered on Sephadex G-100 (29), and eosinophil CSA-containing fractions were pooled and tested in the killing assay (Table II). These fractions enhanced antibody-dependent eosinophil-mediated killing of

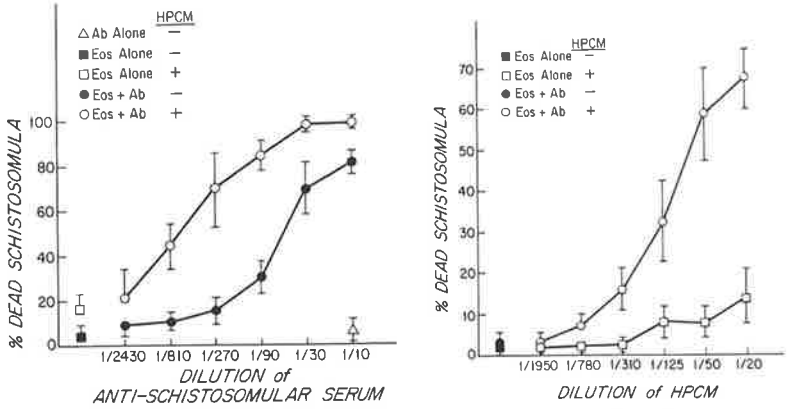

FIG. 1. (left) Enhancement by HPCM of the Ab-dependent eosinophil-mediated killing of schistosomula at various Ab concentrations. Schistosomula were incubated with eosinophils (>89% pure from donors with blood eosinophil counts of 3–13%) and grading concentrations of human antischistosomular antisera. HPCM (1/100) was added to the culture at the beginning of the incubation period. Each point represents the arithmetic mean ± SD of determination in three separate experiments.

FIG. 2. (right) Enhancement by HPCM of the Ab-dependent eosinophil-mediated killing of schistosomula at various HPCM concentrations. Schistosomula were incubated with eosinophils (>85% pure from donors with blood eosinophil counts of 4–10%). HPCM was added to the culture at the beginning of the incubation period. To reveal a maximum HPCM-mediated enhancing effect, antischistosomular sera (from three different patients) were used at dilutions that corresponded to the thresholds that permit eosinophil-mediated killing of schistosomula. Each point represents the mean ± SD of duplicate determinations of the percentage of dead schistosomula observed in three separate experiments.

schistosomula. They did not allow neutrophils to damage Ab-coated larvae (Table II).

All subsequent experiments were performed with the G-100 and phenyl-Sepharose-purified fraction of HPCM. This fraction will be referred to as CSF-α.

Effect of CSF-α on Eosinophil Adherence to Schistosomula. The next experiments were carried out to investigate the mechanism of the enhancement of the eosinophil-mediated killing of schistosomula by CSF-α. It was found that CSF-α enhances eosinophil adherence to antibody-coated schistosomula (Fig. 4). This enhancement was observable 90 min after the addition of CSF-α to the culture. At that time, eosinophils incubated with CSF-α adhered to the larvae twice as well as control eosinophils. Maximum cell adherence was reached after 5–6 h incubation and was 3–10 times higher with CSF-α incubated cells than with control eosinophils. Schistosomula mortality recorded after 20 h was enhanced to the same extent (Fig. 4).

Eosinophils that had been preincubated with CSF-α and then washed and added to antibody-coated schistosomula demonstrated an increase of adherence as early as 30–45 min after addition of the cells to the parasites (Fig. 5). A similar degree of adherence was reached 45–60 min later by eosinophils that had been in contact with CSF-α in the second culture only. This shows that the enhancing effect of CSF-α on cell adherence requires a minimum of 45–60 min to be detectable.

Enhancement of Eosinophil Adherence by CSF-α Occurs in the Absence of Protein Synthesis. Enhancement of eosinophil adherence by CSF-α occurs in the presence of doses of puromycin that totally inhibit eosinophil protein synthesis (see Materials and

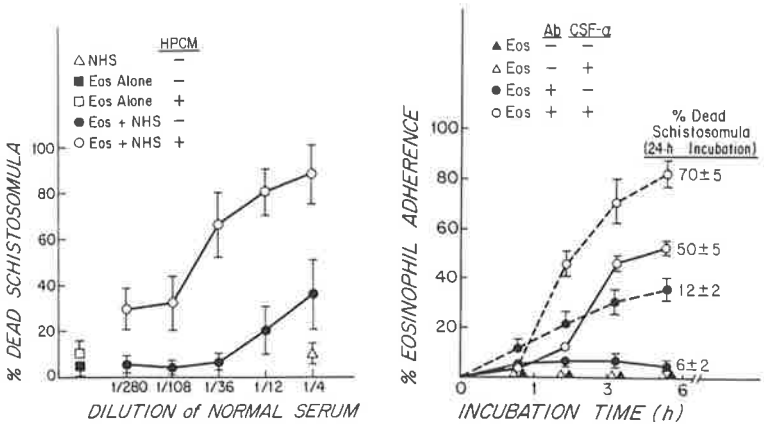

FIG. 3. (left) Enhancement by HPCM of the complement-dependent eosinophil-mediated killing of schistosomula. Schistosomula were incubated with eosinophils (>88% pure from donors with blood eosinophil counts of 4 and 9%) and various dilutions of fresh normal human serum (as source of complement). HPCM (1/100) was added to the culture at the beginning of the incubation period. Each point represents the arithmetic mean ± SD of the schistosomula mortality observed in two separate experiments.

FIG. 4. (right) Effect of CSF-α on Ab-dependent eosinophil adherence to schistosomula. Schistosomula were incubated with human eosinophils (98% pure; donor eosinophilia, 8 and 7%) and antischistosomular serum (1/150 or 1/360 dilutions). CSF-α was added to the culture (1/150) at the beginning of the incubation period. Eosinophil adherence (two experiments) is recorded as the percentage ± SD of organisms bearing >20 cells. Killing was scored after 24 h incubation. - - -, Ab 1/120; ——, Ab 1/360.

TABLE II

Purification of Eosinophil Cytotoxicity-enhancing Activity by Filtration through Phenyl-Sepharose and Sepharose G-100 Columns

HPCM fraction	Percent dead schistosomula			
	Ab	Eosinophils	Eosinophils + Ab	Neutrophils + Ab
None	6 ± 3	5 ± 2	18 ± 5	7 ± 3
Phenyl-Sepharose fractions				
α	4 ± 1	15 ± 4*	70 ± 5*	4 ± 5
β	ND	8 ± 3	20 ± 2	6 ± 4
None	5 ± 3	8 ± 5	16 ± 4	8 ± 3
G-100 (fraction 30,000 mol wt)	7 ± 4	10 ± 3	73 ± 10	7 ± 4

HPCM was fractionated by filtration through phenyl-Sepharose and Sephadex G-100 columns as described in Materials and Methods, and fractions were added (1/100 dilution) to the culture at the beginning of the incubation period. Eosinophils (>89% pure) and neutrophils (99% pure) were obtained from the blood of five patients (blood eosinophil count, 2–15%). Numbers represent arithmetic means of duplicate determinations ± SE obtained in six experiments (upper part of the table) and three experiments (lower part of the table).

* These values differ significantly from vertically adjacent values ($P < 0.01$).

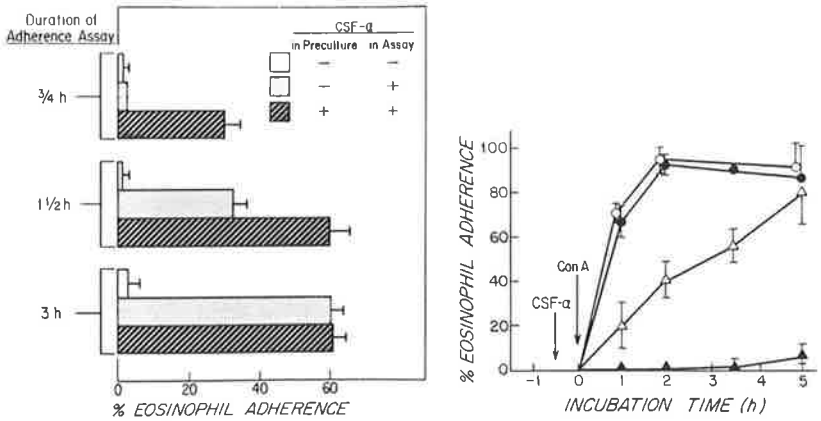

FIG. 5. (left) Time required for enhancement of eosinophil adherence by CSF-α. Eosinophils (95% pure from patients with blood eosinophil counts of 6 and 12%) were incubated in MEM/FCS/D ± CSF-α for 60 min, then Ab-coated schistosomula were added to the culture, and cell adherence was scored at regular time intervals (¾, 1½, and 3 h). Control eosinophils were kept at 37°C in MEM/FCS/D during the preincubation period, and their ability to adhere to Ab-coated schistosomula in the presence or absence of CSF-α (1/100) was tested as above. Eosinophil adherence is expressed as the percentage of schistosomula bearing >20 cells. Each bar represents the arithmetic mean of duplicate determinations ± SD (two experiments).

FIG. 6. (right) Effect of CSF-α on Con A-dependent eosinophil adherence to schistosomula. Eosinophils (2×10^5 per tube) were incubated ½ h at 37°C in MEM/FCS/D ± CSF-α (1/100 dilution), then schistosomula (100/tube) and Con A (100 μg/ml) were added and incubation forwarded at 37°C. At regular time intervals (1, 2, 3½, and 5 h), four tubes in each group were removed from the incubator and αCH_3 mannoside (2×10^{-1} M) was added to two of them. After gently mixing, these tubes were incubated at 37°C for a further 30 min, then eosinophil adherence was scored. Eosinophil adherence is expressed as the percentage of larvae bearing >10 cells. Very few larvae (<5%) bore >20 cells. Each point represents the mean of duplicate determinations in two (1, 2, and 3½ h) and four (5 h) separate experiments. Bar represents standard deviations between experiments. ●, Eos + Con A; ▲, Eos + Con A + αCH_3 mannoside; ○, Eos + CSF-α + Con A; △, Eos + CSF-α + Con A + αCH_3 mannoside.

Methods). As is shown in Table III, this occurs whether puromycin was present during the activation period only (experiments 1 and 2) or during the whole adherence assay (experiments 3 and 4). No effect of puromycin on the enhancement by CSF-α of the eosinophil-mediated killing of schistosomula was detected (Table III, experiments 1 and 2) when puromycin was added during a short activation period. Enhancement by CSF-α of the eosinophil-mediated killing of the larvae was also observed when puromycin was present during the complete (20 h) assay (experiments 3 and 4) but was less dramatic in tubes with puromycin than in tubes without. Puromycin also inhibited the killing reaction in the absence of CSF-α (experiment 5). This phenomenon has been reported for other antibody-dependent cell-mediated cytotoxicity reactions (33) and might reflect the need for a minimum level of protein synthesis for maintenance of the cells in killing reactions (33) rather than a true requirement for newly synthesized proteins for the killing process itself.

Stage of the Killing Reaction Affected by CSF-α. Adherence of eosinophils to antibody-coated schistosomula is a two-step process (28, 34). The first step is a temperature-independent reaction via Fc receptors, whereas the second step is a temperature-dependent reaction, possibly involving cell degranulation that makes the cell adherence irreversible.

TABLE III

Effect of Puromycin on Enhancement by CSF-α of the Ab-dependent Eosinophil Adherence and Eosinophil-mediated Damage to Schistosomula

Experiment	Time with agent		Percent eosinophil adherence*			Percent dead schistosomula‡
	CSF-α	Puromycin	60 min	90 min	150 min	20 h
1	0	0		8	17	19 ± 2
	0	30 min		13	10	17 ± 5
	10 min	0		77	79	41 ± 6
	10 min	30 min		64	70	33 ± 7
2	0	0	23	13	26	13 ± 2
	0	60 min	5	6	4	17 ± 3.5
	40 min	0	52	86	60	43 ± 7
	40 min	60 min	41	64	40	34 ± 1.5
3	0	0	5	21	30	6 ± 2
	0	20 h	4	12	13	3 ± 2
	20 h	0	38	40	65	44 ± 2
	20 h	20 h	56	59	50	17 ± 1§
4	0	0		9		15 ± 5
	0	20 h		2		10 ± 3
	20 h	0		57		52 ± 5
	20 h	20 h		43		43 ± 6
5	0	0				75 ± 13
	0	20 h				43 ± 5§

Eosinophils were incubated in MEM/FCS/D with or without puromycin for 10 min at 37°C, then CSF-α was added at 1/100 dilution. In experiment 1 and 2, cells were washed three times 10 or 40 min later and resuspended for a further 10-min incubation period in MEM/FCS/D ± puromycin; then cells were washed again three times, and their ability to adhere to and to kill Ab-coated larvae was tested as described in Materials and Methods. In experiments 3 to 5, cells were kept during the whole assay in MEM/FCS/D ± puromycin. Eosinophils from four different patients (blood eosinophil count, 4–12%) were used. Eosinophil purity was >90% when eosinophil adherence was tested, and >87% when eosinophil-mediated killing was assayed (see Materials and Methods). Puromycin concentrations were 5 μg/ml in experiment 1, 10 μg/ml in experiments 2, 3, and 4, and 15 μg/ml in experiment 4.

* Single determinations in experiments 1 and 2 and arithmetic mean of duplicate determinations in experiments 3 and 4 (SD, <7%).

‡ Arithmetic mean of duplicate determinations (experiments 1, 2, 3, and 4) and arithmetic mean of duplicate determinations in three separate experiments (experiment 5).

§ These values differ significantly from vertically adjacent values ($P < 0.02$).

Eosinophils that had been preincubated for 2 h at 37°C with CSF-α did not show enhanced adherence to Ab-coated schistosomula when the adherence assay was performed at 4°C (Table IV). This shows that CSF-α does not affect the temperature-independent stage of eosinophil adherence that is mediated by Fc receptors. As soon as the adherence assay was warmed to 37°C, CSF-α preincubated eosinophils adhered much better than control eosinophils to Ab-coated larvae (Table IV in experiments 1 and 3).

CSF-α Incubated Eosinophils Adhere Irreversibly to Con A-coated Schistosomula. The results of the above experiments show that CSF-α affects the temperature-dependent stage of eosinophil adherence possibly by promoting the mechanism(s) that make eosinophil

TABLE IV

CSF-α Enhances the Temperature-dependent Stage of Eosinophil Adherence to Ab-coated Schistosomula

Experiment	CSF-α	Adherence assay		Schistosomula bearing*			
				0–5 cells	>5 cells	>10 cells	>20 cells
		°C	h				
1	−	37	1½	53	49	21	ND
	+			10	90	50‡	ND
	−	4	4	76	24	0	0
	+			79	21	0	0
		then					
	−	37	½	44	57	36	ND
	+			0	100	78‡	ND
2	−	4	12	85	15	0	0
	+			88	11	0	0
3	−	4	14	72	22	0.5	0
	+			83	5	0	0
		then					
	−	37	½	13	87	63	23
	+			5	95	90	62‡

Eosinophils (>96% pure) were incubated (37°C, 2 h, 2 × 10^5/tube) in assay medium with or without CSF-α, then tubes were either kept at 37°C (experiment 1, first two lines) or transferred at 4°C, and 100 schistosomula that had been coated with human antischistosomular antibodies (dilution ⅛, 30 min at 37°C) were added to each tube. Eosinophil adherence was scored after a further 1½ h at 37°C (experiment 1, first two lines) or 4–14-h incubation period at 4°C. Tubes remaining at 4°C (experiments 1 and 3) were then retransferred at 37°C, and eosinophil adherence was scored ½ h later.

* Eosinophil adherence is expressed as the percentage of larvae bearing 0–five cells, >5, 10, or 20 cells. Numbers are arithmetic means of duplicate determinations, and standard deviations were <8%.

‡ Values that differ significantly from their controls (incubations without CSF-α), $P < 0.02$.

adherence irreversible. That hypothesis was tested in the Con A-dependent eosinophil adherence assay. Eosinophils adhere to Con A-coated schistosomula, and this adherence is fully reversible by addition of α-methyl mannoside (28). We found that adherence of eosinophils to Con A-coated schistosomula becomes irreversible when CSF-α is added to the culture (Fig. 6). Maximum irreversibility of the Con A-dependent eosinophil adherence was achieved after 5 h incubation with CSF-α, corresponding to the period required for maximum adherence of CSF-α-activated eosinophils to antibody-coated schistosomula (Fig. 4). In four experiments, CSF-α incubated eosinophils, like normal eosinophils, failed to kill Con A-coated schistosomula (data not shown).

Discussion

The observation that eosinophils from eosinophilic patients have an enhanced helminthotoxicity (8) suggested that the functional activity of mature blood eosinophils might be regulated by factors that also control the proliferation and maturation of eosinophil progenitor cells.

CSF are required for the growth and differentiation of granulocyte and macrophage colonies in vitro (19). They have also been shown to increase RNA (20) and protein synthesis (21) in mature cells and to enhance the killing of leishmania promastigote parasites by macrophages (22). Finally, granulocyte and macrophage CSF serum

levels fluctuate sharply under conditions that involve altered rates of polymorphonuclear cell and monocyte production, such as in granulocyte leukemia (35) and in viral (36) and bacterial (37) infections. This suggests that colony-stimulating factors could have the dual function of regulating granulopoiesis and of controlling the activity of mature granulocytes.

The data presented here show that human placental conditioned medium that contains eosinophil CSF does, in fact, significantly enhance human blood eosinophil helminthotoxicity. This enhancement represents a 4–10-fold increase in the eosinophil schistosomicidal activity and allowed eosinophils to kill Ab- or complement-coated larvae at Ab or complement concentrations ten times lower than the Ab or complement concentratons required by normal eosinophils to damage schistosomula. The activity that enhances eosinophil helminthotoxicity and the eosinophil colony-stimulating activity of HPCM are eluted in the same fraction after chromatography on Sephadex G-100 and phenylsepharose columns, suggesting that both activities might be associated with the same molecule.

The conditions required for colony stimulation and for activation of mature eosinophils are different. Removal of CSF from bone marrow cultures causes an immediate cessation of granulocyte proliferation (38, 39), whereas washing eosinophils a few minutes after the addition of CSF-α does not prevent full activation. Moreover, eosinophil activation when assessed by enhancement of eosinophil adherence, in contrast to cell proliferation and maturation, does not require protein synthesis. This suggests that eosinophil CSA and the activity that enhances eosinophil helminthotoxicity act on eosinophil progenitors and on mature eosinophils by different mechanisms.

We attempted to determine how CSF-α enhances the killing reaction. It was first observed that CSF-α enhances antibody-dependent adherence of eosinophils to the larvae, and the subsequent increase of parasite death was proportional to this enhancement. Because the killing of schistosomula by eosinophils requires the attachment of the cells to the parasite tegument, it is reasonable to assume that the effect of CSF-α on eosinophil adherence accounts, at least partially, for the increase in killing.

CSF-α could modify eosinophil adherence in several ways. First, as Anwar et al. (40) and Capron et al. (5) have reported for eosinophil chemotactic factor of anaphylaxis-activated eosinophils, CSF-α could increase the number of eosinophil CR (40) and Fc receptors (5). This is probably not the case here because the temperature-independent stage of eosinophil adherence that is probably dependent only on the interaction between Fc receptors and Ab bound to schistosomula (28, 34) is not enhanced by CSF-α. Moreover, the number of Fc receptors detectable in a rosetting assay is less on CSF-α-activated eosinophils than on normal cells (J. F. Jakubowitz and M. H. Vadas, manuscript in preparation).

CSF-α does affect, however, the temperature-dependent stage of the eosinophil adherence to Ab-coated larvae. Thus, eosinophils incubated with CSF-α, which adhere as normal eosinophils at 4°C, have enhanced adherence within a few minutes of reaching 37°C. Eosinophil adherence to Ab-coated schistosomula becomes irreversible at 37°C, and it is thought that irreversibility of eosinophil adherence is achieved during cell degranulation. This notion is supported by experiments showing that eosinophil adherence mediated by ligands (like Con A) that fail to trigger eosinophil degranulation is reversible unless degranulating agents are added to the reaction (28). It is shown here that CSF-α incubated eosinophils adhere irreversibly

to Con-A-coated larvae, suggesting that CSF-α affects the mechanism, possibly cell degranulation, that insures irreversibility of cell attachment. This interpretation is consistent with the observation that a small but significant number of eosinophils adhere to schistosomula in the absence of Ab if these cells have been incubated with high doses of CSF-α. Normal eosinophils adhering to Con A-coated schistosomula kill the parasite when the calcium ionophore A23187 is added (28), whereas CSF-α-activated eosinophils do not. This suggests that irreversibility of cell adherence is achieved by mechanism(s) that do not necessarily damage the larvae.

In conclusion, this study suggests that poietic molecules like eosinophil CSF could be the factors that enhance cytotoxicity of blood eosinophils in eosinophilic patients. CSF-α effects on the cell degranulation-associated events that insure irreversibility of eosinophil adherence suggest that these factors could also be responsible for the abnormal tendency of eosinophils to degranulate in the blood of these patients (12, 13). A more definitive demonstration of the involvement of eosinophil CSF-like factors in the regulation of blood eosinophil functions would require, however, a greater purification of eosinophil CSF and a more definite demonstration of its role in eosinopoiesis in vivo.

Summary

Purified human blood eosinophils, when incubated in human placental conditioned medium (a source of colony-stimulating factors [CSF]) demonstrate an enhanced ability to damage antibody- or complement-coated schistosomula. This enhancement represents a 4- to 10-fold increase of eosinophil schistosomicidal ability and a 10-fold lowering of the threshold for antibody or complement required in the killing reaction. The activity that enhances eosinophil cytotoxicity and the eosinophil colony-stimulating activity in the placental conditioned medium are eluted in the same fraction (CSF-α) after chromatography on Sephadex G-100 and phenyl-Sepharose columns, suggesting that these two activities might be associated with the same molecule.

CSF-α enhances the adherence step of the killing reaction: antibody-coated larvae were frequently found covered by several layers of eosinophils in tubes containing CSF-α. Such a degree of adherence was rarely seen in control tubes lacking CSF-α. This enhancement of the eosinophil adherence is detectable 45–60 min after addition of CSF-α to the culture. It is not affected by washing the cells after a short time of preincubation with CSF-α, and it occurs in the absence of protein synthesis, whereas colony-stimulating activity requires continuous protein synthesis and ceases when CSF is removed from the culture. Finally, CSF-α enhances the temperature-dependent reaction that insures the irreversibility of eosinophil attachment to schistosomula.

These observations suggest that eosinopoietic factors could be responsible for some of the modified properties of blood eosinophils in eosinophilic individuals.

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Brief Definitive Report

RECOMBINANT HUMAN INTERLEUKIN 5 IS A SELECTIVE ACTIVATOR OF HUMAN EOSINOPHIL FUNCTION

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Eosinophilia is an easily recognized clinical entity of diverse etiologies including allergic states such as atopy, asthma, and drug reactions, as well as helminth infestations. One of the striking features in many of these conditions is the selective nature of the eosinophilia, with other blood components found essentially in normal numbers. An increase in the state of activation of eosinophils can be observed concomitantly with an increase in eosinophil numbers in some of these syndromes. For example, eosinophils from patients with the hypereosinophilic syndrome show increased binding to antigen–antibody complexes (1), and eosinophils from patients with helminth infections and allergic conditions have a markedly increased capacity to adhere to and kill antibody-coated schistosomula of *Schistosoma mansoni* (2, 3) when tested in vitro.

The observation of selective eosinophilia has led many investigators to postulate the existence of an "eosinophilopoietin" molecule. We have recently shown that a murine cytokine, eosinophil differentiation factor (4), cross-reacted with human cells selectively stimulating the proliferation, differentiation, and function of eosinophils (5), predicting the existence of a human equivalent to this molecule. Although no native human factor has been characterized, a recombinant human (rh) molecule has now been identified and termed IL-5 (6, 7) which stimulates the production of eosinophils in cultures of human bone marrow (7). We show here that rhIL-5 is also a powerful and selective stimulator of human eosinophil function. IL-5 is thus the first hemopoietic factor whose elaboration in vivo can explain the selective eosinophilia and eosinophil activation seen in disease.

Materials and Methods

rhIL-5 and Granulocyte/Macrophage CSF (GM-CSF). The human gene encoding human IL-5 in the expression vector pcEXV-3 was transfected into COS cells by electroporation as previously described (7). The supernatant was collected after 4 d and concentrated by ultrafiltration. Mock-transfected control supernatants were prepared in the same way. rhGM-CSF, 99.4% pure, and with a specific activity of 10^6 U/ml, was a gift from Genetics Institute (Cambridge, MA).

Purification of Human Granulocytes. Peripheral blood was sedimented on dextran, and the leukocyte-rich supernatant was centrifuged on a grandient of hypertonic Metrizamide

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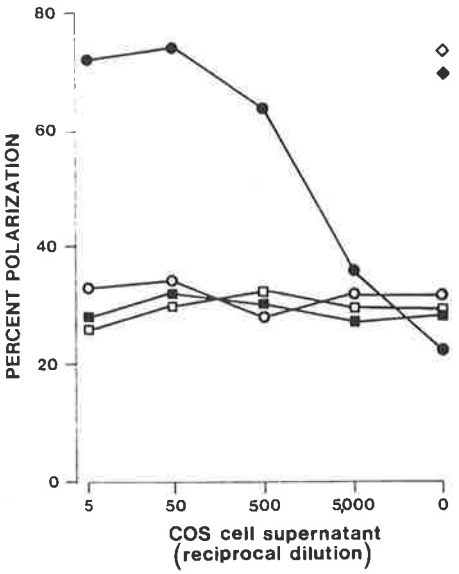

FIGURE 1. Titration of rhIL-5 (*filled symbols*) and mock-transfected COS cell supernatant (*open symbols*) on human eosinophil (*circles*) and neutrophil (*squares*) polarization. The percentage of polarized eosinophils (◇) and neutrophils (◆) in the presence of 10 ng/ml rhGM-CSF is also shown.

(Nyegaard, Oslo, Norway) as previously described (8). The eosinophil and neutrophil preparations were always >90 and >95% pure, respectively.

Functional Assays. The antibody-dependent cell-mediated cytotoxicity, phagocytosis, superoxide anion production, and polarization assays used have been described in detail previously (9–11).

Results

rhIL-5 was found to induce morphological changes on eosinophils. Cells thus stimulated took on an irregular shape, the membrane ruffled, and the granules concentrated on one end (polarization); these are changes typically induced by other established activating agents (11, 12). Eosinophils were polarized by rhIL-5 in a dose-dependent manner and by rhGM-CSF but not by mock-transfected COS cell supernatant (Fig. 1). By contrast, neutrophils were polarized by rhGM-CSF but not by rhIL-5.

The morphological changes induced by rhIL-5 on eosinophils were accompanied by functional activation of these cells. rhIL-5 stimulated eosinophils but not neutrophils to kill antibody-coated tumor cells while rhGM-CSF stimulated both cell types (Fig. 2). rhIL-5 stimulated eosinophils in a dose-dependent manner and the levels of cytotoxicity were similar to those obtained with rhGM-CSF. Similar results were obtained when rhIL-5 was examined for its ability to stimulate phagocytosis of serum-opsonized baker's yeast. Eosinophils but not neutrophils were stimulated by rhIL-5 at 2.5 and 1.2% serum concentrations (Table I). Neutrophils, however, could be stimulated by rhGM-CSF.

The ability of rhIL-5 to stimulate the respiratory burst of eosinophils and neutrophils was examined by measuring the levels of O_2^- production. rhIL-5 stimulated directly the production of O_2^- by eosinophils and to the same extent as rhGM-CSF (Table II). In contrast, neither rhIL-5 nor rhGM-CSF directly stimulated O_2^- production by neutrophils. Because GM-CSF enhances the response of neutrophils to a subsequent stimulus, neutrophils were preincubated

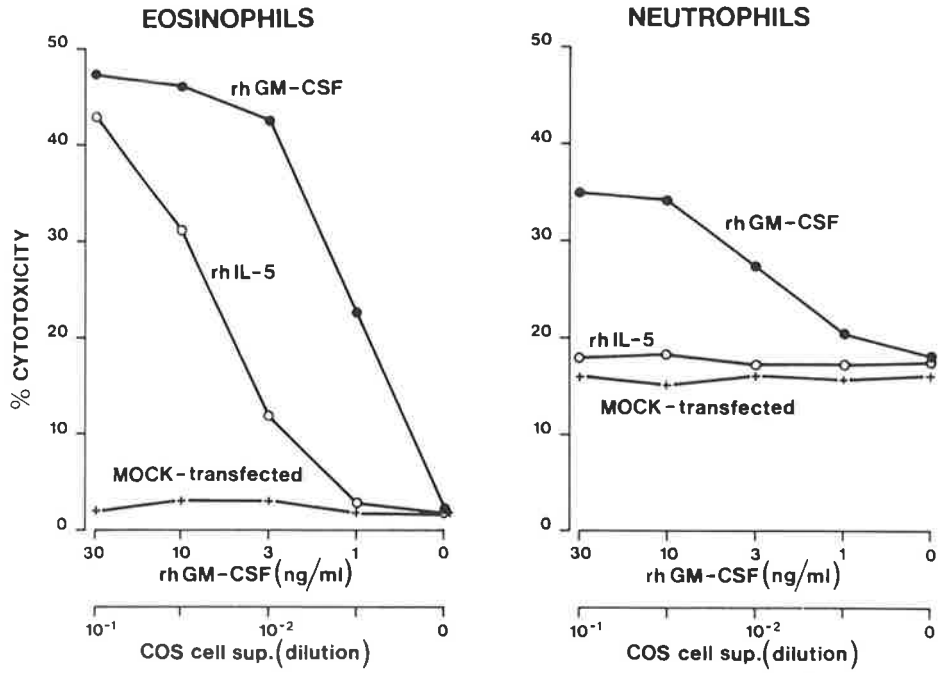

FIGURE 2. Titration of rhIL-5 (○) mock-transfected COS cell supernatant (+) and rhGM-CSF (●) on human eosinophil- and neutrophil-mediated antibody-dependent cell-mediated cytotoxicity of target cells.

TABLE I

rhIL-5 Stimulates Human Eosinophils but Not Neutrophils to Phagocytize Serum-Opsonized Baker's Yeast

Stimuli	Serum concentration	Number of phagocytized baker's yeast per cell					
		Eosinophils			Neutrophils		
		0	1, 2	3, 4	0	1, 2	3, 4
	%						
rhIL-5	2.5	37*	52.5	10.5	58	21	21
Mock-transfected COS cell supernatant	2.5	78.5	18.5	3	62	21.5	16.5
rhGM-CSF	2.5	55	32	13	13.5	31.5	55
rhIL-5	1.2	79.5	18	2.5	81.5	12	6.5
Mock-transfected COS cell supernatant	1.2	90.5	9	0.5	82	13.5	4.5
rhGM-CSF	1.2	80.5	16.5	3	48	27	25

rhIL-5 and mock-transfected COS cell supernatants were tested at 1:10, and rhGM-CSF was tested at 10 ng/ml final concentrations. Determinations were carried out in triplicate. In the case of eosinophils, values obtained with rhIL-5 and rhGM-CSF were significantly different from those obtained with mock-transfected COS cell supernatant ($p < 0.01$). In the case of neutrophils, rhGM-CSF, but not rhIL-5, values were significantly different from those obtained with mock-transfected COS cell supernatant ($p < 0.01$). Neither eosinophils nor neutrophils showed significant phagocytosis in the absence of serum.

* Percentage of cells containing different numbers of phagocytized yeast. A minimum of 200 cells were counted.

for 45 min at 37°C with rhIL-5, mock-transfected COS cell supernatant, or rhGM-CSF, and then stimulated with 10^{-7} M f-Met-Leu-Phe. The O_2^- release by neutrophils was enhanced by rhGM-CSF (15.7 nmol/10^6 cells) compared to

TABLE II

Effect of rhIL-5 on the Direct Production of Superoxide Anion by Human Eosinophils and Neutrophils

Exp.	Eosinophils				Neutrophils			
	rhIL-5	Mock-transfected COS cell supernatant	rhGM-CSF	PMA	rhIL-5	Mock-transfected COS cell supernatant	rhGM-CSF	PMA
1	4.5* ± 0.5‡	1.0 ± 0.4	4.2 ± 0.3‡	41.4 ± 0.9‡	0.8 ± 0.1	1.4 ± 0.2	1.2 ± 0.3	46.5‡ ± 1.6
2	7.6 ± 0.5‡	2.7 ± 0.1	6.3 ± 0.2‡	50.2 ± 1.4‡	1.9 ± 0.3	2.0 ± 0.2	2.2 ± 0.3	60.0‡ ± 0.9

rhIL-5 and mock-transfected COS cell supernatant were tested at 1:10 dilution. rhGM-CSF was used at 10 ng/ml. PMA was used at 30 ng/ml.

* Values in nmol $O_2^-/10^6$ cells followed by SEM.

‡ Values significantly different from mock-transfected COS cells ($p < 0.05$).

medium control (8.5 nmol/10^6 cells), but not by rhIL-5 (8.3 nmol/10^6 cells) or mock-transfected COS cell supernatant (8.1 nmol/10^6 cells).

Discussion

We show here that rhIL-5, in addition to stimulating eosinophil proliferation, is an activator of human eosinophil but not neutrophil function. Its selectivity for human eosinophils makes IL-5 the molecule most likely to be responsible for the increase in eosinophil numbers and for the state of activation of these cells observed in allergy, parasitic infections, and hypereosinophilic syndromes.

In the present experiments, rhIL-5 altered the morphological appearance of eosinophils to cells showing typical features of "polarization" including membrane ruffling, an elongated shape, and the granules concentrated towards one end. These morphological changes have been previously observed with chemotactic factors and rhGM-CSF on neutrophils and eosinophils (11, 12) and probably represent cytoskeletal changes occurring after cell activation (13). In addition, rhIL-5 selectively stimulated several effector functions on human eosinophils but not neutrophils.

Some of the functional effects of rhIL-5 reported in these experiments in vitro appear similar to those taking place in vivo as seen in eosinophils from patients with selective eosinophilia. For example, eosinophils from such patients have been shown to have increased oxidative metabolism (14, 15) and an altered morphology (1). Another feature of these eosinophils is their hypogranular appearance suggestive of degranulation in vivo (1); however, in these studies we have not examined whether IL-5 can directly induce degranulation of eosinophils in vitro.

The production of IL-5 in vivo may have important clinical implications. Firstly, a selective increase in eosinophils, a cell type effective against several parasites in vitro (8, 16), may facilitate the control of parasitic infestations. Secondly, the continuous presence of large numbers of circulating eosinophils may lead to tissue pathology as shown in cases of hypereosinophilic syndromes. In some of these cases peripheral blood eosinophils were degranulated and eosinophilic endomyocardial disease developed (17). Thirdly, IL-5 may be produced by certain tumors. Some cases of carcinoma of the lung are associated with eosinophilia (17, 18) and the extracted tumor can be shown to produce a substance that preferentially stimulates eosinophils in vitro (18). In cases of

lymphomas, eosinophilia has appeared before or at the time of diagnosis of the tumor with peripheral eosinophil counts decreasing during remission but rising again if a relapse occurs (19). Thus, IL-5 may be present in some paraneoplastic syndromes and its detection may serve diagnostic purposes.

Finally, IL-5 becomes the third type of human CSF after GM-CSF (11, 20) and IL-3 (21) capable of stimulating eosinophil proliferation and function in man. The property of activating eosinophils, also shared with TNF-α (22), is not obviously attributable to similarities in the primary structure of these molecules. However, rhIL-5 and rhIL-3 do show a significant homology in their primary sequence at the carboxy-terminal region—48% (including conservative substitutions) over 19 amino acids in a significantly hydrophilic region of the molecule—and it would be interesting to establish the relevance of this region for the eosinophil-activating function of these two molecules.

Summary

Human rIL-5 was found to selectively stimulate morphological changes and the function of human eosinophils. This molecule is thus a prime candidate for the selective eosinophilia and eosinophil activation seen in disease.

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Recombinant Human Granulocyte-Macrophage Colony-stimulating Factor Stimulates In Vitro Mature Human Neutrophil and Eosinophil Function, Surface Receptor Expression, and Survival

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Abstract

A purified recombinant human granulocyte-macrophage colony stimulating factor (rH GM-CSF) was a powerful stimulator of mature human eosinophils and neutrophils. The purified rH GM-CSF enhanced the cytotoxic activity of neutrophils and eosinophils against antibody-coated targets, stimulated phagocytosis of serum-opsonized yeast by both cell types in a dose-dependent manner, and stimulated neutrophil-mediated iodination in the presence of zymosan. In addition, rH GM-CSF enhanced *N*-formylmethionylleucylphenylalanine(FMLP)–stimulated degranulation of Cytochalasin B pretreated neutrophils and FMLP-stimulated superoxide production. In contrast, rH GM-CSF did not promote adherence of granulocytes to endothelial cells or plastic surfaces. rH GM-CSF selectively enhanced the surface expression of granulocyte functional antigens 1 and 2, and the Mo1 antigen. rH GM-CSF induced morphological changes and enhanced the survival of both neutrophils and eosinophils by 6 and 9 h, respectively. These experiments show that granulocyte-macrophage colony stimulating factor can selectively stimulate mature granulocyte function.

Introduction

Colony stimulating factors (CSF)[1] are a group of cell-derived products responsible for the proliferation and differentiation of progenitor cells (1). Recent evidence also suggests that CSF functionally activate mature cells, a process that takes place in a lineage-specific fashion (2–4). In humans, naturally occurring CSF have been only partially purified biochemically (5–7); however, their first definitive identification has been recently achieved by recombinant DNA technology (8, 9).

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1. *Abbreviations used in this paper:* ADCC, antibody-dependent cell-mediated cytotoxicity; CR3, complement receptor type 3 for the C3bi portion of C3; CSF, colony-stimulating factor; FACS, fluorescence-activated cell sorter; FCS, fetal calf serum; FITC, fluorescein-isothiocyanate; FMLP, *N*-formylmethionylleucylphenylalanine; GFA, granulocyte-functional antigen; G, granulocyte; GM, granulocyte-macrophage; HPCM, human placental conditioned medium; LPS, lipopolysaccharide; MAb, monoclonal antibodies; M, macrophage; PMA, phorbol-myristate acetate; rH GM-CSF, recombinant human granulocyte-macrophage colony-stimulating factor.

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One of these recombinant human granulocyte-macrophage CSF (rH GM-CSF) was cloned from a complementary DNA library prepared from Mo cell messenger RNA and was found to stimulate in cultures of human bone marrow the formation of granulocyte-macrophage (GM) and eosinophil (Eo) colonies (4, 8), exhibiting all the functions of the previously described CSF-α obtained from human placenta (6). In all assays used, this was different from CSF-β that has no effect on eosinophil proliferation (6) and from a human-active murine eosinophil-CSF that has no effect on neutrophil progenitor cells (10). In this paper we definitively show that this rH GM-CSF activates mature human neutrophils as well as eosinophils and that this stimulation is selective as rH GM-CSF does not enhance the adherence of these cells to endothelium or plastic surfaces. In addition, we show that activation is associated with an increase in the expression of certain surface antigens associated with function and with a significant prolongation of cell survival.

Methods

Purification of human neutrophils and eosinophils

These cells were obtained from the peripheral blood of healthy volunteers after dextran sedimentation and centrifugation on a gradient of hypertonic Metrizamide (Nyegaard, A/C, Oslo) as previously described (11). The purity was >95% for neutrophils and >92% for eosinophils. The cells were resuspended in Eagle's minimal essential medium supplemented with 10% fetal calf serum (FCS), 20 mM Hepes buffer, and antibiotics. In experiments involving polarization, iodination, and adherence, blood was collected by using 0.2% ethylenediaminetetraacetic acid as anticoagulant. The neutrophils were isolated by density gradient centrifugation in Hypaque-Ficoll, sedimentation in dextran, and hypotonic lysis of erythrocytes as previously described (12). The preparation, which always contained >97% neutrophils with an average purity of 98–99%, was suspended in 0.9% sodium chloride at 5×10^7 neutrophils per milliliter.

rH GM-CSF, conditioned media containing CSF, and recombinant human tumor necrosis factor α (rH TNFα)

rH GM-CSF was obtained from the supernatant of COS cells that had been transfected with cloned human GM-CSF cDNA in the p91203 (B) vector as described (8) and incubated in serum-free medium for 4 d. The supernatant was concentrated by ultrafiltration and the GM-CSF was purified to homogeneity as described (8). Silver-staining of the sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the purified GM-CSF revealed a major band of 19,000 molecular weight. The specific activity of this rH GM-CSF was 40 U/ng of protein. This material contained <0.2 ng/ml of endotoxin as determined by a limulus amoebocyte lysate assay. CSF-α and CSF-β were obtained after fractionation of medium conditioned by the human bladder cell carcinoma line U5637 by phenyl-sepharose chromatography (13), and were a gift from Dr. N. A. Nicola (Walter and Eliza Hall Institute, Melbourne, Australia). rH TNFα was a gift from Genentech, Inc., South San Francisco, CA and contained 3.6×10^7 U/mg, as assayed (by the supplier) for its cytotoxic activity on actinomycin D-treated L929 mouse fibroblast cells. rH TNFα was produced in *Escherichia coli* (14) and purified to 99.8% purity. This material

contained 0.8 ng/ml endotoxin as determined by a limulus amoebocyte lysate assay.

Antibody-dependent cell-mediated cytotoxicity assay (ADCC)

ADCC was performed as previously described (2). In brief, 40 μl of ^{51}Cr-labeled, trinitrophenyl (TNP)-coupled P815 cells (4×10^3) were incubated with 24 μl of rabbit IgG anti-TNP (Miles-Yeda, Rehovot, Israel), 80 μl of purified human neutrophils or eosinophils (1.3×10^5) as effector cells, and 16 μl of rH GM-CSF for 2 h at 37°C in V-bottom microtiter plates. Percent cytotoxicity was calculated from the formula: (test − control/total − control) × 100, where "control" was the ^{51}Cr released by P815 cells in the presence of medium alone, and "total" was the ^{51}Cr released by the addition of 5% Triton X-100.

Phagocytosis assay

This assay measured the phagocytosis of serum-opsonized baker's yeast and was performed as described (15) and modified (4). In brief, 100 μl purified neutrophils (2×10^7/ml) were incubated with 250 μl baker's yeast, 100 μl diluted fresh autologous serum, and 50 μl rH GM-CSF or medium with FCS for 1 h at 37°C. After this, the cells were centrifuged at 4°C, resuspended in 50 μl of cold phosphate-buffered saline (PBS), and smeared onto a slide. When eosinophils were tested, this assay was scaled down because of the relatively low numbers of eosinophils routinely obtained. The volumes were 20 μl of cells (10^7/ml), 50 μl of baker's yeast, 20 μl of freshly obtained human serum, and 10 μl of rH GM-CSF or medium. After incubation, cytocentrifuge preparations were made. Slides were fixed in methanol and stained with Giemsa before being examined for the number of cells showing different numbers of phagocytosed yeast. The data are expressed as percentages after counting a minimum of 200 cells per slide.

Degranulation assay

This was carried out as previously described (16). Briefly, purified neutrophils (5×10^6/ml) pretreated with cytochalasin B (Sigma Chemical Co., St. Louis, MO) for 15 min at 37°C were incubated with different stimuli for 15 min at 37°C. To these mixtures different concentrations of FMLP (Sigma Chemical Co.) were added and the cells incubated a further 30 min at 37°C. After incubation the cells were centrifuged and the released lysozyme was assayed against Micrococcus lysodeikticus (Sigma). The O.D. at 450 nm was measured in triplicate at 10 sec. intervals for 3 min. and the amount of lysozyme released was calculated from a standard curve generated by egg white lysozyme (Sigma Chemical Co.). Maximum values were obtained by using supernatants from neutrophils lysed with Triton X-100.

Iodination

Iodination was measured by the conversion of radioiodide to a trichloroacetic acid (TCA)-precipitable form as previously described (17). Zymosan (ICN Pharmaceuticals, Inc., Cleveland, OH) was prepared by homogenization in water, after which it was boiled for 20 min, washed twice, and suspended in water at 10 mg/ml. The reaction mixture contained 4×10^{-3} M sodium phosphate buffer, pH 7.4, 0.128 M NaCl, 1.2×10^{-2} M KCl, 10^{-3} M $CaCl_2$, 2×10^{-3} M $MgCl_2$, 8×10^{-6} M Na I (4 nmol; 0.05 Ci ^{125}I), 2×10^{-3} M glucose, 0.25 mg albumin, 2.5×10^6 neutrophils, and where indicated, 2 ng/ml rH GM-CSF, 0.5 mg zymosan, or both. The components of the reaction mixture were incubated for 60 min at 37°C in 12 × 75-mm polystyrene test tubes on a Rotor-Rack (Fisher Scientific Co., Pittsburgh, PA) and the reaction was stopped by the addition of 1 ml of cold 10% TCA. The precipitate was collected by centrifugation at 2,500 *g* for 5 min in a refrigerated centrifuge, washed four times with 2 ml of 10% TCA, and the counts per minute were determined in a gamma scintillation counter. A blank containing the standard salt solution, iodine and albumin, was run with each experiment and the results were subtracted from the experimental values. <0.05% of the total added radioiodide was TCA-precipitable in the blank. A standard containing the total ^{125}I added to each experimental tube was counted and the percent iodination was determined as follows: (cpm experimental − cpm blank × 100)/(cpm standard). The results are expressed as nanomoles of iodide converted to a TCA-precipitable form per 10^7 PMN per hour or percent iodide converted to a TCA-precipitable form. Each experimental value was determined in duplicate and averaged for statistical analysis of stated number of experiments.

Superoxide production

Purified neutrophils were incubated with medium or different concentrations of rH GM-CSF for 2 h at 37°C. After this, 150 μl of cells (10^6) were mixed with 100 μl freshly prepared cytochrome-*c* (Sigma Chemical Co., type VI, 12.4 mg/ml), 100 μl FMLP (10^{-6} M), and made up to 1 ml with medium. The mixtures were then incubated at 37°C for 5 min, after which the cells were rapidly cooled, centrifuged at 4°C, and the supernatants were transferred to plastic disposable cuvettes. Superoxide production was measured by the reduction of cytochrome-*c* (18) using an extinction coefficient of 21.1 mM^{-1} (19).

The medium used throughout these assays was RPMI (free of phenol red) containing 2% FCS. In control experiments, rH GM-CSF did not reduce cytochrome-*c* in the absence of cells, and superoxide dismutase (Sigma Chemical Co.) completely abolished the reduction of cytochrome-*c*. The results are expressed as the means of triplicate determinations±1 SD.

Neutrophil adherence assays

These assays measured the adherence of neutrophils to human endothelial cells and to plastic. Neutrophils were first radiolabelled with ^{51}Cr by the method of Gallin et al. (20). Briefly, neutrophils were suspended in Hank's balanced salt solution (HBSS) (Gibco, Grand Island, NY) with 1 mM calcium, 2 mM magnesium, and 0.1% gelatin at 24×10^6/ml and incubated with 24 Ci/ml ^{51}Cr (as sodium chromate, 200–500 Ci/g, New England Nuclear, Boston, MA) at 37°C for 1 h with periodic gentle agitation. After incubation, free ^{51}Cr was removed by washing twice with HBSS. Labeled neutrophils were resuspended in RPMI 1640 medium (MA Bioproducts, Walkersville, MD) or in RPMI 1640 medium with 5% FCS at a final concentration of 10^6/ml.

Adherence to endothelial cells. Endothelial cells were isolated by collagenase treatment of human umbilical cord veins and were maintained in endotoxin-free RPMI 1640 medium with 10% FCS as previously described (21). In the adherence assay, human endothelial cells were plated in RPMI 1640 medium with 20% FCS in 16-mm-diam wells (Costar, Cambridge, MA) at 10^5 cells/cm^2 and grown to confluence. Prior to assay, the medium was decanted and the endothelial cell monolayers were washed once with RPMI 1640 medium containing 5% FCS. To each well, a total volume of 250 μl was added, containing 5×10^5 ^{51}Cr-labeled neutrophils and the test substance. The mixture was then gently agitated and the cells were incubated with the endothelial cell monolayers at 37°C with 5% CO_2. After a 30-min incubation, the supernatant medium and the nonadherent ^{51}Cr-labeled neutrophils were aspirated and each well was washed once with RPMI 1640 medium containing 5% FCS. Aspirated nonadherent neutrophils, incubation medium, and the wash medium from each well were pooled in individual counting tubes and were counted in a gamma spectrophotometer. The endothelial cell monolayers and the adherent ^{51}Cr-labeled neutrophils were then lysed for at least 2 h with 1 M NH_4OH. The NH_4OH lysate and wash from each well were counted in a gamma spectrophotometer. Adherence was determined as the percent of the total ^{51}Cr-cpm added: percent adherence = (^{51}Cr-cpm in adherent neutrophils)/(Total ^{51}Cr-cpm added) × 100. Total ^{51}Cr-cpm added was calculated for each well as the sum of ^{51}Cr-cpm in supernatant medium, washes, and lysate. Total ^{51}Cr-cpm varied between wells by <10%.

Adherence to plastic. 100 μl of ^{51}Cr-labeled neutrophils (5×10^3) in HBSS containing 0.5% bovine serum albumin were added to round-bottom polyvinylchloride microtiter plates together with 5 μl of stimulus and incubated at 37°C. After this, the nonadherent cells were removed as above and adhered and nonadherent cells counted in a gamma counter. Adherence was calculated as above. In each case quadruplicate determinations were made.

Immunofluorescence assay

This was carried out as previously described (22). Briefly, purified neutrophils (10^7/ml) were incubated with medium or 2 ng/ml of rH GM-

CSF for 1 h at 37°C after which they were washed three times and mixed with monoclonal antibodies (MAb) directly conjugated with fluorescein isothiocyanate (FITC) (23). The mouse MAb used were WEM-G1, an IgM directed against granulocyte-functional antigen (GFA)-1 (24), WEM-G11 F(ab')$_2$ directed against GFA-2 (15), Mo1-FITC (Coulter Electronics, Inc., Hialeah, FL), an IgM directed against the Mo-1/OKM1 antigen (25, 26) in the CD_{11} cluster, anti-β_2 microglobulin (an IgG2b anti-β_2m, gift from Dr. I. F. C. Mackenzie), K7 (IgM anti-TNP), and PB10 (an IgG1 against the chicken theta antigen, gift from Dr. P. Bartlett, The Walter and Eliza Hall Institute.) The incubation was carried out in microtiter plates for 45 min at 4°C. After being washed three times the cells were fixed before analysis by flow cytometry as described (22).

Polarization

Changes in cell shape of neutrophils accompanying activation have been described previously (27) and involve an elongation and assumption of irregular shape by the cell. Granulocytes were incubated with rH GM-CSF or medium at 37°C in a 100-μl volume in conical polypropylene tubes. After incubation, the cells were fixed as previously described (22) and duplicate samples of more than 100 cells were examined in a cell-counting chamber. These changes were accompanied by an increase in the flow cytometric parameter of forward or 0° light scatter (28) which were measured in a flow microfluoremeter (Ortho cytofluorograph) and are expressed as the mean of 15,000 cells. Besides the magnitude of the 0° scatter signal, the pulse width of this signal was also significantly increased.

In eosinophils, a shape change was also observed. The cells assumed a triangular shape with the granules clearly concentrated toward the base. The appearance was strikingly similar to candy corn.

Granulocyte survival assay

Details of this assay are described elsewhere (Begley, C. G., A. F. Lopez, N. A. Nicola, D. J. Warren, C. J. Sanderson, and M. A. Vadas, manuscript submitted for publication). In brief, purified neutrophils and eosinophils were placed in Terasaki wells and cultured in the presence of serial two-fold dilutions of rH GM-CSF in medium containing 10% FCS. After regular intervals, the cells were examined by phase contrast and eosin exclusion, and the number of viable cells was recorded. The data are expressed as mean viable cells from duplicate wells.

Statistical analysis

The P values given were derived after comparing the values obtained with cells from the same individual before and after stimulation with CSF by the Student's method of paired differences unless stated otherwise.

Results

Effect of rH GM-CSF on ADCC by human neutrophils and eosinophils. rH GM-CSF was found to stimulate the ADCC of tumor cells by both neutrophils and eosinophils. A typical titration curve with neutrophils and eosinophils purified from the same individual showed (Fig. 1) that rH GM-CSF was active on both cell types over a wide range of concentrations. Eosinophils consistently responded to a greater degree than neutrophils. In other experiments rH GM-CSF increased the percentage of ADCC by eosinophils from 5.8% to 48.6% (mean of four experiments, $P < 0.0001$) and in the case of neutrophils, from 12.5% to 31.5% (mean of four experiments, $P < 0.001$). In the presence of lipopolysaccharide (LPS) at 1 ng/ml the percentage ADCC by neutrophils was 12.8% (mean of two experiments, not significant). The cytotoxicity observed was not due to toxic effects of rH GM-CSF since this material did not induce killing of P815 cells in the absence of either granulocytes or anti-TNP antibody (data not shown).

Effect of rH GM-CSF on neutrophil and eosinophil-mediated phagocytosis. Fig. 2 shows one representative experiment out of four performed in which neutrophils phagocytosed baker's yeast in the presence of different concentrations of normal human serum. Very little phagocytic activity was observed in the absence of serum. The addition of 2 ng/ml of rH GM-CSF resulted in an increase in neutrophil-mediated phagocytosis of baker's yeast at serum concentrations of 2.5% and 1.25% (Fig. 2) but not 5%, a concentration that appeared to saturate this system. This stimulatory effect of rH GM-CSF was seen both in terms of the number of neutrophils showing phagocytosis and in the number of ingested yeast per neutrophil.

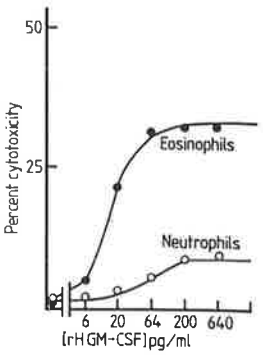

Figure 1. Stimulation by rH GM-CSF of the ADCC of tumor cells by human neutrophils (open circles) and eosinophils (closed circles) from the same individual. Dose-response. Each point is the mean of triplicate determinations.

In order to establish the concentration of rH GM-CSF necessary to stimulate neutrophil-mediated phagocytosis, a titration of rH GM-CSF was carried out using 1.2% serum. In one experiment that was representative of three performed, rH-CSF stimulated phagocytosis by neutrophils at 2 and 0.2 ng/ml, but was ineffective at 0.02 ng/ml (Fig. 3).

To test whether this rH GM-CSF was also able to stimulate phagocytosis of serum-opsonized baker's yeast by mature eosinophils, rH GM-CSF was used at 2 ng/ml over a range of serum concentrations. In two experiments performed, rH GM-CSF activated eosinophil-mediated phagocytosis when baker's yeasts were opsonized with 2.5% or 1.2% human serum. One of these experiments is illustrated in Fig. 4. Similar to its effect on neutrophils, rH GM-CSF increased both the number of eosinophils showing phagocytosis, and the number of ingested yeasts per eosinophil. No stimulation was seen if human serum was omitted or heat-inactivated at 56°C for 40 min. LPS at a concentration between 0.1 and 1 ng/ml (at least 10 times higher than that present in rH GM-CSF) was found not to have an effect in this system (data not shown).

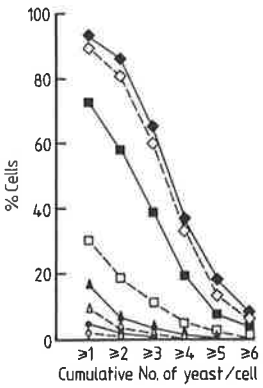

Figure 2. Stimulation by rH GM-CSF of neutrophil-mediated phagocytosis of yeast organisms opsonized with different concentrations of fresh human serum. Closed symbols show the values obtained in the presence of 2 ng/ml of rH GM-CSF and open symbols show those obtained in the presence of medium at serum concentrations of 5% (◇), 2.5% (□), 1.25% (△), and none (○). Each point is the mean of triplicate determinations. The values obtained with neutrophils treated with rH GM-CSF were significantly different from those obtained with neutrophils treated with medium by χ^2 analysis ($P < 0.001$) at a 2.5% serum concentration.

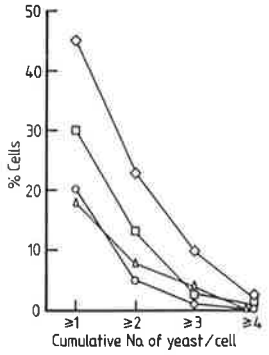

Figure 3. Stimulation by rH GM-CSF of neutrophil-mediated phagocytosis of yeast organisms opsonized with 1.25% human serum: dose response. The concentrations of rH GM-CSF tested were 2 ng/ml (◇), 0.2 ng/ml (□), 0.02 ng/ml (△), and none (○). Each point is the mean of triplicate determinations. The values obtained with neutrophils treated with rH GM-CSF at 2 ng/ml were significantly different from those obtained with neutrophils treated with medium by χ^2 analysis ($P < 0.001$).

Effect of rH GM-CSF on neutrophil degranulation. To test the effect of rH GM-CSF on neutrophil degranulation, a stimulus which is a complete secretagogue for neutrophils was used and the amount of lysozyme released into the supernatant was measured. rH GM-CSF increased FMLP-stimulated degranulation of Cytochalasin B-treated neutrophils (Table I). Semi-purified CSF-α had similar effects as did F(ab')$_2$ fragments of the MAb WEM-G11, which was used as a positive control (15). Neither rH GM-CSF nor CSF-α stimulated lysozyme secretion by themselves (absence of FMLP). Similarly, no lysozyme secretion was detected if neutrophils were not treated with Cytochalasin B or if neutrophils were treated with LPS at 1 ng/ml. In two other experiments rH GM-CSF was found to enhance lysozyme secretion from neutrophils treated with Cytochalasin B and stimulated by 10^{-8} M FMLP in a dose-dependent manner (Table II).

Stimulation of neutrophil-mediated iodination by rH GM-CSF. In the absence of a stimulus no iodination by neutrophils takes place during an incubation period of 1 h. The addition of rH GM-CSF alone induced a very low degree of iodination by neutrophils (Table III). By contrast, and as described previously (29), rH GM-CSF strongly stimulated iodination by eosinophils (data not shown). Similarly to rH GM-CSF, zymosan stimulated very little neutrophil-mediated iodination by itself. However, the addition of rH GM-CSF and zymosan appeared to have a synergistic effect, enhancing iodination by neutrophils to levels significantly greater ($P < 0.001$) than those obtained by either stimulus alone.

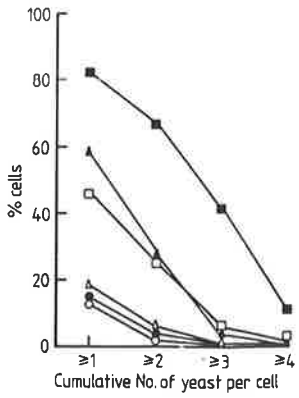

Figure 4. Stimulation by rH GM-CSF of eosinophil-mediated phagocytosis of yeast organisms opsonized with different concentrations of human serum. Values obtained in the presence of 2 ng/ml of rH GM-CSF are shown as closed symbols, and those obtained in the presence of medium were shown as open symbols. The serum concentrations used were 2.5% (□), 1.2% (△), and none (○). Each point is the mean of triplicate determinations. The values obtained with eosinophils treated with rH GM-CSF were significantly different from those obtained with eosinophils treated with medium by χ^2 analysis ($P < 0.001$) at 2.5% and 1.2% serum concentrations.

Table I. rH GM-CSF Enhances Lysozyme Secretion from Cytochalasin B-treated Human Neutrophils Stimulated with FMLP

	Lysozyme*			
	Preincubation with:			
Stimulus:FMLP	rH GM-CSF (2 ng/ml)	CSF-α (1:100)	WEM-G11 F(ab')$_2$ (60 ng/ml)	Medium
10^{-7} M	340±9‡	193±3	205±9	105±10
10^{-8} M	245±10	145±0	167±6	60±0
None	0±0	0±0	20±10	0±0

* Units of measure, U/ml per minute per 10^6 cells.
‡ Arithmetic means of triplicate determinations followed by 1 SD. Maximum, 372±12 U/ml per minute per 10^6 cells.
Purified neutrophils (5×10^6/ml), pretreated with 5 μg/ml cytochalasin B for 15 min at 37°C were incubated with different stimuli for 30 min at 37°C. To these mixtures different concentrations of FMLP were added and the cells were incubated a further 30 min at 37°C. After incubation the cells were centrifuged and the released lysozyme was assayed against Micrococcus Lysodeikticus. The O.D. at 450 nm was measured in triplicate at 10 s intervals for 3 min and the amount of lysozyme released was calculated from a standard curve generated by egg white lysozyme. Maximum values were obtained by using supernatants from neutrophils lysed with Triton X-100.

Stimulation of neutrophil superoxide production by rH GM-CSF. A titration of rH GM-CSF showed that neutrophils responded with an increased production of O_2^- upon stimulation with FMLP as judged by the increased reduction of cytochrome-C observed. Fig. 5 shows one representative experiment out of six performed. Very little reduction of cytochrome-C was detected with neutrophils preincubated with rH GM-CSF without the addition of FMLP. LPS at 1 ng/ml did not enhance the neutrophil response to FMLP. That rH GM-CSF but not LPS was responsible for the stimulatory effect on neutrophils was further demonstrated by one experiment in which preincubation of rH GM-CSF with polymyxin B did not reduce its stimulatory activity.

Effect of rH GM-CSF on neutrophil adherence. The effect of rH GM-CSF on the ability of neutrophils to adhere to human endothelial cells from umbilical cord veins and plastic surfaces was measured, as these phenomena appear to be the earliest steps leading to neutrophil migration into inflammatory sites. rH GM-CSF was unable to stimulate neutrophil adherence to

Table II. Enhancement of Lysozyme Secretion by Cytochalasin B-treated, FMLP-stimulated Neutrophils by rH GM-CSF at Different Concentrations

	Lysozyme*			
Experiment	[rH GM-CSF], 2 ng/ml	[rH GM-CSF], 0.2 ng/ml	[rH GM-CSF], 0.02 ng/ml	None
1	230±9‡	225±4	115±3	70±3
2	207±6	200±4	127±0	105±6

* Units of measure, U/ml per minute per 10^6 cells.
‡ Arithmetic means of triplicate determinations followed by 1 SD. Maximum for experiment 1, 268±12, and for experiment 2, 248±10. Details are as for Table I. Neutrophils were stimulated with FMLP at 10^{-8} M.

Table III. rH GM-CSF Enhances Iodination by Neutrophils Stimulated with Zymosan

Iodination	rH GM-CSF	Zymosan	rH GM-CSF + zymosan	P*
nmol/h per 10^7 cells	0.10±0.04 (5)	0.44±0.19 (4)	5.98±0.84 (3)	<0.001
Percent‡	0.64±0.26 (5)	2.65±1.2 (4)	38.4±5.50 (3)	<0.001

The reaction mixture contained 4×10^{-3} M sodium phosphate buffer, pH 7.4, 0.128 M NaCl, 1.2×10^{-2} M KCl, 10^{-3} $CaCl_2$, 2×10^{-3} $MgCl_2$, 8×10^{-6} NaI (4 nmol; 0.05 μCi ^{125}I), 2×10^{-3} M glucose, 0.25 mg albumin, 2.5×10^6 neutrophils, and 2 ng/ml rH GM-CSF, 0.5 mg zymosan, or both. The final volume was 0.5 ml and the incubation was for 1 h. The data are expressed as the mean±SE of (n) experiments.

* P value for the difference between zymosan and rH GM-CSF + zymosan.

‡ Percent iodide converted to a TCA-precipitable form.

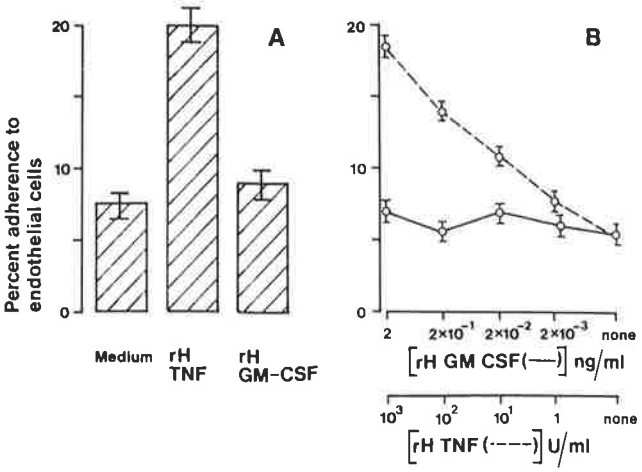

Figure 6. Adherence of neutrophils to endothelial cells. (A) Mean adherence±SE of neutrophils from five different individuals in the presence of medium, rH GM-CSF (2 ng/ml), or rH TNF-α (10^3 U/ml). (B) Dose response of neutrophils from one individual in the presence of different concentrations of rH GM-CSF (solid line) and rH TNF (dashed line). Each point is the mean of duplicate determinations. In this experiment 10 ng/ml PMA induced 75.9% adherence.

endothelial cells (Fig. 6 *A*). This was in contrast to the effects of PMA, and of rH-TNFα, which increased neutrophil adherence to endothelial cells in a dose-dependent manner (Fig. 6 *B*). LPS at 1 ng/ml was also a strong stimulus for neutrophil adherence (not shown).

rH GM-CSF was also a very poor stimulus at promoting adherence of neutrophils to plastic (Fig. 7). In six individuals, the mean adherence in the absence of any stimulus (control) was 4.3±2.2 (percentage±SE). The addition of rH GM-CSF into the reaction increased adherence to 6.4±2.5 (not significant). By contrast, rH TNF stimulated adherence by 18.9±4.2 (different from control, $P < 0.001$). In two individuals, however, neutrophils adhered to a relatively larger extent in the absence of stimulus, and in one of these the adherence could be increased by the addition of rH GM-CSF. PMA, used as a positive control, increased adherence to 50.7±9.6 ($n = 8$, different from control, $P < 0.001$).

Regulation of expression of granulocyte functional antigens by rH GM-CSF. rH GM-CSF was found (Fig. 8) to enhance the expression of GFA-1, GFA-2, and Mo1 as measured by the binding of FITC-labeled MAb WEM-G1, WEM-G11 $F(ab')_2$ and anti-Mo1 to these antigens. This assay, monitored by flow cytometry, showed that the mean and median values obtained with these MAb increased when neutrophils were incubated with rH GM-CSF. Thus, the mean fluorescence values obtained with these MAb increased by 40% (range, 20–77%) with WEM-G1, by 38% (range, 11–59%) with WEM-G11 $F(ab')_2$, and by 81% (range, 52–119%) with anti-Mo1 upon stimulation of neutrophils with 2 ng/ml of rH GM-CSF. The increase in the binding of MAb was rapid (evident at 10 min) and appeared to reach a plateau at 1 h of incubation at 37°C (data not shown). In contrast, very little or no increase was seen in the binding of MAb anti-β_2m, or the isotype matched but nonbinding controls K7 or PB10.

The selective upregulation of functional antigens was also demonstrable on human eosinophils. Thus, rH GM-CSF enhanced the expression of GFA-1 by 58% and of Mo1 by 68% but no increase was seen in the binding of anti-β_2m, K7, or PB10. LPS at a concentration of 3 ng/ml had no effect on the upregulation of neutrophil and eosinophil functional antigens.

Effect of rH GM-CSF on granulocyte morphology. Upon incubation with rH GM-CSF at 37°C a change in the shape of granulocytes was noted. The neutrophils appeared irregular in shape and elongated and eosinophils assumed a triangular shape with the granules concentrated near the base. These changes were interpreted as polarization. The change in shape in neutrophils was similar to that seen with FMLP and other stimuli. A titration of rH GM-CSF showed that most neutrophils were polarized at 15 min at rH GM-CSF concentrations of 0.2 ng/ml and above (Fig. 9 *A*). Eosinophils and neutrophils showed a

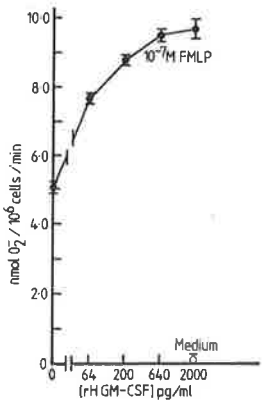

Figure 5. Effect of rH GM-CSF on the superoxide anion production of neutrophils upon stimulation with FMLP 10^{-7} M (●) or medium (○). Each point is the mean of triplicate determinations. The bars show the standard error of the mean.

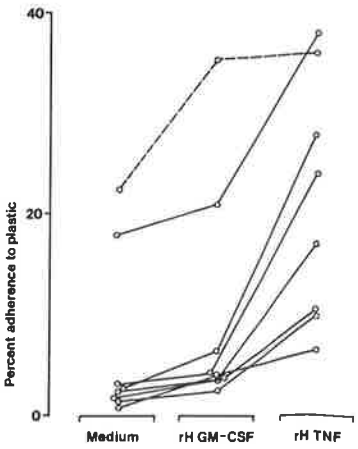

Figure 7. Adherence of neutrophils from eight individuals to plastic in the presence of medium, rH GM-CSF (2 ng/ml), and rH TNF-α (10^3 U/ml). Each point is the mean of quadruplicate determinations. The lines join the values obtained with neutrophils from the same individual. The broken line shows the values obtained with neutrophils from a high responder individual.

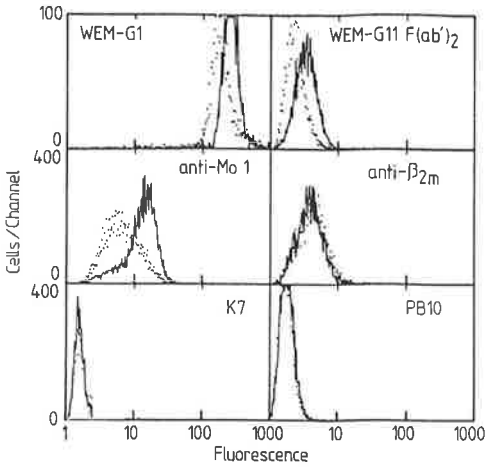

Figure 8. Fluorescence-activated cell sorter (FACS) profiles of the binding of MAb WEM-G1, WEM-G11 F(ab')$_2$, anti-Mo1, anti-β_2, K7, and PB10 to human neutrophils preincubated with medium (dotted line) or rH GM-CSF (continuous line) at 2 ng/ml. The increase in binding obtained with MAb WEM-G1, WEM-G11, F(ab')$_2$, and anti-Mo1 was significantly higher ($P < 0.001$) than the coefficient of variation of the FACS (<3%).

similar dose response with rH GM-CSF. Four other experiments gave similar results but with up to a fourfold variation in the dose of rH GM-CSF required to induce maximal responsiveness depending on the individual tested. The polarization of neutrophils by rH GM-CSF was seen within 5 min (Fig. 9 *B*). The changes observed by light microscopy were also reflected by an increase in the 0° light scatter of neutrophils as measured by flow cytometry (Fig. 9 *B*). The 0° light scatter also reflected the dose response relationship (data not shown).

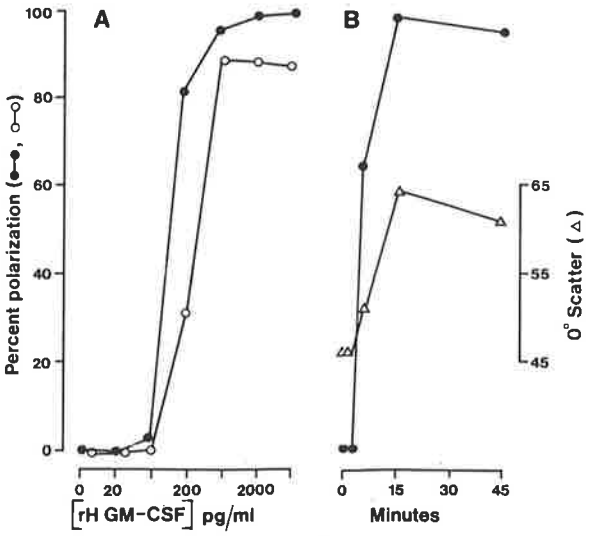

Figure 9. Morphological changes induced on human neutrophils (●) and eosinophils (○) by rH GM-CSF at 15 min of incubation. (A) Dose response of rH GM-CSF expressed as percent polarized cells after visual examination. (B) Time course of the effect of rH GM-CSF (2 ng/ml) on neutrophils expressed as percent polarized cells based on visual examination (●) and as mean channel number in the 0° scatter measured by flow cytometry (△). Each point is the mean of duplicate determinations.

Effect of rH GM-CSF on the survival of mature neutrophils and eosinophils. rH GM-CSF increased the survival of mature neutrophils as well as eosinophils in vitro. In four experiments performed, determinations of the time points at which 50% of plated cells were still alive revealed that rH GM-CSF significantly enhanced the median survival in vitro of eosinophils from 30 h (medium control) to 39 h ($P < 0.005$), and that of neutrophils from 22 h (medium control) to 28 h ($P < 0.0025$). In each case a titration of rH GM-CSF was carried out. A representative experiment out of eight performed is shown in Fig. 10 where neutrophils were examined after 28 h and eosinophils were examined after 31 h of incubation. rH GM-CSF at a concentration as low as 5 pg/ml was enough to support maximum levels of survival of both neutrophils and eosinophils. As the rH GM-CSF was diluted out, the number of viable cells rapidly decreased. The percentage of viable cells in cultures containing only medium with FCS was ~25% of that seen with optimal amounts of rH GM-CSF at these time points.

Discussion

In this paper we definitively show that a purified recombinant human GM-CSF (rH GM-CSF) stimulates the function of mature neutrophils as well as eosinophils, enhances the expression of functional antigens, and prolongs their survival in vitro.

Previous experiments in the mouse have shown that macrophages could be stimulated by GM-CSF to kill parasites (30) and by macrophage-CSF (M-CSF) to produce plasminogen activator (31). Furthermore, both granulocyte-CSF (G-CSF) and GM-CSF purified to homogeneity were shown to activate mouse neutrophils and G-CSF also stimulated human neutrophil but not eosinophil function (3).

In the human, early experiments had strongly suggested that one of the chief effects of CSF was the activation of mature granulocytes. For example, human placental conditioned medium (HPCM), a rich source of CSF, stimulated the killing of parasites by eosinophils (32), and HPCM as well as semipurified CSF-α and CSF-β enhanced the ADCC of tumor cells by human neutrophils (2). In addition, human mononuclear cell supernatants with CSF activity were found to enhance the helminthotoxic capacity of eosinophils (33) and stimulated neutrophils to kill microorganisms and tumor cells (34, 35). More impor-

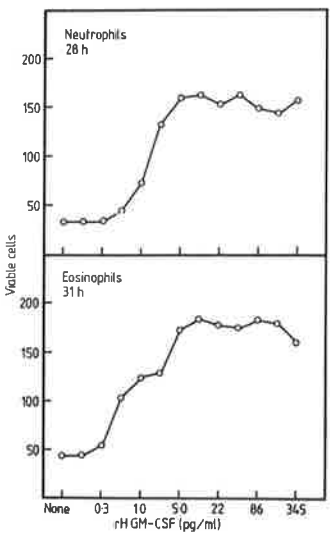

Figure 10. Stimulation by rH GM-CSF of the survival of mature human neutrophils and eosinophils. Purified cells were dispensed in Terasaki plates at ~200 per microwell in medium containing 10% FCS and different concentrations of rH GM-CSF. After the specified time the number of viable cells in each well was recorded. Each point shown is the mean of duplicate determinations.

tantly, fractionation of these mononuclear cell supernatants showed that the fractions with CSF activity co-chromatographed with fractions showing stimulation of killing by neutrophils and eosinophils (35).

Subsequent studies showed that GM-CSF purified from Mo cells–conditioned medium inhibited the migration of human neutrophils in agarose (7), and that the purified recombinant GM-CSF molecule increased superoxide anion production by neutrophils stimulated with FMLP (36). Preliminary experiments also showed that this material enhanced the cytotoxic and phagocytic activities of neutrophils (4). In this paper we confirm some of these findings and extend these observations to show that rH GM-CSF stimulates several but not all parameters of granulocyte function and is active on eosinophils as well as on neutrophils.

The first point of interest was that rH GM-CSF stimulated both neutrophil and eosinophil function (Figs. 1–5, Tables I–III). This is notable since the stimulation of function in both cell types previously seen with CSF-α (2) can now be attributed to a single molecule. It should also be pointed out that in some assays (e.g., ADCC), the stimulation of eosinophil function was stronger than that of neutrophil function, a pattern not seen with CSF-α (2; and our unpublished observations). Thus, it is still possible that another stimulatory molecule was present in CSF-α which was responsible for the strong stimulation of neutrophil function.

A second point of interest was the enhancement of the expression of GFA-1, GFA-2, and Mo-1 but not β_2m by rH GM-CSF, which suggests that CSFs, like the bacterial products LPS and FMLP (22), and the complement component C5a (37), selectively up-regulate the surface expression of functionally important molecules. In fact, the increase in binding of both radiolabeled C3bi (38) and of antibody to the complement receptor type 3 (CR3) for C3bi (39) on neutrophils incubated with Raji cell supernatants may be due to the up-regulation of this receptor (which is located on the Mo1 antigen) by CSF present in such supernatants. The upregulation of granulocyte functional antigens may be one important mechanism by which CSF and other factors play a role in vivo.

The stimulation of granulocytes by rH GM-CSF involved two types of functions. One was opsonin-dependent and encompassed ADCC (Fc-mediated) and fresh serum-dependent phagocytosis (complement-mediated). Enhancement of this type of function may be useful in vivo where activation of granulocytes by GM-CSF may not take place until granulocytes encounter the appropriate target. Although it can be argued that stimulation of this type of function is secondary to an increase in the relevant receptors (i.e. Fc, CR3) on granulocytes (Fig. 8), the magnitude of the different responses and the lack of effect of rH GM-CSF on adherence (see below) would suggest that this is not the only mechanism by which rH GM-CSF exerts its function. The second type of functions stimulated by CSF depends on a secondary stimulus, FMLP in the case of O_2^- production and degranulation, and zymosan in iodination experiments. While the stimulation of these functions may reflect changes in FMLP and zymosan receptor numbers or behavior, note that the stimulation observed is much greater than that seen with maximal FMLP doses, which suggests an increased intrinsic responsiveness to this agent.

The failure of rH GM-CSF to enhance the adhesiveness of neutrophils to plastic or endothelial surfaces was a notable negative finding, showing that the stimulation of granulocyte function by rH GM-CSF is selective.

The adherence of granulocytes to vascular endothelium is the initial step in the emigration of these cells into the tissues, and as such is central to the process of inflammation (40). This adherence of granulocytes to endothelium has been shown to be influenced by bacterial products and components of the complement cascade (41), platelet products (42), and more recently by rH TNFα (43). These data suggest that while GM-CSF may be of great importance in stimulating human neutrophil and eosinophil function at the site of inflammation, cell-derived products other than GM-CSF are responsible for stimulating granulocyte adherence leading to their emigration into inflammatory sites. This is an important point in future considerations involving the therapeutic administration of CSF, as it would suggest that the clinical use of CSF may be safe inasmuch as thrombotic reaction would not be predicted to occur.

Since these adhesive phenomena appear to involve the surface glycoproteins with C3bi receptor function (43), our negative findings are also important when contrasted with the results with phagocytosis also apparently dependent on the same neutrophil structure (44). This would support the notion that the stimulatory phenomena involve mechanisms apart from up-regulation of these surface receptors.

The rapid induction of morphological changes was similar to those noted in neutrophils incubated with FMLP (45) and phorbol myristate acetate (PMA) (27), and in the latter case these changes could be correlated with superoxide release and activation of protein kinase C. FMLP was also shown to cause polarization of human neutrophils which parallelled F-actin depolymerization and redistribution (46). The polarization of neutrophils by rH GM-CSF could also be detected by flow cytometry as judged by the increase in forward light scatter, an increase also seen when neutrophils were stimulated with FMLP and PMA (28). Taken together, these data suggest that rH GM-CSF induces morphological changes in neutrophils also seen with other activating agents and that these may be a reflection of cytoskeleton modifications and the state of activation of the cells.

The last parameter examined of granulocyte stimulation by rH GM-CSF was the survival in vitro. CSF is known to prolong the survival of bone marrow progenitor cells (1), and mouse M-CSF (CSF-1) has been shown to be required for the survival of bone marrow, resident peritoneal, and cultured peritoneal exudate macrophages (47). The demonstration that rH GM-CSF can extend the survival of both human neutrophils and eosinophils is in agreement with the specificity shown in functional assays. This effect may be beneficial in vivo by giving granulocytes a longer life span to carry out their effector function.

rH GM-CSF prolonged the survival of neutrophils for ~6 h and that of eosinophils for ~9 h. While the in vivo relevance of this phenomenon is not yet known the prolongation of granulocyte survival in peripheral blood may account for rapid changes in blood neutrophil numbers not previously explicable by demargination or bone marrow production. The low amounts of rH GM-CSF required to induce this phenomenon points to its possible use in therapy.

Also note, however, that CSF may be harmful if present in high concentration at sites of inflammation where it could play a role in the pathogenesis of certain diseases. Preliminary experiments have suggested that this may be the case in rheumatoid arthritis where CSF can be found in the fluid of diseased joints

(Williamson, D. J., C. G. Begley, M. A. Vadas, and D. Metcalf, unpublished observations).

The effects of rH GM-CSF on mature cells clearly encompasses activation, enhanced antigen expression, and survival. The degree to which these three parameters are related is not yet clear and it is possible that most parameters of activation may be explicable by the effect on receptor numbers and on survival. Regardless of the exact mechanism involved, the functional effects of rH GM-CSF on mature cells is likely to be biologically significant, and one that could be exploited therapeutically. For example, CSF may be a useful adjunct in the treatment of infections as well as in the ex vivo prolongation of granulocyte survival. In addition, the effect on mature cells will have to be taken into account when these substances are administered systemically to stimulate the proliferation and differentiation of bone marrow cells.

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Stimulation of proliferation, differentiation, and function of human cells by primate interleukin 3

(colony-stimulating factor/eosinophil)

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ABSTRACT **Cloned gibbon interleukin 3 (gIL-3) was found to stimulate the proliferation and differentiation of human bone marrow cells to produce day-14 granulocyte, macrophage, granulocyte–macrophage, and eosinophil colonies in semisolid agar. In the presence of normal human plasma, gIL-3 stimulated megakaryocytes. In methylcellulose cultures, it stimulated erythroid colonies in the presence, but not in the absence, of erythropoietin. When mature human leukocytes were used, gIL-3 stimulated the function of purified mature eosinophils as measured by the capacity to kill antibody-coated target cells, to produce superoxide anions, and to phagocytize opsonized yeast particles in a manner similar to recombinant human granulocyte–macrophage colony-stimulating factor. In contrast, gIL-3 did not significantly stimulate any of the neutrophil functions tested, whereas human recombinant granulocyte–macrophage colony-stimulating factor was active in these assays. Among cytokines that are active on human hematopoietic cells, gIL-3 thus has a distinct set of functions and may predict the range of actions of the human molecule.**

The murine (m) cytokine interleukin-3 (IL-3) (1, 2), also known as multipotential colony-stimulating factor (3) and persistent cell-stimulating factor (4), is distinct among the cloned hematopoietic-stimulating factors in having the capacity to stimulate progenitor cell renewal. This, as well as its relative lack of lineage restriction, suggested that mIL-3 is active at a more primitive level than the colony-stimulating factors (CSF) granulocyte–macrophage (GM)-CSF, macrophage (M)-CSF, granulocyte (G)-CSF, or eosinophil differentiation factor (EDF) and that it may have a special role in leukemogenesis (5).

We have cloned the gene encoding a molecule active in hematopoiesis from a gibbon (g) cell line that is 29% homologous at the amino acid level and has a genomic organization similar to that of mIL-3 (6). These properties led to the designation of this molecule as gIL-3.

In this communication we describe some of the biological effects of gIL-3 and show that, to our knowlege, its spectrum of activities on human cells differs from all described hematopoietic factors.

METHODS

Cytokines. The gIL-3 used was in a COS cell-conditioned medium obtained by transfecting COS-1 cells with 5 μg of plasmid DNA containing the gIL-3 cDNA (pMLA-CSF) and harvesting the gIL-3-containing medium 72 hr after transfection (6).

Recombinant human (rh) GM-CSF, with endotoxin at <0.2 ng/ml, was purified from the conditioned medium of COS cells that had been transfected with cloned human GM-CSF cDNA in the p91203(B) vector as described (7). Silver-staining of the $NaDodSO_4$/polyacrylamide gels of the purified GM-CSF revealed a major band of 19 kDa.

rh tumor necrosis factor type α was a gift from Genentech, (South San Francisco, CA) and contained cytotoxic activity (3.6×10^7 units/mg) on actinomycin D-treated L929 mouse fibroblast cells and endotoxin at 0.8 ng/ml. rh tumor necrosis factor type α was produced in *Escherichia coli* (8) and purified to 99.8% purity.

Bone Marrow Cultures. *Erythroid colony-forming unit assay.* Light-density nonadherent bone marrow cells were obtained by separation on a Ficoll/Paque (Pharmacia, Sweden) density gradient followed by a 60-min incubation with carbonyl iron [200 mg of carbonyl iron per 15×10^6 cells in 10 ml of RPMI with 15% (vol/vol) fetal calf serum (FCS)] and removal of monocytes (containing attached or phagocytized iron particles) with a magnet. Cells were cultured in 0.9% methylcellulose (Fluka, Sweden) with Iscove's modified Dulbecco's medium (Commonwealth Serum Laboratories, Australia), 30% (vol/vol) FCS (GIBCO), 0.66% bovine serum albumin (fraction V, Sigma), and 20 μM 2-mercaptoethanol at a concentration of 5×10^4 cells per ml of culture medium. The cultures were stimulated with 1 unit of high-purity human urinary erythropoietin (EPO) per plate and phytohemagglutinin-stimulated leukocyte-conditioned medium (PHA-LCM) [5% (vol/vol)], rhGM-CSF (100 ng/ml), or gIL-3 (1:200 dilution). Control cultures with no added stimulus or with EPO alone were also prepared. The cultures were incubated in an atmosphere of 5% CO_2/95% air. Hemoglobin-containing colonies present after 14 days containing >100 cells were scored as large erythroid colonies, and those containing 40–100 cells were scored as small erythroid colonies.

Myeloid colony-forming unit assay. The same culture system was used except that 0.3% agar (Difco) replaced 0.9% methylcellulose and that EPO was omitted. Aggregates of >40 cells were scored as colonies after 14 days incubation. The agar discs were then fixed with 3% (vol/vol) glutaraldehyde and transferred onto individual 5×8 cm glass slides. The discs were dried at room temperature and stained with luxol fast blue and a combined specific and nonspecific esterase stain.

Megakaryocyte colony-forming unit assay. The same culture system was used except that EPO was omitted and that

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Abbreviations: CSF, colony-stimulating factor; EDF, eosinophil differentiation factor; EPO, erythropoietin; FCS, fetal calf serum; g, gibbon; G, granulocyte; h, human; HUVE, human umbilical vein endothelium; IL-3, interleukin 3; m, murine; M, macrophage; PHA-LCM, phytohemagglutinin-stimulated leukocyte conditioned medium; r, recombinant.

Table 1. Morphological types of colonies in human marrow cultures after 14 days of stimulation in agar

Marrow sample	Stimulus	Total number of colonies	Morphological type, % of colonies			
			G	GM	M	Eo
1	Medium	0	—	—	—	—
	PHA-LCM	303	44	9	32	16
	gIL-3	181	33	4	43	20
2	Medium	0	—	—	—	—
	PHA-LCM	96	55	10	18	17
	rhGM-CSF	103	59	7	28	5
	gIL-3	91	66	1	12	11

Eo, eosinophil.

Table 2. Enhancement of erythroid colonies by gIL-3

Stimulus	Mean number of erythroid colonies (large/small)		
	1	2	3
EPO	0/1	0/0	4/30
EPO/PHA-LCM	6/18	9/12	85/61
EPO/rhGM-CSF	2/14	0/5	4/53
EPO/gIL-3	0/4	1/7	20/65

The mean number of erythroid colonies from three experiments is reported. A small colony had 40–100 cells; a large colony had >100 cells.

15% (vol/vol) human plasma and 15% (vol/vol) FCS were substituted for 30% (vol/vol) FCS. After a 14-day incubation, each 1-ml culture was resuspended in Dulbecco's phosphate-buffered saline (PBS), and cytosmears were prepared on a cytocentrifuge. The cytosmears were examined for the presence of megakaryocytes using the alkaline phosphatase–monoclonal anti-alkaline phosphatase technique (9). The monoclonal antibody 25E11, which recognizes the platelet glycoprotein IIb/IIIa complex (10) served as the primary antibody.

Purification of Human Neutrophils and Eosinophils. Peripheral blood of healthy volunteers was centrifuged on a hypertonic gradient of metrizamide (Nyegaard, Oslo) as described (11) after dextran sedimentation. The purity was >95% for neutrophils and >92% for eosinophils. The cells were resuspended in Eagle's minimal essential medium supplemented with 10% (vol/vol) FCS, 20 mM Hepes buffer (pH 7.4), and antibiotics. In experiments involving adherence, blood was collected using 0.2% EDTA as anticoagulant. The neutrophils were isolated by density-gradient centrifugation in Ficoll/Paque, followed by sedimentation in dextran and hypotonic lysis of erythrocytes. The preparation contained >97% neutrophils and was suspended in 0.9% NaCl at 5×10^7 neutrophils per ml.

Antibody-Dependent Cell-Mediated Cytotoxicity Assay. ^{51}Cr-labeled, trinitrophenyl-coupled P815 cells (4×10^3 cells in 40 μl) were incubated with 24 μl of rabbit anti-trinitrophenyl (Miles-Yeda, Rehovot, Israel), 80 μl of purified human neutrophils or eosinophils (1.3×10^5 cells) as effector cells, and 16 μl of rhGM-CSF, gIL-3, or medium for 2.5 hr at 37°C in V-bottom microtiter plates. The percent cytotoxicity was calculated as described (12).

Phagocytosis Assay. Eosinophils were suspended at 2×10^7 cells per ml in PBS. Dried bakers' yeast (Tandaco, Melbourne, Australia) was suspended in PBS to an OD_{540} of 1.6. A 250-μl aliquot was mixed with 50 μl of stimulator (gIL-3, rhGM-CSF, or medium control), with 100 μl of fresh autologous serum, and with 100 μl of cells (13). Tubes were incubated for 1 hr at 37°C, placed on ice, and then centrifuged at 4°C. Cell pellets were resuspended in 50 μl of cold PBS. Smears were fixed in methanol and stained with Giemsa. The number of phagocytized yeast cells were counted in at least 100 eosinophils per preparation.

Neutrophil Adherence Assay. First- or second-passage human umbilical vein endothelial (HUVE) cells were isolated and maintained as described (14). HUVE cells were plated in RPMI 1640 medium with 20% (vol/vol) FCS into 641-mm diameter wells (Costar, Cambridge, MA) at 2×10^4 cells per well and grown to confluence. Prior to assay, the medium was removed, and the HUVE monolayer was washed once in RPMI 1640 containing 10% (vol/vol) FCS. To each well was added a total volume of 200 μl containing neutrophils and the activating substance. The monolayers were incubated at 37°C for 30 min in an atmosphere of 5% CO_2/95% air, and then the nonadherent cells were removed by aspiration, and each well was washed once. All medium was then removed, and 100 μl of a 0.25% solution of rose bengal in PBS, pH 7.3, was added

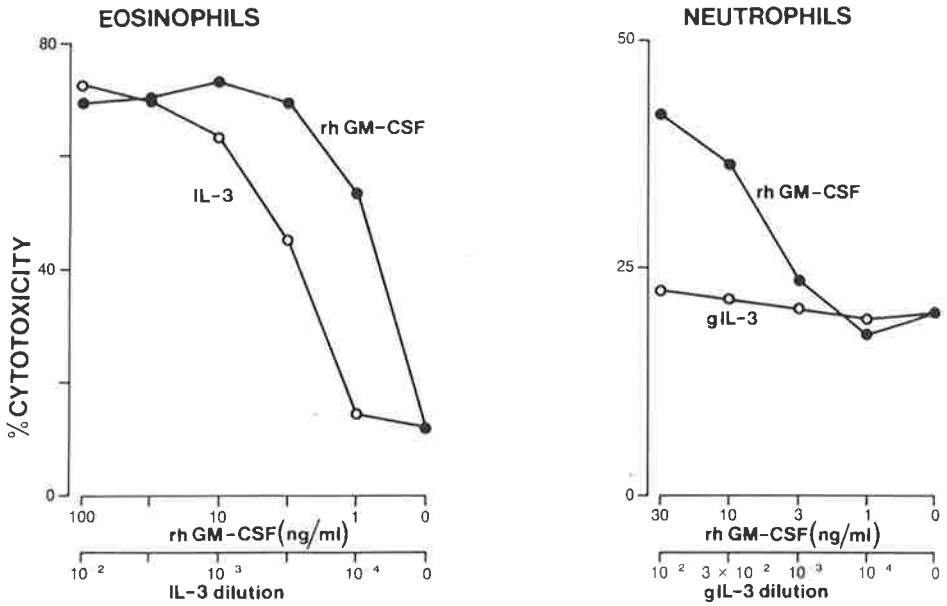

FIG. 1. Antibody-dependent cytotoxicity of trinitrophenyl-coupled P815 cells by human eosinophils and neutrophils in the presence of various concentrations of gIL-3 (○) and rhGM-CSF (●). The anti-trinitrophenyl IgG dilution was 1:300 for eosinophils and 1:3000 for neutrophils. No cytotoxicity was observed in the absence of antibody with or without gIL-3 or rhGM-CSF.

Table 3. gIL-3 stimulates human eosinophils to phagocytize serum-opsonized bakers' yeast

Fresh autologous human serum*	Stimulus	% of eosinophils containing phagocytized yeast cells 0	1	2
5% (vol/vol)	None	94.7 ± 0.9	4.3 ± 0.7	1 ± 0.6
	gIL-3†	84.5 ± 4.5‡	10.3 ± 1.2‡	5.3 ± 0.7‡
	rhGM-CSF	84 ± 1.1‡	12 ± 0‡	4 ± 1‡
2.5% (vol/vol)	None	97 ± 0.7	2 ± 0.6	0.7 ± 0.3
	gIL-3	84 ± 3.5‡	12.7 ± 2.3‡	3.3 ± 1.2‡
	rhGM-CSF	90 ± 1.5‡	7.7 ± 1.9‡	2.3 ± 0.3§

The percent of eosinophils containing zero, one, or two yeast cells is shown as the arithmetic mean ± SEM of triplicate determinations.
*In the absence of serum no phagocytosis was observed with any stimulus.
†gIL-3 was used at a final dilution of 1:300, while rhGM-CSF was used at 30 ng/ml.
‡Differs from no stimulus samples by $P \leq 0.05$.
§In this group alone there were several eosinophils with three particles.

to each well for 10 min at room temperature (15). After aspiration of the stain, each well was washed twice in assay medium and 200 μl of a 1:1 (vol/vol) solution of ethanol/PBS was added. When a uniform release of stain had occurred, 30 min later, the OD_{570} of each well was determined using an ELISA reader. Adherence is proportional to the difference between the OD of each well minus the OD of wells containing HUVE cells only.

Superoxide Production. Purified neutrophils or eosinophils were incubated with gIL-3, rhGM-CSF, or medium for various times at 37°C. Cells (150 μl) were then added to a mixture of 100 μl of freshly prepared cytochrome c (Sigma, type VI; 12.4 mg/ml), 100 μl of fMet-Leu-Phe (Sigma), and medium to 1 ml. The mixtures were incubated at 37°C for 5 min, after which the cells were rapidly cooled and pelleted at 4°C. The supernatants were transferred to plastic, disposable cuvettes. Superoxide production was measured in duplicate by the reduction of cytochrome c as described (16). In each experiment superoxide dismutase (Sigma) inhibited all superoxide generation.

Chemotaxis. The chemotactic response of neutrophils in a gradient of fMet-Leu-Phe was tested under agarose (17). Agarose (5 ml of 0.5% agarose; type II, Sigma) in RPMI 1640 with 2% (vol/vol) FCS and 20 mM Hepes was poured into a plastic Petri dish (50 mm, Kayline), and wells 2.4 mm in diameter were formed 2.4 mm apart in a horizontal line from the center to the edge of the plate. Neutrophils at 2.5×10^7 cells per ml in RPMI and 2% (vol/vol) FCS were preincubated for 45–60 min at 37°C in the presence or absence of rhGM-CSF at 100 ng/ml or gIL-3. Ten microliters of these cells was added to the center well; 10 μl of medium was added to the inner well; and 10 μl of fMet-Leu-Phe was added to the outer well. The Petri dish was then incubated for 2 hr at 37°C in 5% CO_2/95% air. Then, the cells were fixed in methanol at 4°C overnight, followed by 47% (vol/vol) formalin for 30 min at 25°C. Migration was measured under ×40 magnification with a calibrated graticule (1 division = 0.04 mm). Chemotaxis was the difference between directed and random movement.

RESULTS

Stimulation of Bone Marrow Cells. gIL-3 stimulated the formation of myeloid colonies in agar after 14 days of incubation (Table 1) and of very few clusters of cells after 7 days. Morphological examination of the stained agar cultures showed that gIL-3, like rhGM-CSF, stimulated granulocyte, macrophage, granulocyte–macrophage, and eosinophil colonies (Table 1). In two experiments megakaryocytes were identified in cytosmear preparations of gIL-3- but not of GM-CSF-stimulated colonies. gIL-3 also stimulated the formation of erythroid colonies in the presence but not in the absence of pure EPO (Table 2).

Stimulation of Human Granulocyte Function. *Antibody-dependent cell-mediated cytotoxicity assay.* gIL-3 stimulated eosinophils to kill antibody-coated tumor target cells in a dose-dependent manner (Fig. 1). The degree of stimulation was similar to that of rhGM-CSF, and comparable levels of killing were obtained with a 1:1000 dilution of gIL-3 and rhGM-CSF at 1 ng/ml. In contrast, neutrophil-mediated killing was not significantly enhanced by gIL-3 over the same dose range, whereas neutrophils did respond to stimulation by rhGM-CSF.

Phagocytosis. gIL-3 stimulated eosinophils to phagocytize serum-opsonized bakers' yeast (Table 3). The degree of

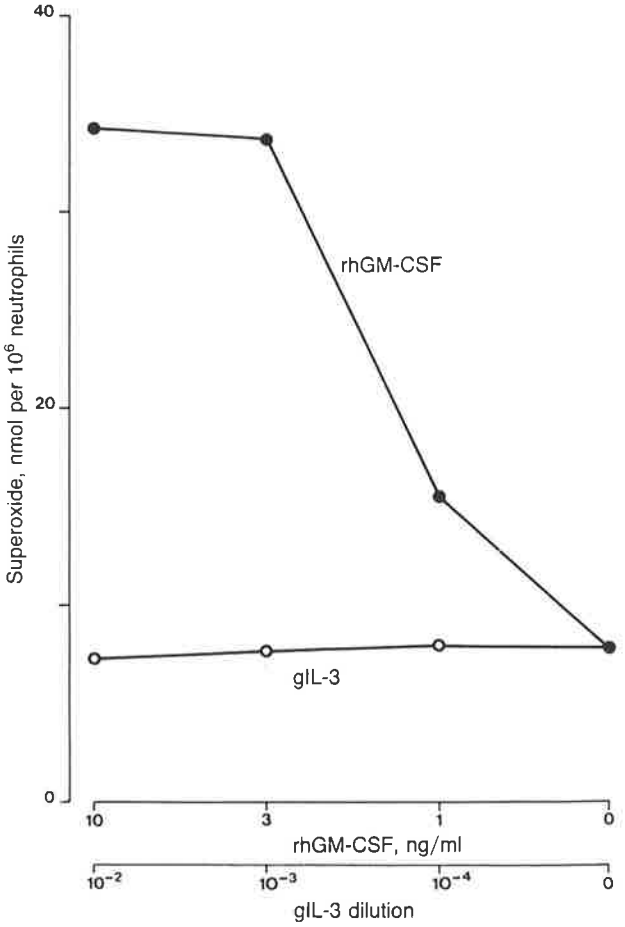

FIG. 2. rhGM-CSF but not gIL-3 enhanced fMet-Leu-Phe-stimulated superoxide production by human neutrophils. Points are arithmetic means of duplicate determinations, and SDs were always <15% of means.

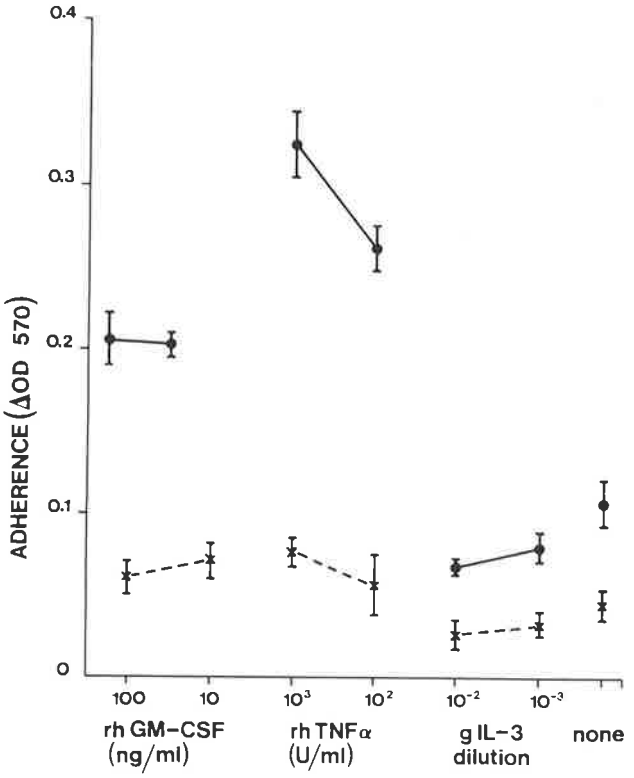

FIG. 3. Stimulation of neutrophil (●) and eosinophil (×) adherence to HUVE cells by rhGM-CSF, recombinant human tumor necrosis factor type α (rhTNF-α), and gIL-3. Each point is the arithmetic mean of three replicates ± SEM. Adherence is given as the change in OD_{570} of each well.

phagocytosis was similar to that obtained with rhGM-CSF, at the two concentrations of serum used.

Superoxide anion production. gIL-3 directly stimulated superoxide production by eosinophils (10.7 nmol per 10^6 cells per 15 min), which was comparable to the effect of rhGM-CSF (11 nmol per 10^6 cells per 15 min). By contrast neither cytokine directly stimulated superoxide production from neutrophils. Preincubation of neutrophils with rhGM-CSF, but not gIL-3, strongly enhanced their capacity to respond to fMet-Leu-Phe (Fig. 2).

Adherence. Because some cytokines have been shown to influence the adherence of neutrophils to endothelial cells, gIL-3 was tested in the HUVE cell adherence assay. gIL-3 did not stimulate either eosinophils or neutrophils to adhere to HUVE cells (Fig. 3). The control cytokines tumor necrosis factor type α and, to a lesser degree, rhGM-CSF stimulated neutrophil adherence, while phorbol myristate acetate was the only stimulus tested that was effective on eosinophils.

Table 4. Effect of rhGM-CSF and gIL-3 on neutrophil migration

Stimulus	Migration, mm			
	1 μM	0.1 μM	0.01 μM	None
	Total			
None	6.44 ± 0.06	5.4 ± 0.0	4.2 ± 0.1	4.00 ± 0
rhGM-CSF	5.86 ± 0.08*	4.54 ± 0.1*	4.14 ± 0.1	
gIL-3	6.5 ± 0.1	5.2 ± 0.1	4.1 ± 0.1	
	Chemotactic			
None	2.68 ± 0.06	1.38 ± 0.08	0.48 ± 0	0.32 ± 0
rhGM-CSF	2.18 ± 0.03*	0.84 ± 0.1*	0.18 ± 0.06	
gIL-3	2.5 ± 0.17	1.52 ± 0.06	0.24 ± 0.0	

Neutrophils were incubated with no stimulus, rhGM-CSF, or gIL-3, and fMet-Leu-Phe at 1 μM, 0.1 μM, or 0.01 μM was added as chemoattractant. As a control, medium without fMet-Leu-Phe was also used. Total migration and chemotactic migration [total migration − (random migration plus well diameter)] was measured.
*Differs from control sample by $P \leq 0.05$.

Table 5. Effect of various CSFs on human myeloid cells

CSF	Bone marrow colony type(s)	Neutrophil function	Eosinophil function
hGM-CSF	G, M, Eo	+	+
m- or hG-CSF	G	+	−
mEDF/Eo-CSF	Eo	−	+
gIL-3	G, M, Eo, megakaryocytic	−	+

Eo, eosinophil. +, Function present; −, function absent.

Chemotaxis. The ability of gIL-3 to influence neutrophil movement was tested in a migration assay. gIL-3 did not stimulate or inhibit random migration of neutrophils or their unidirectional movement to a chemotactic gradient of fMet-Leu-Phe (Table 4). These functions, however, could be inhibited by rhGM-CSF.

DISCUSSION

Our results indicate that gIL-3 is a multipotential proliferative stimulus for human cells and strongly stimulates some functions of mature eosinophils but not of neutrophils.

gIL-3 stimulated normal human nonadherent bone marrow cells to produce predominantly day-14 colonies similar in morphology to those produced by GM-CSF (Table 1), thus suggesting that gIL-3 acts on a relatively primitive type of progenitor cell. In the presence of human plasma, gIL-3 also stimulated the formation of megakaryocytes, an effect comparable to that of mIL-3 on mouse bone marrow. On the other hand, its capacity to stimulate mast cell colonies remains to be elucidated. In the presence of EPO, gIL-3 stimulated erythroid colonies. The spectrum of activities of gIL-3, therefore, appears to be similar to GM-CSF except for the megakaryocyte-stimulating property. However, because only a single dose of each cytokine was used in these experiments and because gIL-3 was not purified, no conclusions can be drawn about their relative potency.

gIL-3, like rhGM-CSF, was a powerful stimulator of mature human eosinophil function as judged by enhancement of antibody-dependent cell-mediated cytotoxicity, superoxide production, and phagocytosis (Fig. 1 and Table 3). In contrast to the rhGM-CSF, however, gIL-3 had no detectable effect on any neutrophil function we studied. In this regard it resembled mEDF, although EDF is known to stimulate only eosinophil colonies from human bone marrow cells (18) whereas gIL-3 clearly stimulated neutrophil and macrophage maturation as well.

The failure of gIL-3 to stimulate mature neutrophil function was surprising in view of its ability to produce day-14 granulocyte colonies. However, since no day-7 granulocyte colonies were produced either, it is possible that human neutrophils lose their receptors or their responsiveness to IL-3 as they mature. In the mouse, autoradiographic studies with radiolabeled mIL-3 revealed a decreasing number of receptors on neutrophils and eosinophils during maturation; however, mature neutrophils and eosinophils were clearly labeled—eosinophils bound twice as many mIL-3 molecules as did neutrophils (19). An alternative, though unlikely, explanation for the lack of human neutrophil stimulation by gIL-3 is the fact that gIL-3 differs from hIL-3 by 11 amino acids (6), which may affect the interactions of the gibbon molecule with the human receptor.

It is clear from our experiments that IL-3, like other CSFs, has the property of stimulating granulocyte function. mIL-3 can stimulate murine macrophages *in vitro* (20) and *in vivo* (21), although no effect was observed on mature neutrophil

Table 6. Regions of homology between human IL-3 and mouse T-cell replacing factor/B-cell growth factor 2

	61	78
hIL-3	G E D - - - Q D I L M E N N L R R P N L E	
	* * * * * . . * . *	
mTRF	G E I F G G L D I L K N Q T V R G G T V E	
	63	83
	125	138
hIL-3	E F R R K L T F Y L K T L - E	
	* * * * * * *	
mTRF	E R R R T R Q F - L D Y L Q E	
	106	119

Identical amino acids are indicated by asterisks and conserved changes with dots. mTRF, T-cell replacing factor.

and eosinophil function (21). Thus it remains to be seen whether there are fundamental differences between mouse and primate IL-3 in their capacity to bind and functionally activate mature neutrophils and eosinophils.

The biological activities of gIL-3 are compared to those of other CSFs known to stimulate human cells in Table 5. Clearly this molecule has a characteristic range of actions. It has broad specificity on relatively undifferentiated bone marrow cells, whereas it is active only on mature eosinophils and not mature neutrophils. It is also apparent that three cytokines have now been described with the capacity to stimulate mature human eosinophil function: rhGM-CSF (13, 16), mEDF (18), and gIL-3. We found some homologies between g- or hIL-3 and mouse T-cell replacing factor/B-cell growth factor 2, a molecule that is probably identical to mEDF (22). A computer comparison of hIL-3 (or gIL-3) and mouse T-cell replacing factor amino acid sequences revealed two regions that were 47% and 46% homologous (Table 6). The percentage homology was calculated over regions of >15 amino acids and includes conserved amino acid changes. It would be of interest to determine whether these regions of homology are related to eosinophil stimulation.

An eosinophil stimulatory molecule is produced by human mononuclear cells, probably monocytes, after culture (23, 24). It has been proposed that this molecule is similar but not identical to GM-CSF, or CSF-α (25). Our present results support the hypothesis that IL-3 is at least one of the factors produced under these culture conditions and is responsible for controlling some aspects of eosinophil stimulation. It is also possible that GM-CSF and IL-3 may regulate different aspects of eosinophil function; if this is the case, eosinophilia in allergy and in parasitism may be stimulated by different molecules and result in different clinical conditions reflecting the different roles of the eosinophil.

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ACTIVATION OF ANTIBODY-DEPENDENT CELL-MEDIATED CYTOTOXICITY OF HUMAN NEUTROPHILS AND EOSINOPHILS BY SEPARATE COLONY-STIMULATING FACTORS

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Semi-purified human colony-stimulating factors (CSF) powerfully enhanced the antibody-dependent cell-mediated cytotoxicity (ADCC) by metrizamide gradient-purified human neutrophils and eosinophils. The stimulation was observed on three different tumor targets, was rapid (less than 1 hr) in onset, and CSF-stimulated cells needed direct contact with targets for killing. A subspecies of human CSF, CSF-α, with eosinophil and granulocyte-macrophage (GM) colony-stimulating activity enhanced both eosinophil and neutrophil killing. In contrast, another subspecies of human CSF, CSF-β, having only GM colony-stimulating activity, only enhanced neutrophil-mediated ADCC. These results support the notion that human CSF have two sites of action: i) the progenitor cell, where they stimulate a relatively slow process of differentiation, and ii) the mature cell, where they have a rapid action of increasing functional capacity. Furthermore, it seems the pattern of CSF receptors on progenitor cells is maintained throughout the lineage of such cells and serves to regulate the function of mature cells.

Although granulocytes are the most abundant of peripheral white cells, there is minimal knowledge of the physiologic modulation of their function or of the nature and potency of the regulators involved. The observation that eosinophils obtained from the blood of patients with eosinophilia had enhanced helminthocidal (1) and other metabolic functions (2) suggested the function of these mature granulocytes might be regulated by identifiable factors. Subsequently, it was shown that a substance that co-purifies with a subspecies of human colony-stimulating factor (CSF),[2] CSF-α (3), has a powerful effect on human eosinophils *in vitro*, greatly enhancing their capacity to adhere to and kill schistosomula of *S. mansoni* (4, 5). These findings suggested that in man, as well as in the mouse (6–8), CSF-like molecules serve a dual purpose, namely, the stimulation of maturation of progenitor cells and the activation of mature cells of the same lineage. It was also noted in the above experiments that another subspecies of human CSF, CSF-β, had no effect on mature eosinophils, judged by their interaction with antibody-coated schistosomula.

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[1] Dr. Vadas is supported by a grant from the National Health & Medical Research Council.
[2] Abbreviations used in this paper: CSF, colony-stimulating factor; CSA, colony-stimulating activity; EO, eosinophil; GM, granulocyte-macrophage; HPCM, human placental conditioned medium; HEM, HEPES-buffered Eagle's medium; MTPBS, mouse tonicity phosphate-buffered saline; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; ADCC, antibody-dependent cell-mediated cytotoxicity; NRS, normal rabbit serum; E:T, effector to target; SPA, Staph protein A.

The main difference between CSF-α and CSF-β is in their colony-stimulating activity (CSA). Of the two CSF, only CSF-α has eosinophilopoietic activity, whereas both have the capacity to stimulate the production of granulocyte-macrophage (GM) colonies from different subsets of progenitor cells (3). In this communication, we use an antibody-dependent cell-mediated cytotoxic (ADCC) assay in which both human eosinophils and neutrophils are active to examine the specificity of CSF. We show that CSF-β does have the capacity to activate mature cells, but its activity is restricted to mature neutrophils. In contrast CSF-α, with both eosinophilopoietic and neutrophilopoietic activity, stimulates killing by mature eosinophils as well as neutrophils to a similar extent. These findings make it likely that CSF is the factor responsible for the activation of mature granulocytes, and that receptors for a certain type of CSF are maintained during the maturation of that lineage. In addition, the magnitude of stimulation of ADCC by CSF suggests these molecules have the important physiologic property of regulating granulocyte function.

MATERIALS AND METHODS

Preparation and purification of cells. The methods have been described in detail (9). Briefly, purified eosinophils and neutrophils were obtained from the same subjects by centrifugation of unpurified leukocytes over a slightly hypertonic metrizamide gradient. Purities ranged from 90 to 100%. The subjects were either scientists at The Walter and Eliza Hall Institute selected for having slightly elevated eosinophil counts or patients with allergic conditions. In both cases, informed consent was obtained before blood was taken. The cells were then washed twice in minimal essential medium supplemented with 10% fetal calf serum (FCS), 100 U penicillin G, 100 μg/ml streptomycin, 1% glutamine, 25 mM HEPES[2] buffer, and 30 mg/liter DNase (MEM-FCS).

Colony-stimulating factors (CSF). CSF-α and CSF-β were prepared from human placental conditioned medium (HPCM) by gel filtration (Ultrogel AcA44, LKB Produkter, Sweden) and hydrophobic chromatography (phenyl Sepharose CL-4B, Pharmacia, Sweden) as described, (3, 10). All batches were tested for CSA and for functional selectivity. Thus, CSF-α formed day 14 colonies that were either eosinophilic or of the neutrophil-macrophage type. CSF-β stimulated only neutrophil-macrophage colonies that formed by day 7. When a single concentration of CSF was used, it was chosen to be approximately three- to fivefold maximal in the colony formation assays.

Antibody-dependent cell-mediated cytotoxicity (ADCC). P815 (DBA/2 mastocytoma), BW (AKR thymoma) and EL-4 (C57BL thymoma) target cells were incubated with 150 μCi/ml ^{51}Cr (at 5×16^6 cells/ml) for 1½ hr, were washed once in HEPES-buffered Eagle's medium and 1% FCS (HEM-FCS), and then twice in mouse tonicity phosphate-buffered saline (MTPBS) before incubation with 10 mM trinitrobenzene sulfonic acid (TNBS) in MTPBS for 20 min at 22°C and pH 7.3. The reaction was stopped by dilution with HEM-FCS and cells were then washed twice in HEM-FCS, the last centrifugation being through a 1-ml underlay of FCS. Five thousand target cells were then aliquoted into V-bottomed microtiter plates that contained some of the following: one of two batches of rabbit anti-DNP (cross-reacting with TNP) IgG (Miles-Yeda, Rehovot, Israel), normal rabbit serum (NRS), CSF-α, CSF-β, and detergent (Triton X-100) to obtain maximum release. After 2½ hr incubation (a time previously determined to be optimal) one-half (80 μl) of the supernatant was removed and ^{51}Cr gamma emission was counted. Percent cytotoxicity was determined according to the formula:

$$\text{Percent cytotoxicity} = \frac{\text{Experimental cpm} - \text{spontaneous release cpm}}{\text{Maximum cpm} - \text{spontaneous release cpm}} \times 100$$

in which spontaneous release was determined by incubation of target cells alone.

Most experiments were performed by using at least three different effector to target (E:T) ratios; however, due to limitations of space, in some instances only single E:T ratios are reported. In some experiments, 10 μl of 0.5 mg/ml solution *Staphylococcus aureus* protein A (SPA) (Pharmacia, Sweden) were added to inhibit Fc receptor-mediated interactions.

Adherence assay. A typical assay as described above was set up and was terminated after 30 min of incubation. The cells were centrifuged for 3 min at 400 × G, the supernatant was removed, and cells were resuspended in 20 to 40 μl medium and placed on a microscope slide previously coated with toluidine blue. Each tumor cell bearing three or more attached granulocytes was counted as a rosette.

Statistics and controls. In general, standard deviations of replicates were less than 10% of total counts. Data from a typical experiment are shown in Table 1, together with some controls that are not shown with each experiment in *Results*. These show that cytotoxicity is only seen when cells and antibody are present together with target cells, and that various additives to the reaction do not by themselves cause ^{51}Cr release. Arithmetic means, standard deviations, and p values (with a two-tailed *t*-test) were determined on a HPCV pocket calculator.

RESULTS

Effect of CSF-α and CSF-β on eosinophil and neutrophil-mediated ADCC. Purified eosinophils or neutrophils were incubated at various E:T ratios with TNP and ^{51}Cr-labeled P815 cells, rabbit anti-DNP IgG, CSF-α, CSF-β, or medium control. The ^{51}Cr release after 2½ hr incubation (Fig. 1) shows that under these conditions, CSF-α enhanced both neutrophil and eosinophil-mediated ADCC, but CSF-β enhanced only neutrophil-mediated ADCC.

This experiment has been repeated more than 20 times, with 15 different individuals, and similar results were obtained. A summary of the first six experiments is shown in Figure 2. Two observations are of note: i) there is considerable variability between individuals in both the resting and CSF-stimulated ADCC; and ii) in all cases, CSF-α enhanced both neutrophil and eosinophil-mediated ADCC, whereas CSF-β only enhanced neutrophil-mediated ADCC. In some individuals (I, II) CSF-β did increase eosinophil-mediated ADCC, but it was calculated from cell titration experiments, always performed simultaneously, that the killing by contaminating neutrophils could entirely account for this small increase.

Effect of various concentrations of CSF on neutrophil and eosinophil-mediated killing. Purified cells were incubated, at an E:T ratio of 32:1, with rabbit anti-DNP IgG and serial dilutions of CSF. Figure 3 shows that CSF-α powerfully influenced ADCC by both cell types in a dose-dependent manner, whereas the main effect of CSF-β was on neutrophils. The

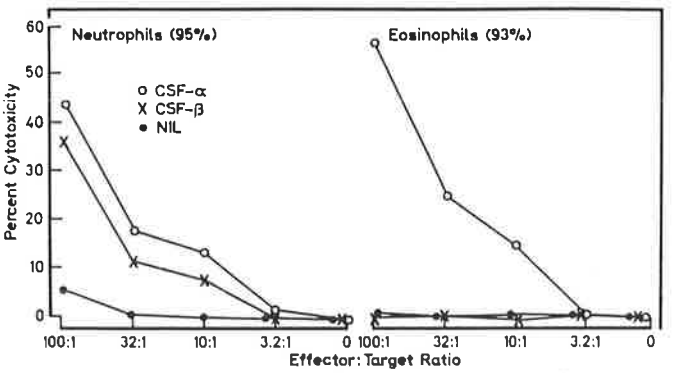

Figure 1. Antibody-dependent neutrophil or eosinophil-mediated cytotoxicity (percent cytotoxicity) of TNP and ^{51}Cr-labeled P815 mastocytoma cells, at various E:T ratios in the presence of CSF-α (○), CSF-β (X), or medium alone (●). Rabbit anti-TNP IgG was used in 1/3000 dilution. Each point is the arithmetic mean of quadruplicate determinations, and at E:T of 10:1 to 100:1 CSF-α incubated neutrophils differ from values at the same E:T ratios incubated without CSF by $p < 0.01$, 0.02, 0.01, respectively. For neutrophils and CSF-β, those figures are <0.02, 0.05, 0.05, and for eosinophils and CSF-α, <0.05, 0.05, and 0.001, respectively.

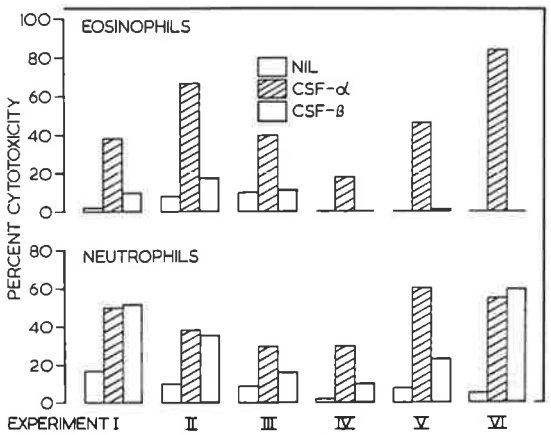

Figure 2. Effect of CSF-α and CSF-β on the antibody-dependent eosinophil- (*top panel*) or neutrophil- (*bottom panel*) mediated cytotoxicity of TNP and ^{51}Cr-labeled P815 target cells. Effector cells were used at an E:T ratio of 100:1 (I, V, VI) or 32:1 (II–IV). Each bar is the arithmetic mean of quadruplicate determinations. Eosinophils were 98, 90, 90, 100, 99, and 100, and neutrophils were 95, 98, 97, 100, 98, and 98% pure in Experiment I to VI, respectively. ^{51}Cr release by eosinophils stimulated by CSF-α differed from unstimulated values by $p = 0.0003$ or less. ^{51}Cr release by eosinophils stimulated by CSF-β was not significantly different from unstimulated values except in experiments I and II ($p < 0.07$ and 0.01, respectively) in which cases the increase was explicable by CSF-β stimulation of contaminant neutrophils. ^{51}Cr release by neutrophils stimulated by CSF-α or CSF-β differed from unstimulated values by $p = 0.007$ or less.

TABLE I

Raw data from a typical experiment

Group	Target	Incubations: Neutrophil 100:1 E:T	Incubations: αDNP[a] IgG	Incubations: NRS[b]	Incubations: CSF-α[c]	^{51}Chromium Release (Counts/2 min ± SD)	Percent Cytotoxicity
I	+	−	−	−	−	275 ± 42	0
II	+	−	−	−	+	282 ± 64	0.1
III	+	+	−	−	−	269 ± 39	−0.1
IV	+	+	−	−	+	223 ± 44	−0.7
V	+	+	+	−	−	1,000 ± 41	30.3[d]
VI	+	+	+	−	+	2,028 ± 93	75.1[d]
VII	+	−	+	−	−	300 ± 57	1.0
VIII	+	−	+	−	+	335 ± 247	3.5
IX	+	+	−	+	−	324 ± 62	2.0
X	+	−	−	+	+	322 ± 32	2.0
XI	+	Maximum release[e]				2,666 ± 144	100

[a] αDNP-IgG was used at 1/3000 dilution.
[b] Normal rabbit serum used at 1/50 dilution.
[c] CSF-α used at 1/10 dilution.
[d] Differs from group I by $p < 0.0001$.
[e] Measured by release due to Triton X-100.

minor effect of CSF-β on eosinophils could again be accounted for by its effect on contaminating neutrophils. Similar results were obtained with another batch of CSF: CSF-β caused neutrophil stimulation similar in magnitude (from 10 to 40% cytotoxicity) and titration (down to 1/300) to that by CSF-α. Eosinophil stimulation by CSF-β was only seen at a 1/10 dilution (from 9 to 18% cytotoxicity), which was small in comparison with that stimulated by CSF-α (9 to 66% cytotoxicity).

Effect of antibody concentration on ADCC by neutrophils and eosinophils. Neutrophils or eosinophils were incubated with targets and serial dilutions of anti-DNP IgG in the presence or absence of CSF-α. Figure 4 shows that in the absence of CSF, at a given concentration of antibody, neutrophils were slightly more efficient in killing than eosinophils; however, in the presence of CSF the cells performed approximately equally. In other experiments, it was shown that CSF-α and CSF-β had similar effects on neutrophil-mediated ADCC at all dilutions of antibody (Fig. 4, legend) and that CSF-β had no net effect on

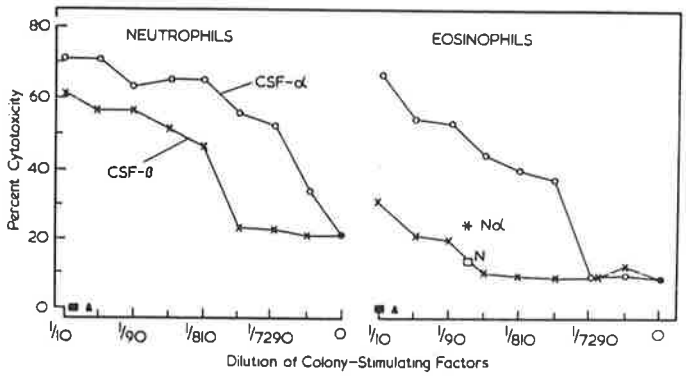

Figure 3. Effect of various doses of CSF-α (○) and CSF-β (X) on the antibody-dependent neutrophil or eosinophil-mediated cytotoxicity (percent cytotoxicity) of TNP and ^{51}Cr-labeled P815 mastocytoma cells at an E:T ratio of 32:1, and rabbit antI-TNP dilution of 1/3000. Each point is the arithmetic mean of quadruplicate determinations. The cytotoxicity in the absence of CSF (●) and in the absence of antIbody but not CSF-α (■) or CSF-β (▲) at 1/10 dilution are also shown. Eosinophils and neutrophils were 98% pure. Also shown is the contribution to eosinophil-mediated killing by contaminating neutrophils estimated from cell dose-response experiments with CSF-α (*) and CSF-β (□).

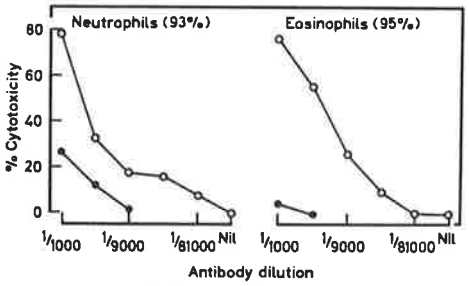

Figure 4. Effect of different dilutions of rabbit-anti-DNP IgG on cytotoxicity by neutrophils or eosinophils in the absence (●) or presence (○) of CSF-α. CSF-β did not increase eosinophil-mediated killing at any dilution of antibody, whereas neutrophil-mediated kill was increased to a level similar to CSF-α (the percent neutrophil-dependent cytotoxicity with CSF-α, CSF-β, and medium control being 62, 59, and 33 (1/1000 antibody), 56, 52, and 19 (1/3000 antibody), and 39, 39 and 2 (1/9000), respectively).

eosinophil-mediated ADCC at any dilution of antibody (data not shown).

Effect of CSF-α on ADCC by neutrophils and eosinophils on various target cells. To show that CSF-mediated effects apply to more than one type of target, EL-4 and BW thymomas were labeled with TNP and ^{51}Cr and were compared with P815 in the granulocyte-mediated ADCC system. The results in Table II show that both eosinophils and neutrophils had the capacity to kill these targets and the killing was significantly increased by CSF-α.

Effect of the killing reaction on bystander targets. To show that contact between effector cells and targets is necessary for CSF-α-stimulated target destruction, three simultaneous experiments were carried out with neutrophil effectors, anti-DNP serum, and P815 targets that were either labeled with ^{51}chromium and TNP, only ^{51}chromium, or only TNP. The isotope release from doubly labeled P815 targets served as a positive control that was compared with either only ^{51}chromium-labeled (bystander) targets or a mixture of ^{51}chromium-labeled targets and TNP-labeled targets. The results (Fig. 5) show that no significant isotope release took place from targets labeled only with ^{51}chromium even when mixed with TNP-labeled targets, demonstrating an absence of killing of bystander targets.

Effect of preincubation with CSF-α on ADCC by eosinophils and neutrophils. Purified cells were preincubated for 30 min at 37°C or 4°C with CSF-α, were washed thoroughly, and were tested for the capacity to mediate ADCC. Cells preincubated with CSF-α at 37°C (Table III), but not at 4°C (data not shown) killed better than control cells, indicating that activation at 37°C takes place rapidly and that CSF does not need to be present during the killing reaction. The preincubation did not damage the cells because the subsequent addition of CSF resulted in full activation, and extracellular carry-over of CSF did not account for the phenomenon because the supernatant from the last wash was shown to lack the capacity to activate cells.

Effect of addition of SPA on ADCC. To examine the critical time in CSF-α stimulated killing further, SPA was added at various times to the killing reaction. As noted previously in a different system (11), SPA prevents Fc receptor-mediated effector-target interactions and also reverses some established interactions. The results (Fig. 6) show that when SPA was added at the beginning of the culture all killing was prevented, whereas when the addition was delayed 1 hr, there was no observable inhibitory effect. A duplicate set of cultures was terminated, and ^{51}chromium release was measured at the time of addition of SPA. This showed that although after 1 hr incubation, SPA could no longer prevent killing, this was not due to maximal ^{51}chromium release having taken place, but to

TABLE II

Effect of CSF-α on neutrophil and eosinophil-mediated ADCC on various targets

Target	Rabbit Anti-DNP IgG	Percent Cytotoxicity[a]					
		Neutrophils			Eosinophils		
		No CSF	CSF-α	p Value[b]	No CSF	CSF-α	p Value
P815	+	18.2	53.4	< 0.001	3.7	67.8	< 0.001
	−		3.1			2.8	
EL-4	+	32.7	53.2	< 0.01	14.9	66.6	< 0.001
	−		0.9			1.7	
BW	+	30.2	79.0	< 0.02	26.4	45.8	< 0.04
	−		+2.3			−2.2	

[a] Arithmetic mean of triplicate determinations.
[b] p Value refers to differences between values with and without CSF.

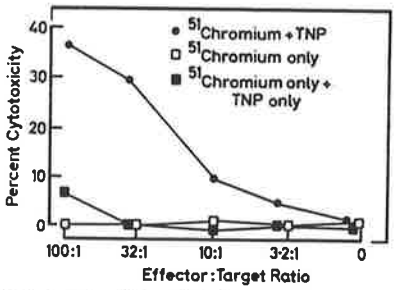

Figure 5. Effect of neutrophil-mediated ADCC on bystander targets. Purified neutrophils (97% pure), CSF-α, and rabbit anti-DNP IgG were added to targets labeled either with ^{51}chromium and TNP (●), only ^{51}chromium (□), or a mixture of targets labeled only with TNP and only with ^{51}chromium (■). The percent cytotoxicity was measured after a 2½ hr incubation.

TABLE III

Effect of preincubation for 30 min at 37°C of neutrophils or eosinophils with CSF-α on ADCC

E:T Ratio	CSF-α	Percent Cytotoxicity					
		Neutrophils[a]			Eosinophils[b]		
		Medium	CSF-α	p Value[d]	Medium	CSF-α	p Value
100:1	−	29.1[c]	55.9	<0.01	17.2	46.7	<0.02
32:1	−	17.0	32.2	<0.01	4.4	36.2	<0.01
10:1	−	6.7	16.1	<0.02	1.4	22.4	<0.01
32:1	+	59.9	64.2	NS	48.4	−	−

[a] Neutrophils were 93% pure.
[b] Eosinophils were 96% pure.
[c] Arithmetic mean of triplicate determinations.
[d] p Values refer to differences between preincubations with and without CSF-α.

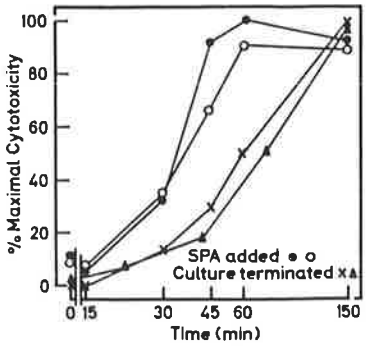

Figure 6. Effect of the addition of 10 μl of 0.5 mg/ml SPA at various times to incubations of ^{51}chromium and TNP-labeled P815 targets, rabbit anti-DNP IgG, CSF-α, and purified (97% pure) neutrophils. To show two experiments, the cytotoxicity is expressed as the percentage of killing obtained at 2½ hr (absolute values being 53 and 83%). At various times (ordinate) either SPA was added to the cultures (○, ●) and killing was measured at 2½ hr, or cultures were terminated and killing was measured at those times (▲, ×). The values at 30, 45, and 60 min differed by at least p = 0.004, 0.01, and 0.001, respectively.

an irreversible effect on the interaction between effectors and targets.

Effect of CSF-α and SPA on neutrophil-target cell rosettes. The cells in a typical experiment were examined morphologically after 30 min incubation at 37°C. In the presence of antibody, rosettes of tumor cells with effector cells were observed, and it was noted that the presence of CSF enhanced slightly the percentage of tumor cells with rosettes and the number of cells adherent to each target. SPA prevented the effector-target interaction (the formation of rosettes) and also reversed established interactions (dissociated formed rosettes). The degree of dissociation of rosettes formed in the presence of CSF-α was however less than that of rosettes formed in its absence (Table IV).

TABLE IV

Effector-target interaction examined morphologically

Incubations with TNP Labeled P815 Cells	Percent Rosettes at 30 Min[a]	
	No Additions	SPA Added at 15 Min
Neutrophils	0	0
Neutrophils + rabbit anti-DNP IgG	91 ± 7[b] (15)[c]	3.5 ± 0.7 (4)
Neutrophils + CSF-α + rabbit anti-DNP IgG	97.5 ± 4.4 (50)	19.7 ± 7.9 (3)

[a] Rosette defined as tumor target with three or more cells around it.
[b] Arithmetic mean ± S.D. of triplicate determinations.
[c] Numbers in parentheses indicate percentage of rosettes with more than five granulocytes.

Effect of CSF-α and CSF-β on Eosinophils and Neutrophils and their Progenitor Cells

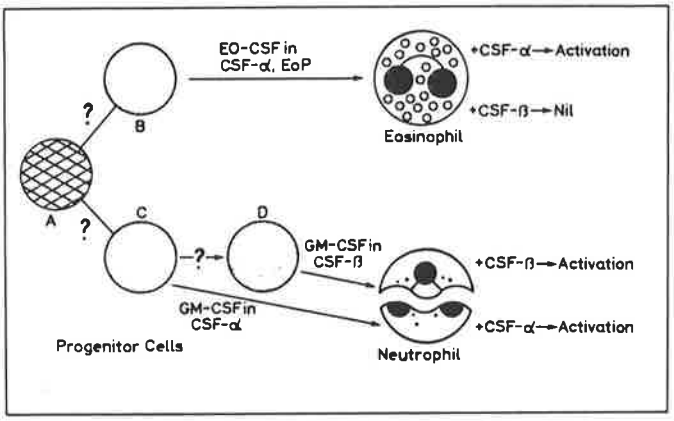

Figure 7. Schematic representation of the action of CSF on human granulocyte series. A multipotential stem cell, A, under unknown influences gives rise to progenitor cells B, C, and D. Progenitor cell B, under the influence of eosinophil-(EO) CSF in CSF-α, and perhaps eosinophilopoietin (EoP) gives rise to eosinophil colonies. These mature cells are activated in function by a substance in CSF-α. Progenitor cell C is different from progenitor cell D in physical properties (18) and response to regulators (3). C gives rise to GM colonies under the influence of GM-CSF in CSF-α, and D gives rise to GM colonies under the influence of GM-CSF in CSF-β. Mature neutrophils are activated by both CSF-α and by CSF-β. It is possible that neutrophils generated by GM-CSF in CSF-α and GM-CSF in CSF-β are not identical, being activated as a mature cell only by the regulator that stimulated their differentiation.

DISCUSSION

This paper confirms that human granulocytes are able to mediate ADCC (12–17) and makes the new observation that the level of ADCC is powerfully enhanced by materials containing CSF. The enhancement was demonstrable on three different targets (Table II), was mediated by direct contact of effector with target (Fig. 5), and was well developed after 30 min of incubation (Table III; Fig. 6). The mechanism of this action is uncertain, but because CSF-α powerfully increases resting and stimulated superoxide production and iodination by eosinophils,[3] it is possible that oxygen-dependent pathways are involved. The enhancement was especially marked at low levels of antibody and low E:T ratios, conditions that are likely to prevail early in the course of an immune response. Thus the local release of CSF may be of critical importance in enhancing the body's immune capacity to deal with foreign substances.

Because human CSF has not been purified to homogeneity, at this stage, we cannot definitively claim that the same molecule is responsible for the stimulation of progenitor cells and for the activation of mature cells. Nevertheless, there are compelling arguments that support such a notion. First, as far as eosinophils are concerned, the two activities have been shown to co-purify on Ultrogel AcA44 and on phenyl Sepharose chromatography (Reference 5; and M. Vadas *et al.*, unpublished observations). Secondly, there exists a parallel between the type of mature cells activated by CSF and the type of granulocyte formation that CSF promotes from progenitor cells (Fig. 7). Thus, CSF-α stimulates the formation of both eosinophil and GM colonies in semi-solid agar and stimulates ADCC by both mature cell types. CSF-β, on the other hand, has neither eosinophilopoietic nor eosinophil-stimulating activity, but has both neutrophilopoietic (GM) activity and neutrophil-stimulating activity. This suggests the pattern of CSF receptors in progenitor cells is maintained throughout their lineage and serves to regulate the function of mature cells (Fig. 7).

The progenitor cell target (cell C, Fig. 7) of GM-CSF in CSF-α differs from the target of GM-CSF in CSF-β (cell D, Fig. 7) in both sedimentation velocity and rate of proliferation necessary for colony formation (3, 19). This raises the possibility that the two CSF may also act on different subpopulations of mature neutrophils, and this suggests another level of control of mature cells. It is envisaged that the selective secretion of one subspecies of CSF would activate only a subpopulation of granulocytes. For example eosinophil-CSF may activate only eosinophils, and GM-CSF in CSF-α activate only those neutrophils that matured under its influence, the rest of the neutrophils responding to GM-CSF in CSF-β. Preliminary evidence (M. Vadas and N. Nicola, in preparation) showing that the actions of CSF-α and CSF-β are additive does suggest there may be different cellular targets of these two regulators.

The general observation that CSF-like molecules have a dual site of action is supported by data from murine systems in which pure CSF was shown to alter the function of mature

[3] Vadas, M. A., G. Varigos, N. Nicola, *et al.*, Eosinophil activation by colony-stimulating factor in man: metabolic effects and analysis by flow cytometry. Submitted for publication.

macrophages (8) or neutrophils (7). The argument that CSF is the active molecule in these systems is made even stronger by the recent observation (20) that monospecific anti-CSF antibody blocks the activation of murine macrophages by pure macrophage-CSF. Our observations in man suggest: 1) this is a general phenomenon; 2) a physiologic basis for the rapid and transient increase of CSF levels in the serum seen after the administration of bacterial products such as lipopolysaccharide (LPS) (21); and 3) the primary function of LPS-induced CSF could be an immediate effect on preformed mature cells rather than the more slow induction of the generation of mature cells by stimulating progenitor cell proliferation.

Finally, it should be noted that the variability among granulocytes from different individuals in resting killing capacity and in the response to CSF (Fig. 2) could be due to genetic or environmental factors. Regardless of the cause of the variability, it is striking and may in the future explain some of the individual variation in diseases in which neutrophils or eosinophils play a protective role.

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Three Essential Promoter Elements Mediate Tumour Necrosis Factor and Interleukin-1 Activation of the Granulocyte-Colony Stimulating Factor Gene

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Granulocyte-colony stimulating factor (G-CSF) is a haemopoietic growth factor produced by mesenchymal cells but not T lymphocytes after stimulation with specific cytokines or mitogens. A 330 bp promoter fragment of the human G-CSF gene induced reporter gene expression in human embryonic lung fibroblasts in response to tumor necrosis factor-α (TNF-α) or interleukin-1β (IL-1β). The same promoter fragment was not active in Jurkat T cells nor did it respond to phorbol ester in either cell type. At least three distinct elements, the CK-1 sequence, a decanucleotide present in haemopoietic growth factor genes, an NF-IL-6 consensus sequence and a consensus octamer sequence, were essential in the G-CSF promoter for TNF-α and IL-1β response. Mutation of any of these sequences abolished promoter function. In contrast, mutation of two other consensus protein binding sequences, i.e. a Pu-1 site and a CK-2-like sequence, did not eliminate promoter function. Both the CK-1 and octamer sequences acted independently as TNF-α and IL-1β responsive elements upstream of a heterologous promoter. The response of the octamer sequence and the 330 bp promoter but not the CK-1 sequence was greater with IL-1β than TNF-α reflecting a similar response of the endogenous gene.

KEYWORDS: G-CSF, gene transcription, TNF-α, IL-1β, CK-1, octamer

INTRODUCTION

Granulocyte-colony stimulating factor (G-CSF) is a member of a family of glycoproteins that control the survival, proliferation and differentiation of haemopoietic progenitor cells and the functional activation of mature cells (Clark and Kamen, 1987; Metcalf, 1989). G-CSF function is restricted to cells of the neutrophilic granulocyte lineage (Metcalf, 1989), and *in vivo* G-CSF has been shown to be implicated in normal neutrophil maturation (Hammond et al., 1991). G-CSF has been shown to release haemopoietic stem cells into the circulation and to be a powerful cofactor in stem cell proliferation (Ikebuchi et al., 1988; Sheridan et al., 1989). During inflammation, G-CSF is released into the serum and probably mediates the marked increase in granulocyte cell numbers (Cheers et al., 1988).

G-CSF production is induced in mesenchymal and myeloid cells in response to inflammatory mediators. Fibroblasts and endothelial cells express G-CSF when treated with tumour necrosis factor-α (TNF-α) and interleukin 1β (IL-1β) as well as phorbol ester (e.g. PMA) (phorbol-12-myristate-13-acetate) (Broudy et al., 1987; Koeffler et al., 1987; Kaushansky et al., 1988) or modified low density lipids (Rajavashisth et al., 1990). Monocytes and macrophages produce G-CSF in response to lipopolysaccharide (LPS) and phorbol ester (Vellenga et al., 1988; Ernst et al., 1989). Both increased transcription from the G-CSF gene (Seelentag et al., 1987) and alteration of

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G-CSF mRNA stability (Ernst et al., 1989) have been implicated in the inducible nature of G-CSF expression.

The G-CSF promoter region contains a decamer sequence, CK-1, found in several CSF and interleukin genes, at approximately 200 bp from the G-CSF transcription start site (Tsuchiya et al., 1987). We have previously shown that the G-CSF CK-1 sequence acts as a TNF-α and IL-1β inducible element when multiple copies were cloned upstream of a heterologous promoter in human embryonic lung (HEL) fibroblasts (Shannon et al., 1990; Kuczek et al., 1991). However, a fragment of DNA containing an identical CK-1 sequence from GM (granulocyte-macrophage)-CSF was not functional in a heterologous system (Kuczek et al., 1991). This CK-1 element from both genes specifically binds a nuclear protein, NF-GMa, the level of which is induced by TNF-α and IL-1β in fibroblasts and endothelial cells (Shannon et al., 1990; Kuczek et al., 1990). Because of the discrepancy in the function of the G-CSF and GM-CSF CK-1 sequences and the fact that multimerization of elements generate an artificial promoter we decided to study the relevance of the CK-1 sequence in the context of the G-CSF promoter. We have mutated the CK-1 sequence and other potential factor binding sites in the G-CSF gene and analysed the effect on promoter function. These other sites include a consensus octamer sequence (Schaffner, 1989), a potential NF-IL6 binding site (Akira et al., 1990), a potential PU.1 factor binding site (Klemsz et al., 1990) and a CK-2-like sequence, a conserved sequence also identified in GM-CSF and IL-3 (Shannon et al., 1988). Some of these sites have previously been shown to be important for mouse G-CSF expression in LPS induced macrophages (Nishizawa and Nagata, 1990).

In this study, we show that a 330 bp fragment of the human G-CSF promoter is TNF-α and IL-1β responsive in HEL fibroblasts. Point mutations in the CK-1 sequence could abolish the inducibility of the promoter. Mutations in the octamer sequence and the NF-IL6 site, but not the PU.1 or the CK-2 sequences, also abolished promoter function. The G-CSF octamer sequence independently conferred TNF-α and IL-1β responsiveness on a tk promoter cloned upstream of the CAT reporter gene. Thus, it appears that induction of the G-CSF promoter is mediated via the activation and potential interaction of at least three essential elements.

MATERIALS AND METHODS

Cells

The Jurkat T lymphoblastoid cell line was grown at a density of 5×10^5 cells/ml in RPMI 1640 with 10% fetal calf serum (FCS). Human embryonic lung fibroblasts (HEL) cells were obtained from CSL Laboratories (Melbourne, Australia) and grown in DMEM and 10% FCS. These cells were used from passage 14–20 in all experiments. Der-

A

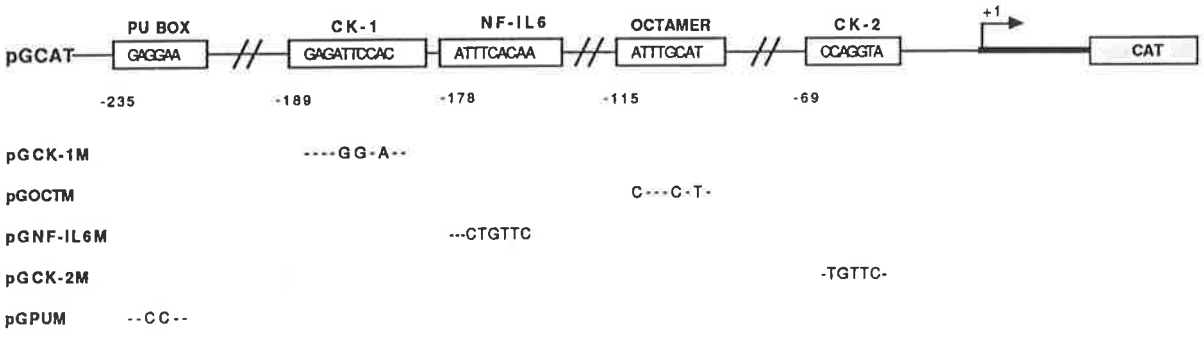


FIGURE 1. (A) A schematic representation of the 330 bp fragment (–311 to +18) of the human G-CSF promoter cloned upstream of the CAT reporter gene in pBLCAT3. Sequences boxed and named represent consensus binding sites for known transcription factors. The transcription start site is indicated at +1 and the numbers on the boxed sequences represent the distance of each element from +1. Mutations were introduced into each element to generate the plasmids shown. In each case the rest of the promoter is identical to pGCAT.

mal fibroblasts were obtained by collagenase treatment of dermal foreskin tissue, grown in RPMI+10% FCS and used at passages 3–5.

RNA Isolation and RNase Protection

Cells for RNA isolation were treated with TNF-α (100 U/ml), IL-1β (10 U/ml, defined as thymocyte mitogenesis units (Kronheim et al., 1985)) or PMA (20 ng/ml) for 6 hr before extraction. Total cellular RNA was isolated using guanidinium isothiocyanate (Chomczynski and Sacchi, 1987). RNase protection was performed as previously described (Goodall and Filipowicz, 1989), except that 10 μg of total HEL cell RNA was hybridized with approximately 1 fmol of ^{32}P-labelled RNA complementary to nucleotides 1232 to 1508 of the human G-CSF mRNA. ^{32}P-labelled RNA complementary to 120 nucleotides of human β-actin mRNA was included as an internal standard. After hybridisation the RNA was digested with RNase A at 10 μg/ml.

B

OCT

5'AATTCTGGACACCAAATTTGCATAAATCCTGGGG3'
3'GACCTGTGGTTTAAACGTATTTAGGACCCCTTAA5'

OCTM

5'AATTCTGGACACCAACTTTCCTTAAATCCTGGGG3'
3'GACCTGTGGTTGAAAGGAATTTAGGACCCCTTAA5'

CK-1

5'AATTCAAAGGAGGATCAGAGATTCCACAATTTCACAAAACG3'
3'GTTTCCTCCTAGTCTCTAAGGTGTTAAAGTGTTTTGCTTAA5'

IgK-kB

5'AACAGAGGGGACTTTCCGAGGCCATCT3'
3'TTGTCTCCCCTGAAAGGCTCCGGTAGA5'

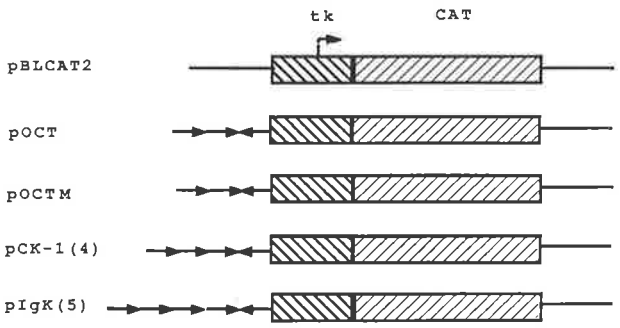


FIGURE 1. (B) Sequences of oligonucleotides used either in transfection plasmids or gel retardation assays. Sequences of a 34 bp fragment across the G-CSF octamer (OCT), a mutant version of this sequence (OCTM), a 40 bp fragment spanning the CK-1 sequence from G-CSF (CK-1) and a 27 bp sequence spanning the NF-κB site from the IgK gene (IgK-κB) are shown. The number and orientation of the copies of these oligonucleotides cloned into pBLCAT2 are shown.

Plasmid Constructs

A 330 bp fragment (–311 to +18) of the G-CSF promoter was cloned from genomal DNA prepared from Jurkat T cells by polymerase chain reaction (Sambrook et al., 1989). The amplified fragment was digested with EcoR1 and cloned into SP72 to create pSPG-CSF. Following confirmation of the sequence a Hind III/Bam HI fragment was cloned into the vector pBLCAT3 (Luckow and Shutz, 1987) to generate pGCAT (Fig. 1a). Site directed mutagenesis (Zoller and Smith, 1984) was used to generate the mutant G-CSF promoters as described in Fig. 1a.

Oligonucleotides containing the G-CSF octamer or mutant octamer sequence were synthesized on an Applied Biosystems model 381A DNA synthesiser and full length material purified by polyacrylamide gel electrophoresis (Sambrook et al., 1989). Following 5' phosphorylation and annealing, double stranded fragments were cloned into Bluescript SK(–) (Stratagene, Inc) using EcoRI ends. Clones containing 3 copies of the wild type and mutant sequences were selected and cloned with Bam HI/Hind III digestion into pBLCAT2 (Luckow and Shutz, 1987) to generate pOCT and pOCTM respectively (Fig. 1b). A plasmid containing 5 copies of the Igκ NF-κB binding site was generated in a similar manner and called pIgκ(5). A reporter plasmid (pCK-1(4)) containing 4 copies of the G-CSF CK-1 sequence was previously described (Shannon et al., 1990). The sequences of the oligonucleotides used to generate these plasmids and the orientation and number of sites in each plasmid are shown in Fig. 1b.

Transfection of Cells

HEL or dermal fibroblasts were transfected using DEAE Dextran and 10 μg plasmid DNA as previously described (Shannon et al., 1990). Transfection conditions were optimized using pSU2-CAT as a positive control. Twenty-four hours later cells were treated with TNF-σ (100 V/ml), IL-1β (10 U/ml) or PMA (20 ng/ml). Cells were grown for a further 16–24 hr before harvesting.

Jurkat cells were transfected by electroporation. 5 $\times 10^6$ cells were electroporated (260 V, 970 μF) in 300 μl DMEM and 30% FCS with 10 μg plasmid DNA. The cells were stimulated with TNF-α (100 U/ml), PMA (20 μg/ml), or phytohaemaglutinin (PHA) (1 μg/ml) and PMA (20 μg/ml) 24 hr later and harvested 2 days later. Cytoplasmic extracts were prepared and chloramphenicol acetyl transferase (CAT) assays carried out as previously described (Shannon et al., 1990). Percentage CAT conversion was determined by liquid scintillation counting of TLC plate areas containing ^{14}C-chloramphenicol and its acetylated derivatives. Relative CAT activity was determined within each experiment from the % CAT conversion value obtained from the unstimulated pGCAT or pBLCAT3 control.

Preparation of Nuclear Extracts and Gel Retardation Assays

Nuclear proteins were prepared from HEL fibroblasts or Jurkat T cells and enriched for NF-GMa by heparin-sepharose chromatography as previously described (Shannon et al., 1990). Oligonucleotide probes were ^{32}P-labelled using polynucleotide kinase (Sambrook et al., 1989). Binding reactions for NF-GMa were as previously described (Shannon et al., 1990). Protein DNA complexes were separated on 12% polyacrylamide gel in 0.5×TBE (Shannon et al., 1990). Protein binding to the G-CSF octamer and mutant octamer sequences was carried out with 3 μg crude nuclear extract from either HEL fibroblasts or Jurkat T cells. The binding reaction contained TM buffer (Shannon et al., 1990), 50 mM KCl and 3 μg polydI:dC. Protein DNA complexes were separated on 6% polyacrylamide gels in tris/glycine buffer. NF-κB protein binding to the Igκ NF-κB oligonucleotides from crude nuclear extracts of HEL fibroblasts was as previously described (Shannon et al., 1990).

RESULTS

Expression of the G-CSF Gene in Human Embryonic Fibroblasts

Human embryonic lung (HEL) fibroblasts, grown to 70–80% confluency in DMEM and 10% FCS, were treated for 6 hr with TNF-α (100 U/ml), or IL-1β (10 U/ml) or PMA (20 ng/ml). Total RNA was isolated from the cells and G-CSF mRNA levels determined by RNase protection. G-CSF mRNA was undetectable in unstimulated cells but was induced in cells treated with either TNF-α, IL-1β or PMA (Fig. 2). Dose response titrations showed that interleukin-1β always induced higher maximum levels of G-CSF mRNA than TNF-α (data not shown). The level of β-actin mRNA in the same RNA samples did not change with any of the treatments.

The Human G-CSF Promoter is Induced by TNF-α and IL-1β in Fibroblasts

We cloned a 330 bp fragment of the G-CSF promoter, generated by the polymerase chain reaction, upstream of the chloramphenicol acetyl transferase (CAT) reporter gene in pBLCAT3 (Luckow and Shutz, 1987) to generate pGCAT. This plasmid was transfected into HEL fibro-

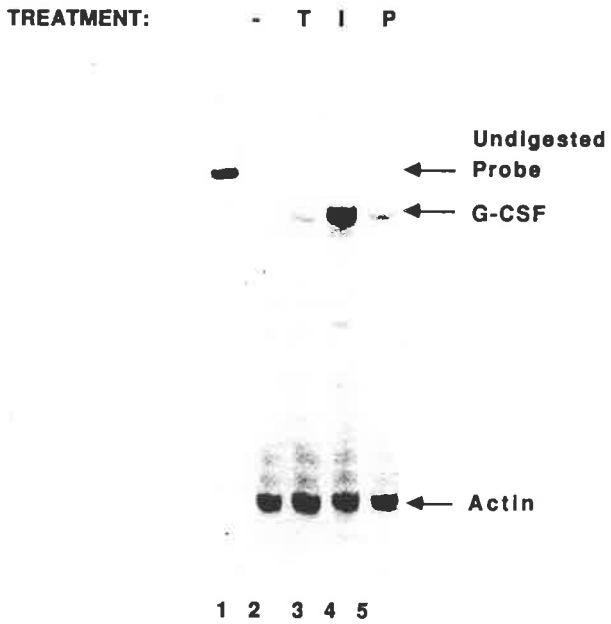


FIGURE 2. G-CSF mRNA expression in fibroblasts is induced by TNF-α, IL-1β and PMA. Total cellular RNA was prepared from HEL fibroblasts either unstimulated (lane 2) or stimulated with TNF-α (100 U/ml) (lane 3), IL-1β (10 U/ml) (lane 4) or PMA (20 ng/ml) (lane 5) for 6 hr. RNase protection assays were carried out using a radiolabelled G-CSF single stranded antisense probe to generate a protected fragment of 276 bp. A single stranded probe for β actin, generating a protected fragment of 120 bp, was included as a control for RNA quality and integrity. The undigested G-CSF probe (lane 1) as well as the protected G-CSF and actin bands are indicated.

blasts using DEAE dextran. The transfected cells were treated with either TNF-α (100 U/ml), IL-1β (10 U/ml) or PMA (20 ng/ml) for 24 hr prior to harvesting for CAT activity measurement. The G-CSF promoter activity was not higher than vector alone in instimulated cells (Fig. 3). An average increase of 2.5 fold and 4.0 fold (average of 4 experiments) in CAT activity was observed following TNF-α or IL-1β treatment respectively (Fig. 3). The increased transcriptional activity of the G-CSF promoter was both time and dose dependent with maximum levels of expression observed with 100 U/ml of TNF-α and 10 U/ml IL-1β 24 hr after addition of the stimulus (data not shown). Treatment of transfected cells with phorbol ester (PMA) (20 ng/ml) did not induce G-CSF promoter activity (Fig. 3). The G-CSF promoter did not respond to TNF-α or PMA (20 ng/ml) and PHA (2 μg/ml) treatment in Jurkat T cells (data not shown). A plasmid containing 5 copies of the Igκ NF-κB site responded at least 100 fold to both TNF-α and PMA/PHA in Jurkat T cells (data not shown). These results show that the 330 bp fragment of the G-CSF promoter parallels the response of the endogenous G-CSF gene to induction by TNF-α and IL-1β but not in its response to PMA in fibroblasts.

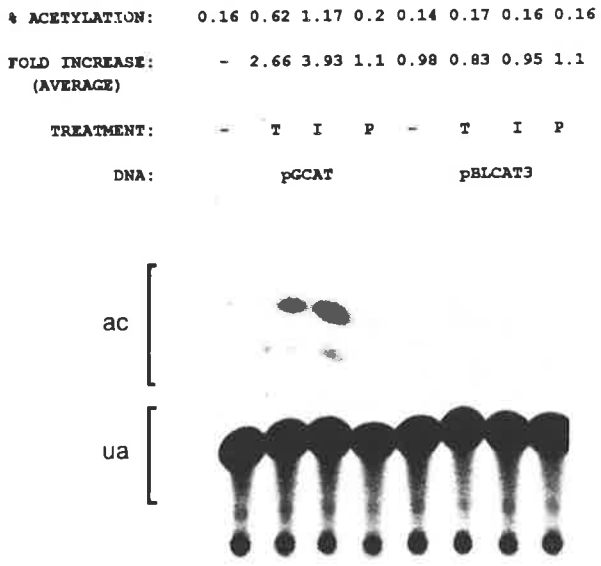


FIGURE 3. The G-CSF promoter is responsive to TNF-α and IL-1β but not PMA. Embryonic fibroblasts were transfected with pGCAT (lanes 1–4) or pBLCAT3 (lanes 5–8). Twenty-four hours after transfection the cells were left unstimulated (–) or stimulated with TNF-α (T; 100 U/ml), IL-1β (I; 10 U/ml) or PMA (P; 20 ng/ml) for 12–16 hr prior to harvesting. Cytoplasmic extract (20–50 μg) was assayed for 1 hr for CAT activity. The average fold induction for each treatment from at least three experiments as well as the % chloramphenicol acetylation is shown. Unacetylated (ua) and acetylated (ac) forms of CAT are indicated.

The CK-1 Sequence is Required for the TNF-α and IL-1β Response of the G-CSF Promoter

We have previously shown that a 40 bp oligonucleotide spanning the CK-1 sequence (–189 GAGATTCCAC –180) from the G-CSF gene, cloned upstream of the thymidine kinase (tk) promoter in pBLCAT2 mediated increased transcriptional activity in response to TNF-α and IL-1β treatment in HEL fibroblasts but not Jurkat T cells (Shannon et al., 1990; Kuczek et al., 1991). Mutations in the CK-1 sequence which abolished the *in vitro* binding of a transcription factor, NF-GMa, also abolished the response to TNF-α and IL-1β (Kuczek et al., 1991). To determine the role of the CK-1 sequence in the context of the 330 bp intact G-CSF promoter, 3 base positions were mutated in the G-CSF CK-1 sequence to generate the plasmid pGCK-1M (Fig. 1a). A comparison of the CAT activity from cells transfected with pGCAT and pGCK-1M showed that mutation of the CK-1 sequence abolished almost all detectable response to TNF-α and IL-1β (Fig. 4a).

We examined the ability of the NF-GMa protein to bind to the mutated CK-1 sequence used above. Double stranded oligonucleotides spanning the wild type G-CSF CK-1 sequence or the mutated CK-1 sequence (Fig. 1a) were used as probes in gel retardation assays. Binding of NF-GMa to the mutant CK-1 sequence was reduced to approximately 10% of that to wild-type CK-1 (Fig. 4b, compare lanes 1 and 2). For comparison, a single base mutation (T→G at position 6) which we had previously shown to dramatically reduce NF-GMa binding (Shannon et al., 1990) is shown (lane 4) whereas a second single base substitution (G→T at position 3) reduced binding by approximately 40% (lane 3). These data show that CK-1, and by inference from the binding data, NF-GMa, are essential components of the TNF-α and IL-1β activation machinery of the G-CSF promoter.

The NF-IL6 and Octamer Consensus Sequences Are also Required for G-CSF Promoter Induction

Examination of the G-CSF promoter sequence

showed several other potential transcription factor binding sites. These include an octamer sequence (−115 to −108), an NF-IL6 binding site (−178 to −170), a PU.1 binding site (−235 to −230) and a CK-2 like sequence (−69 to −63) (Fig. 1a) (Shannon et al., 1988). The NF-IL-6 binding site was of particular interest since the CK-1 DNA fragments which conferred TNF-α and IL-1β responsiveness to the tk promoter also contained this more recently described potential functional site (Shannon et al., 1990). Mutations, designed to abolish binding of the cognate transcription factors, were introduced into each of the potential transcription factor binding sites in the whole promoter (Fig. 1a). Transfection of these mutant plasmids into HEL fibroblasts, followed by treatment with either TNF-α or IL-1β showed that the octamer sequence and NF-IL6 concensus sequence were both required for promoter response to these cytokines (Fig. 5). On the other hand, mutation of the CK-2-like sequence (pGCK-2M) had no effect on promoter inducibility (Fig. 5). Mutation of the potential PU.1 binding site (pGPUM) also did not abolish promoter function. It did, however, reduce the IL-1β response of the promoter (4 fold) to the level seen for TNF-α (2.5 fold) (P=0.022 from paired t-test on each experiment). The PU.1 mutation was designed to abolish potential PU.1 binding (Klemsz et al., 1990) and the mutation in the CK-2 sequence was based on our previous observation that binding of a protein, NF-GMb, to the CK-2

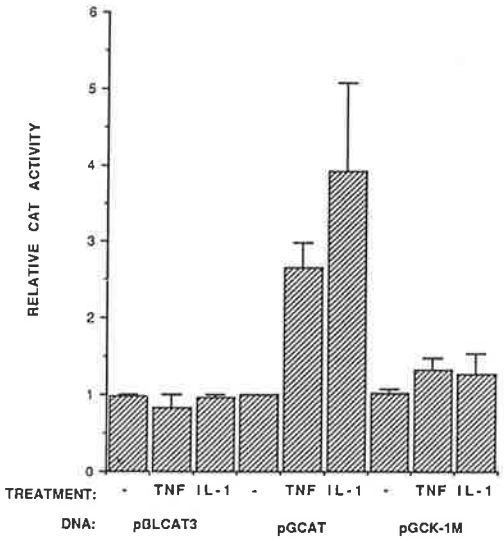


FIGURE 4. (A) The CK-1 sequence is required for G-CSF promoter response to TNF-α and IL-1β. Embryonic fibroblasts were transfected with pBLCAT3, pGCAT or pGCAT containing a mutant CK-1 sequence (pGCK-1M). Cells were stimulated with TNF-α and IL-1β and CAT assays performed as described in the legend to Fig. 3. The level of CAT activity in the unstimulated pGCAT samples was assigned a value of 1 and the levels in all other samples are shown relative to this value. The relative CAT levels are the mean (±SE) of at least three experiments.

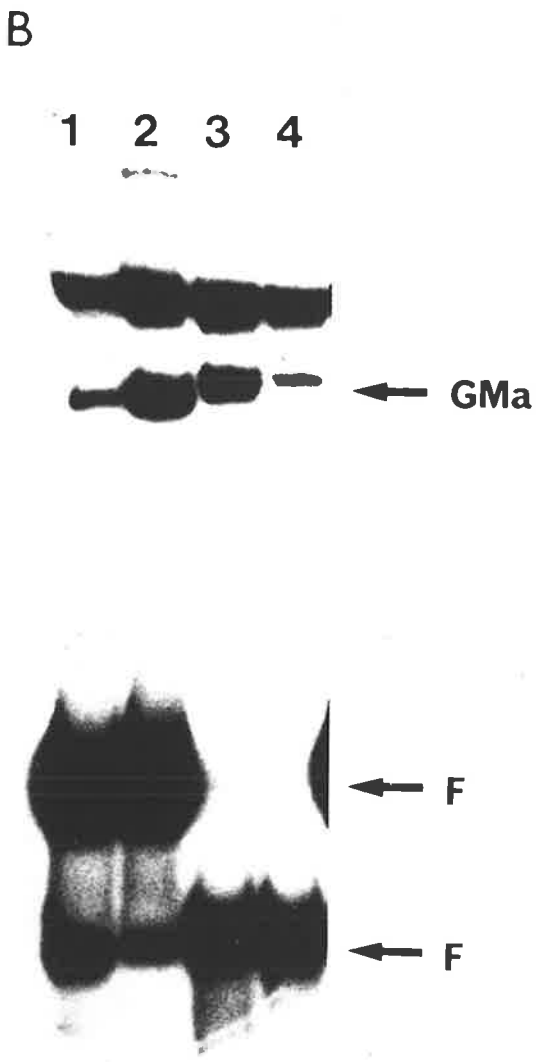


Figure 4. (B) Mutations in the CK-1 sequence reduce NF-GMa binding. Gel mobility shift assay using 6 μg of heparin sepharose enriched extract from HUT78 T cells showing binding of NF-GMa to a 41 bp radiolabelled fragment spanning the G-CSF CK-1 sequence (5' GAGATTCCAC 3', lane 2) and to the CK-1M sequence (5' GAGAGGCAAC 3', lane 1). Binding to 30 bp oligonucleotides containing single base mutations in the G-CSF CK-1 sequence (G3→T; lane 3 and T6→G; lane 4) are also shown.

sequence in the GM-CSF gene could be abolished by this mutation (Shannon et al., 1988).

These results show that at least three promoter elements, namely the CK-1 sequence, the octamer sequence and the NF-IL6 consensus sequence are all essential for TNF-α and IL-1β induction of the human G-CSF promoter.

The G-CSF Octamer Sequence Is an Inducible Element

To further test the octamer sequence function, 3 copies of a 34 bp sequence spanning the octamer were cloned upstream of the tk promoter in pBLCAT2 to generate pOCT (Fig. 1b). pOCT showed no transcriptional activity in unstimulated HEL fibroblasts but treatment with either TNF-α (100 U/ml), IL-1β (10 U/ml) or PMA (20 ng/ml) led to averages of 7.5, 74.5 and 3.5 fold increases in transcriptional activity respectively (Fig. 6a). pOCTM containing 3 copies of a mutant octamer sequence (Fig. 1b) was only very weakly inducible compared to the wild type sequence (Fig. 6a).

pOCT was transfected into dermal fibroblasts to determine if the induction phenomenon was general in fibroblast type cells. pOCT showed high constitutive activity in these cells with a

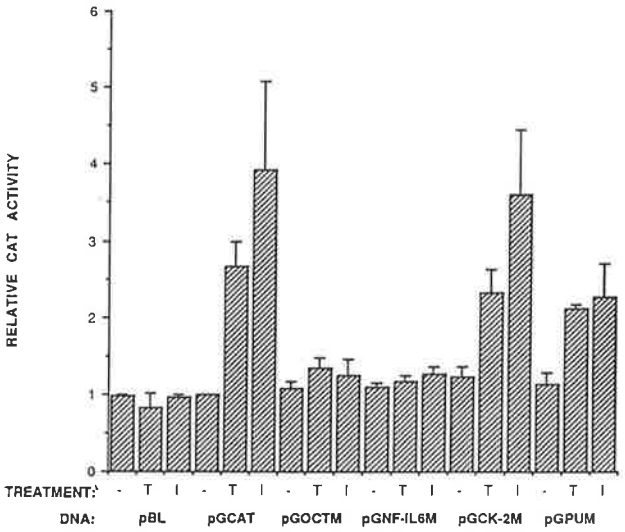
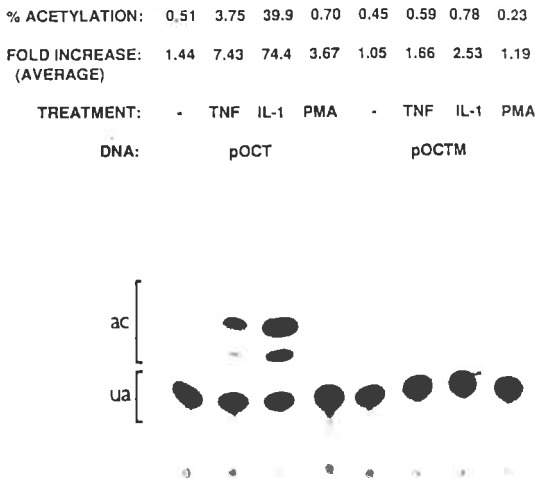


FIGURE 5. The effect of specific sequence mutations on G-CSF promoter function. Embryonic fibroblasts were transfected with pBLCAT3 (pBL), pGCAT and mutant version of pGCAT shown in Fig. 1a. Cells transfected with each plasmid were stimulated with TNF-α (T) or IL-1β (I) for 12–16 hr prior to harvesting. Within each experiment all CAT activity levels were expressed relative to a value of 1 assigned to the unstimulated pGCAT sample. The relative CAT levels shown are the mean (±SE) for at least 3 experiments.

A



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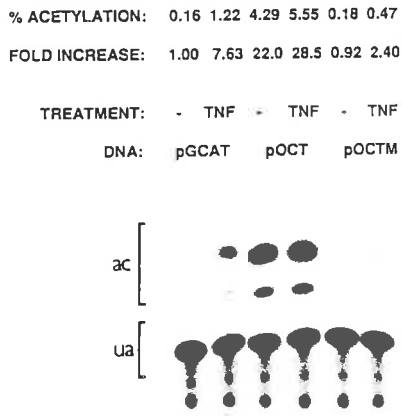

FIGURE 6. The G-CSF octamer sequence is TNF-α and IL-1β responsive in embryonic fibroblasts. (A) Embryonic fibroblasts were transfected with pOCT and pOCTM (Fig. 1b) and cells stimulated with TNF-α, IL-1β and PMA as described in the legend to Fig. 3. The % chloramphenicol acetylation is shown for each treatment. The average (3 experiments) fold increase from the value obtained for untreated pBLCAT2 is also shown. (B) Dermal foreskin fibroblasts were transfected with pGCAT, pOCT and pOCTM using DEAE Dextran as described. Transfected cells were left unstimulated (–) or stimulated with TNF-α (TNF) for 12–16 hr before harvesting. The % chloramphenicol acetylation and the fold increase in CAT activity over the value obtained for pBLCAT2 are shown for each plasmid and treatment. Unacetylated (ua) or acetylated (ac) forms of chloramphenicol are indicated.

minimal level of induction by TNF-α (Fig. 6b). In the same experiments the G-CSF promoter showed no constitutive activity and showed an even greater response (7.5 fold) to TNF-α than described for HEL fibroblasts (Fig. 6b). The constitutive activity observed in these cells was abolished by mutation of the octamer sequence (Fig. 6b). Similar results were obtained with IL-1β (data not shown). In Jurkat T cells transfected with pOCT or pOCTM only low levels of constitutive activity with no inducibility was detected with TNF-α (data not shown). In the same experiments multiple copies of the IgK NF-κB site responded to >50 fold to TNF-α treatment.

We attempted to determine if a TNF-α and IL-1β inducible octamer binding protein was present in HEL fibroblasts. Gel retardation assays were carried out using the octamer and octamer mutant radiolabelled oligonucleotides with extracts from unstimulated or TNF-α, IL-1β or PMA stimulated HEL fibroblasts. No specific protein binding to the octamer probe was detected in these cells (Fig. 7a, lanes 1–8). The complex marked x was not specific for octamer binding in competition experiments (data not shown). We also examined binding to the octamer probe with extracts from Jurkat T cells either unstimulated or treated with PMA, PMA/PHA or TNF-α. These Jurkat cells contained a protein which comigrated with a known Oct1 protein band from HeLa cells (Fig. 7a). This protein bound specifically to the G-CSF octamer probe but not to the mutant octamer probe (Fig. 7a, lanes 9–16). The HEL fibroblast extracts were analysed for the presence of a second inducible DNA binding protein, NF-κB. NF-κB binding was detected in IL-1β, TNF-α and PMA stimulated HEL fibroblasts but not in unstimulated cells (Fig. 7b) showing that the HEL cells responded to treatment with the various agents and that the extracts were not degraded. These results show that the G-CSF octamer sequence can bind a protein which appears to be Oct1 and that the HEL fibroblasts contain undetectable

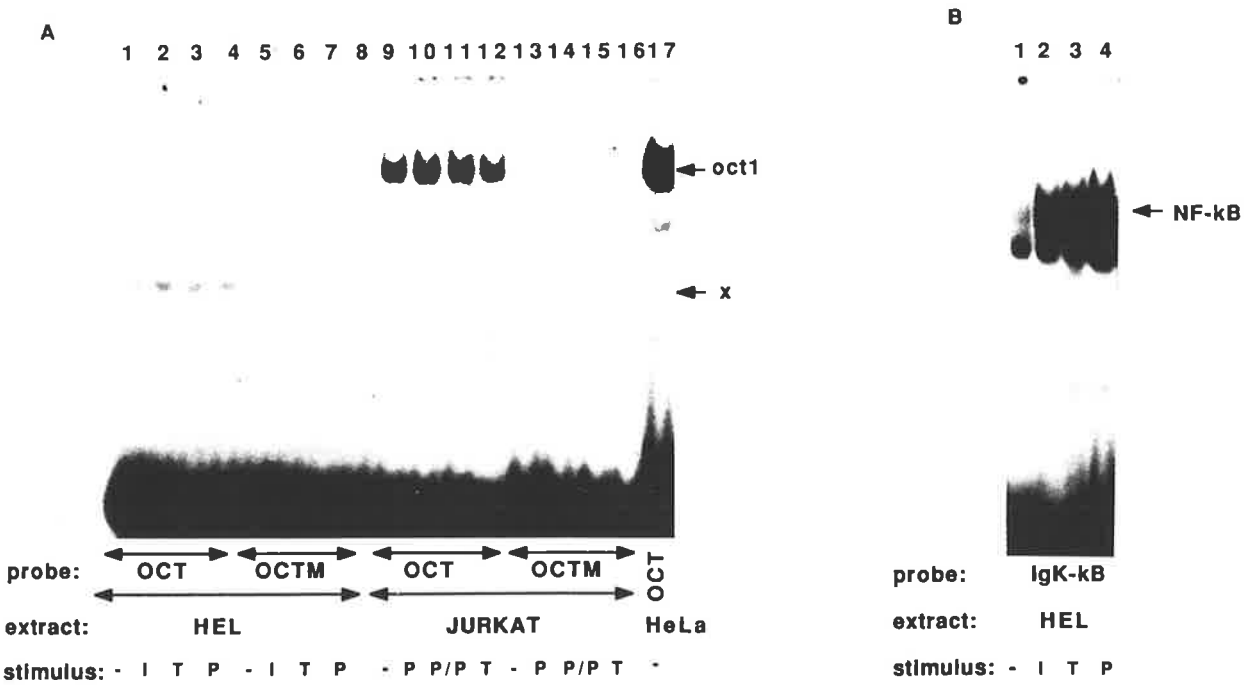

FIGURE 7. (A) Binding of nuclear proteins to the G-CSF octamer sequence. Gel mobility shift assay showing binding to a 34 bp double stranded oligonucleotide spanning the G-CSF octamer sequence (Fig. 1b) (lanes 1–4, 9–12 and 17) or a mutant version of the sequence (lanes 5-8 and 13-16). 3 μg of crude nuclear extract from HEL fibroblasts (lanes 1–8), Jurkat T cells (lanes 9–16) or HeLa cells (lane 17) were used in the binding reactions. The HEL fibroblasts were unstimulated (lanes 1 and 9) or stimulated with IL-1β (I, lanes 2 and 5), TNF-α (T, lanes 3 and 6) or PMA (P, lanes 4 and 8) for 6 hr prior to harvesting. The Jurkat cells were unstimulated (lanes 9 and 13) or stimulated with PMA (P, lanes 10 and 14), PMA/PHA (P/P, lanes 11 and 15) or TNF-α (T, lanes 12 and 16) for 6 hr. The complex generated by Oct1 is indicated as is the non-specific complex, "x". (B) Gel mobility shift assay showing binding of proteins from crude nuclear extract of HEL fibroblasts to the IgK NF-κB sites. HEL fibroblasts were stimulated and prepared as in A.

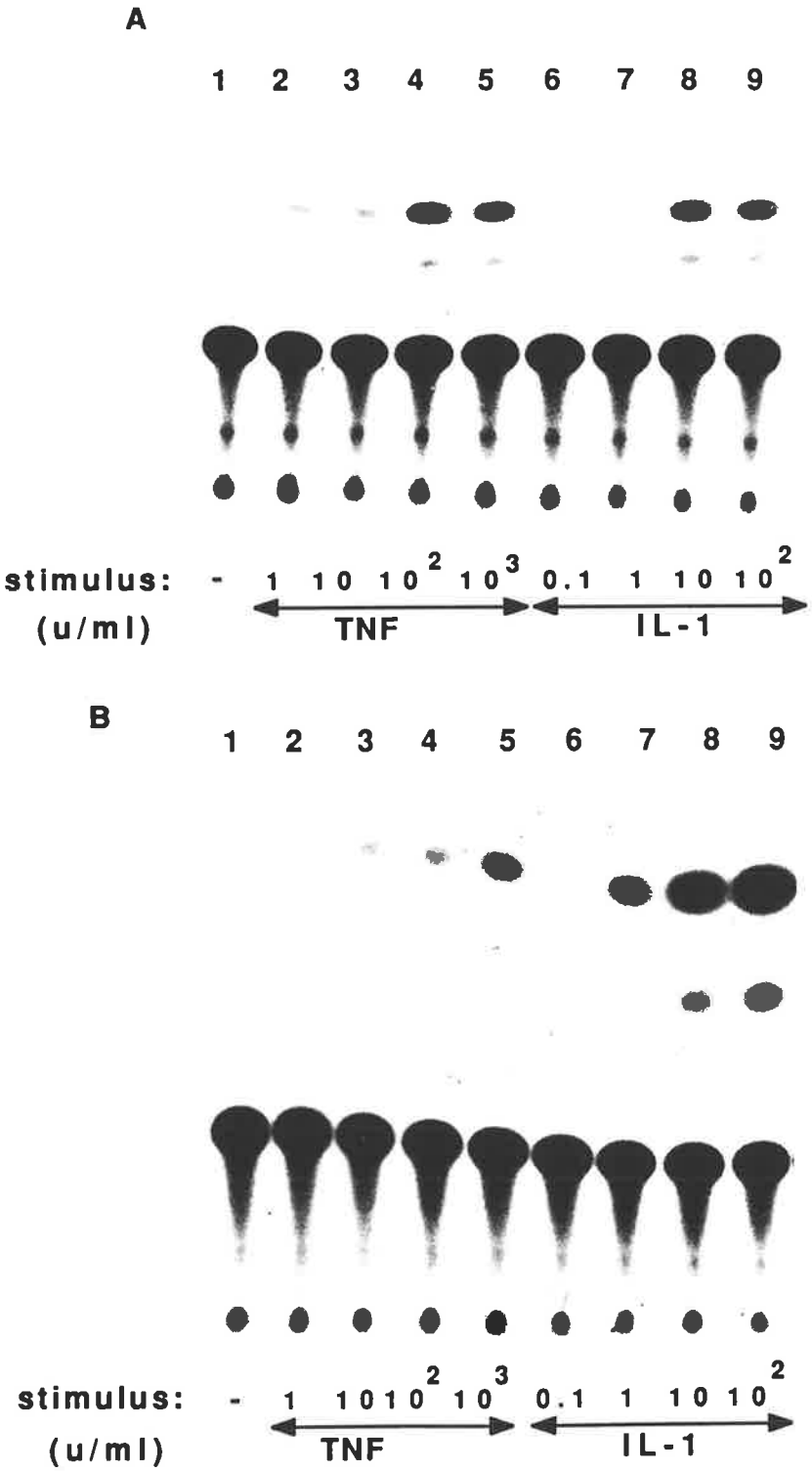

FIGURE 8. TNF-α and IL-1β activation of the CK-1 and octamer sequences from the G-CSF promoter. (A) HEL fibroblasts were transfected with pCK-1(4) and 24 hr later treated with the amounts of TNF-α (lanes 1–5) or IL-1β (lanes 6–8) indicated. Cells were harvested for CAT activity measurements 24 hr later. (B) pOCT plasmid was transfected into HEL fibroblasts and the cells treated as described in A. 30 μg cytoplasmic extracts were assayed for CAT activity in each track.

levels of this protein. Also, no inducible protein was detected binding to the G-CSF octamer sequence.

Differential Response of Promoter Sequences to TNF-α and IL-1β

Initial experiments had shown that IL-1β induced much higher maximum levels of G-CSF mRNA than did TNF-α (Fig. 2). This same observation was also true for the 330 bp G-CSF promoter (Fig. 3a) as well as pOCT containing multiple copies of the G-CSF octamer sequence (Fig. 6a). Our previous work had shown that multiple copies of the CK-1 sequence did not show this differential response to the two cytokines (Kuczek et al., 1991). To confirm the different response of the CK-1 and octamer sequences, dose response curves to TNF-α and IL-1β were determined with pCK-1(4) containing 4 copies of the G-CSF CK-1 sequence and pOCT (Fig. 1b). The experiment showed that pCK-1(4) gave the same maximum CAT activity with TNF-α and IL-1β (Fig. 8a) whereas pOCT always gave at least a 5 fold greater response to IL-1β than TNF-α (Fig. 8b).

DISCUSSION

The experiments described here show that a 330 bp fragment of the human G-CSF promoter is responsive to TNF-α and IL-1β in human embryonic lung fibroblasts and that at least three distinct sequence elements are essential for promoter function. The level of response of the promoter, i.e. 2–4 fold induction, is the same as that seen for the GM-CSF promoter in endothelial cells in response to IL-1 (Kaushansky, 1989) and for the promoters of some other HGFs. It may be possible to obtain a larger fold induction with a longer upstream fragment but this was not the case for the mouse G-CSF gene in macrophages (Nishizawa and Nagata, 1990). Nuclear run on experiments with the endogenous GM-CSF gene in embryonic fibroblasts have also shown a 2-3 fold increase (Koeffler et al., 1988). The transcriptional activity of the G-CSF promoter fragment appears to be cell specific since no activity was observed in Jurkat T cells. This cell specificity corresponds to that seen for the endogenous G-CSF gene (Clark and Kamen, 1987). The response of the 330 bp G-CSF promoter fragment to TNF-α and IL-1β in transfection experiments reflects the response of the endogenous gene to these agents as shown here by measuring G-CSF mRNA levels. The G-CSF promoter appears not to respond to PMA induction whereas endogenous G-CSF mRNA levels are increased. PMA may require additional sequences not present in the 330 bp fragment used here or may function through stabilisation of mRNA and not induction of gene transcription.

We have shown here that the CK-1 sequence (5' GAGATTCCAC 3') in the human G-CSF promoter is essential for its transcriptional response to TNF-α and IL-1β in HEL fibroblasts. This finding extends our earlier observation that multiple copies of the CK-1 sequence confers TNF-α and IL-1β responsiveness on a heterologous promoter (Shannon et al., 1990; Kuczek et al., 1991). Mutations in the CK-1 sequence of the mouse G-CSF promoter have also been shown to abolish LPS inducibility in macrophages (Nishizawa and Nagata, 1990) and also constitutive activity in carcinoma lines (Nishizawa et al., 1990). We have previously identified a nuclear protein, NF-GMa, that binds to the G-CSF CK-1 sequence (Shannon et al., 1990). The level of this protein, as measured by DNA binding, is increased by TNF-α and IL-1β treatment of HEL fibroblasts (Shannon et al., 1990; Kuczek et al., 1990). Mutations in the CK-1 sequence which abolish transcriptional activity also reduce NF-GMa binding capacity (Kuczek et al., 1991). These results imply that the TNF-α and IL-1β response is mediated via the NF-GMa protein binding to CK-1. However, in mouse macrophages protein binding to the CK-1 sequence was not observed and despite the observation that mutation of the CK-1 sequence abolishes LPS inducibility in these cells, multiple copies of the CK-1 sequence were not LPS inducible upstream of a minimal G-CSF promoter (Nishizawa and Nagata, 1990). It is possible that the CK-1 sequence is functionally distinct in the mouse and human promoters or there is a cell specificity in the proteins which bind to the CK-1 sequence.

A more plausible explanation for the differences observed between macrophages and fibroblasts is the length of the DNA fragments used to generate the plasmids for transfection. The pCK-1(4) fragment (Fig. 1b) contains not only the CK-1 sequence but also a potential binding site for the more recently described NF-IL6 (Akira et al.,

1990). NF-IL6 is a member of the C/EBP family of transcription factors that binds to the IL-6 gene promoter and mediates the response of the gene to IL-1 in a glioblastoma cell line (Akira et al., 1990). Although mutations in the CK-1 sequence abolished the inducible function of pCK-1(4) (Shannon et al., 1990) it is possible that the NF-IL6 site also contributes to function by interaction between the two binding proteins. In support of this, Nishizawa and Nagata, (1990), showed that the fragment spanning both CK-1 and the NF-IL-6 binding site was LPS responsive but that as mentioned above CK-1 alone was not. By mutating the NF-IL6 site in the whole promoter context, we have also shown here that it is required for TNF-α and IL-1β induction in fibroblasts but the involvement of the NF-IL6 factor has not been shown directly.

It is possible that the CK-1 element and the NF-IL6 site form an inducible promoter unit. Binding of proteins to these two adjacent sequences may be cooperative and required for promoter function. In support of this hypothesis we have previously shown that a fragment of DNA spanning the CK-1 sequence in GM-CSF (identical to G-CSF, CK-1) did not respond to TNF-α and IL-1β in HEL fibroblasts (Kuczek et al., 1991) despite the fact that it can bind the inducible transcription factor NF-GMa (Shannon et al., 1990). The GM-CSF fragment does not contain a good match to the NF-IL6 consensus binding site, perhaps explaining the lack of activity. A search of sequences in the HGF genes has revealed that CK-1 and NF-IL6 consensus sequences exist within 20 bp of each other in the promoters of the IL-6 (Yasukawa et al., 1987), interleukin-8 (IL-8) (Mukaida et al., 1989) and TNF-α (Nedwin et al., 1985) genes as well as the above mentioned G-CSF gene (Table 1). The human IL-8 and TNF-α NF-IL6 consensus sequences have been shown to bind NF-IL6 (Akira et al., 1990). It will be of interest to determine if any interaction occurs between NF-GMa and NF-IL6 in mediating the response of the G-CSF or other promoters to TNF-α and IL-1β.

The octamer consensus sequence at −115 upstream from the start of the transcription is also essential for TNF-α and IL-1β induction of the G-CSF promoter. This sequence has also been shown to contribute to inducible G-CSF promoter function in mouse macrophages (Nishizawa and Nagata, 1990) and to constitutive activity in carcinoma cells (Nishizawa et al., 1990). It was possible that the upstream inducible elements such as CK-1 and NF-IL6 mediated their effects via the octamer element where the octamer as such was not an inducible element.

The human G-CSF octamer sequence, however, functioned independently as a TNF-α and IL-1β inducible element when cloned upstream of the tk promoter in HEL fibroblasts. Inducibility was not seen in Jurkat T cells nor in dermal fibroblasts. In mouse macrophages, it has been shown that this element is not inducible when cloned upstream of a truncated G-CSF promoter (Nishizawa et al., 1990). The response of this element may be cell specific, perhaps evident only in embryonic cells.

A number of octamer binding proteins have been described in mouse embryonic stem cells and embryonic tissues the levels of which are reduced upon differentiation (Monuki et al., 1989; Scholer et al., 1989a; Scholer et al., 1989b). It is possible that one of these embryonic octamer proteins is responsive to TNF-α and IL-1β and that its reduced levels in non-embryonic dermal fibroblasts, Jurkat T cells and macrophages diminishes the response of the octamer

TABLE 1
CK-1 and NF-IL6 Consensus Sequences in Haemopoietic Growth Factor Genes

Gene[a]	CK-1 (1)[b]			NF-IL6 (2)			Order[c]
IL-6	−139	AGGTTTCCAA	−130	−156	ATTGCACAA	−148	2 1
G-CSF	−189	GAGATTCCAC	−180	−178	ATTTCACAA	−170	1 2
IL-8	−79	GAATTTCCTC	−70	−56	TTTTCATTA	−64	1 2R
TNF-α	−223	GGGTATCCTT	−214	−194	CTTTCCAAA	−186	1 2
CONSENSUS		GRGNTTNCNN			ATTNCNNAA C C		

[a]The gene names are abbreviated as in text.
[b]The numbers in parenthesis (1) and (2) are used to indicate the order in which the CK-1 and NF-IL6 sequences occur (5' to 3') in the gene promoter. The nucleotides which match the G-CSF CK-1 and NF-IL6 sequences are indicated by a dot. The numbering of the sequence refers to number of bases upstream from the start of transcription of each gene.
[c]The order of occurrence is indicated 5' to 3' in the gene. R indicates that the consensus sequence is found in reverse orientation.

sequences. Alternatively a second protein interacting with an octamer binding protein or sequestered to the DNA by the octamer protein could mediate TNF-α and IL-1β induction. Such a situation has been shown recently, for the NF-IL2A octamer binding site in the IL-2 gene (Ullman et al., 1991). This sequence binds Oct-1 as well as a PMA/calcium ionophore inducible octamer associated protein (OAP). This OAP is thought to be involved in the PMA/ionophore response of the element in Jurkat T cells (Ullman et al., 1991). Preliminary transfection experiments with the NF-IL2A site have shown that it has constitutive activity but is not TNF-α and IL-1β inducible in HEL fibroblasts indicating that OAP is probably not involved in the response of the G-CSF octamer to these agents. Nuclear extracts from HEL fibroblasts did not have detectable levels of Oct-1 binding activity nor could we detect any binding activity inducible by TNF-α, IL-1β or PMA under standard octamer DNA-protein binding conditions. It is of interest to note that only low levels of Oct-1 mRNA have been detected in WI-38 cells, another embryonic lung fibroblast cell, compared to other human cell lines (Sturm et al., 1988).

It was of interest to note that IL-1β induced a greater response than TNF-α from the endogenous G-CSF gene, the G-CSF promoter and the G-CSF octamer sequence. In agreement with our results it has been previously reported that IL-1 was a more potent stimulus for CSF production than TNF (Hamilton et al., 1992). Dose response titrations for both IL-1β and TNF-α confirmed that pOCT gave a greater response to IL-1β than TNF-α whereas the response of pCK-1(4) was the same for both activators. This result implies that the octamer sequence may be involved in the greater IL-1β response of the G-CSF gene promoter. In the whole promoter context mutation of the PU.1 consensus sequence reduced the IL-1β response to the level seen for TNF-α suggesting that PU.1 or a related protein in fibroblasts may be involved in IL-1β but not TNF-α induction of the G-CSF gene. These results imply possible cooperative interaction between the upstream PU.1 element and the octamer sequence.

At least three distinct promoter elements are required for induction of the G-CSF proximal promoter in HEL fibroblasts. It will be of interest to determine if any cooperative interactions take place in binding of transcription factors to these elements. Related signalling pathways from the TNF-α or IL-1β cell surface receptors may activate a group of transcription factors each of which contributes to promoter function.

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STIMULATION OF NEUTROPHILS BY TUMOR NECROSIS FACTOR[1]

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Human recombinant tumor necrosis factor (TNF) was shown to be a weak direct stimulus of the neutrophil respiratory burst and degranulation. The stimulation, as measured by iodination, H_2O_2 production, and lysozyme release, was considerably increased by the presence of unopsonized zymosan in the reaction mixture, an effect which was associated with the increased ingestion of the zymosan. TNF does not act as an opsonin but, rather, reacts with the neutrophil to increase its phagocytic activity. TNF-dependent phagocytosis, as measured indirectly by iodination, is inhibited by monoclonal antibodies (Mab) 60.1 and 60.3, which recognize different epitopes on the C3bi receptor/adherence-promoting surface glycoprotein of neutrophils. Other neutrophil stimulants, namely *N*-formyl-methionyl-leucyl-phenylalanine, the Ca^{2+} ionophore A23187, and phorbol myristic acetate, also increase iodination in the presence of zymosan; as with TNF, the effect of these stimulants is inhibited by Mab 60.1 and 60.3, whereas, in contrast to that of TNF, their stimulation of iodination is unaffected by an Mab directed against TNF. TNF may be a natural stimulant of neutrophils which promotes adherence to endothelial cells and to particles, leading to increased phagocytosis, respiratory burst activity, and degranulation.

Tumor necrosis factor (TNF)[4] was first described by Carswell et al. (1) as a factor in serum obtained from mice, rats, or rabbits which had been injected with viable bacillus Calmette-Guerin (BCG) and which, 14 to 21 days later, were injected with endotoxin. This factor, which reached maximal levels 2 hr after endotoxin administration, caused regression of some transplanted tumors in vivo and was cytostatic or cytocidal to some tumor cells in culture. It was proposed that the cell of origin was the macrophage, based on the massive hyperplasia of macrophages after BCG administration and the disruption of this cell population after endotoxin administration, at a time when the TNF level in serum was high.

TNF can be produced in vitro by macrophages from normal and, in particular, BCG-injected animals or by certain macrophage-derived cell lines after treatment with endotoxin (2, 3). A human cell line, HL-60, treated with phorbol myristic acetate (PMA) produces large amounts of TNF, and this cell preparation has been employed for the purification of human TNF to homogeneity (4, 5) and for the molecular cloning of the TNF gene (5, 6). Cloning and expression of the murine TNF gene also has been achieved (7). TNF has an m.w. of 17,300 and has about 30% homology with the functionally similar lymphocyte product lymphotoxin (6). Recently, TNF was found to be identical to cachectin (8), a monokine which suppresses the lipoprotein lipase activity of adipocytes and certain other cells, leading to marked hypertriglyceridemia associated with a severe wasting diathesis (9). The lethal effects of endotoxin appear to be mediated at least in part by TNF/cachectin because antibody to TNF/cachectin is protective (10).

TNF also has been found to affect neutrophil function. Neutrophil adherence to endothelial cells is increased by TNF, and this effect is due in part to a rapid, direct affect on the neutrophil associated with the increased surface expression of the C3bi receptor/adherence glycoprotein (designated CD11 complex in World Health Organization nomenclature (11), and in part to an effect on the endothelial cell (12). The latter effect is maximally expressed after 4 hr of endothelial cell culture and is dependent on both RNA and protein synthesis (12). The closely related lymphokine lymphotoxin (also referred to as TNF-β to distinguish it from the monokine TNF-α) stimulates phagocytosis by neutrophils, as measured by the uptake of fluoresceinated latex beads, and both TNF-α and TNF-β enhance neutrophil-mediated antibody-dependent cellular cytotoxicity against chicken erythrocytes (13).

Iodination is a convenient measure of neutrophil function because, with a particulate stimulus, optimal iodination is dependent on phagocytosis, the presence and release of myeloperoxidase (MPO) by degranulation, and the formation of H_2O_2 during the respiratory burst (14). The MPO reacts with H_2O_2 to form a complex, designated compound I, which oxidizes iodide to a form that binds in covalent linkage, generally to tyrosine residues of proteins, but also to unsaturated lipids (15) and other constituents. The iodination is in part intracellular, largely in the phagosome containing the ingested particle, and in part extracellular, where the released MPO and H_2O_2

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[4] Abbreviations used in this paper: fMLP, *N*-formyl-methionine-leucine-phenylalanine; LDH, lactate dehydrogenase; Mab, monoclonal antibody; MPO, myeloperoxidase; PMN, polymorphonuclear leukocytes; ZYM, zymosan; TNF, tumor necrosis factor.

iodinate extracellular protein or other components (16, 17). When a soluble stimulus is employed (e.g., chemotactic factors, PMA), the requirements for iodination are the same except that phagocytosis is not involved.

This report describes the stimulatory effect of recombinant human TNF on iodination by human neutrophils. The effect is small but statistically significant when TNF is added alone, and the stimulation is greatly increased when unopsonized zymosan is added. The stimulation of iodination by TNF was associated with increased zymosan ingestion, H_2O_2 production, and degranulation, and was abolished by monoclonal antibodies (Mab) directed against the C3bi receptor/adherence-promoting surface glycoprotein of neutrophils.

MATERIALS AND METHODS

Special reagents. Recombinant human TNF was kindly provided by Genentech, Inc., South San Francisco, CA. The preparation, assayed (by the supplier) by its cytolytic activity on actinomycin D-treated L929 mouse fibroblast cells, contained 3.6×10^7 U/mg. The TNF preparation (2×10^7 U/ml), stored at 4°C, was diluted in 0.9% sodium chloride containing 0.5% bovine serum albumin to 2×10^4 U/ml weekly, and a daily stock solution was prepared by a 10-fold dilution with 0.9% sodium chloride. TNF-D, kindly provided by Genentech, Inc., is a murine Mab directed against TNF. The preparation purified from ascites fluid had a neutralization titer of 2700 U/mg of antibody. Mab 60.1, 60.3, 60.5, and H11 (18–20) were kindly provided by Dr. P. Beatty, Fred Hutchinson Cancer Research Center, Seattle, WA. Mab 60.1 recognizes the α-subunit (αM) of the heterodimer CD11 with C3bi receptor and adherence-promoting properties (also designated Mac1 or Mo1). Mab 60.3 immunoprecipitates a family of heterodimers with World Health Organization designation CDw18 (one of which is CD11) (11) through recognition of a common chain or a common quarternary structure associated with the α- and β-subunits of each heterodimer. Mab 60.5 reacts with the framework region of HLA Class I antigens present on all human leukocytes. Mab H11 reacts with a 120,000 m.w. protein present on all neutrophils and some T lymphocytes. The latter two antibodies, although binding to neutrophils, do not react with any of the components of the CDw18 complex, and were used as controls.

Zymosan (ZYM; ICN Pharmaceuticals, Inc., Cleveland, OH) was suspended in water by homogenization, was boiled for 20 min, was washed twice, and was suspended in water at 10 mg/ml. Where indicated, the ZYM was opsonized by incubation with an equal volume of pooled human serum for 20 min at 37°C with shaking. The opsonized ZYM was washed twice and was suspended at 10 mg/ml in water. Catalase (Worthington Biochemical Corp., Freehold, NJ; CTR 64,773 U/mg) was dialyzed against water before use. PMA and lipopolysaccharide from *E. coli* 055:B5 (endotoxin) were obtained from Sigma Chemical Corp., St. Louis, MO; the calcium ionophore A23187 was from Calbiochem-Behring, La Jolla, CA; and *N*-formylmethionine-leucine-phenylalanine (fMLP) was from Peninsula Laboratories, San Carlos, CA.

Isolation of polymorphonuclear leukocytes (PMN). Venous blood was collected from normal volunteers just before cell separation, using 0.2% ethylenediaminetetraacetic acid (EDTA) as anticoagulant. The PMN were isolated by density gradient centrifugation in Hypaque-Ficoll, sedimentation in dextran, and hypotonic lysis of erythrocytes as described (21). The preparation, which always contained greater than 97% PMN with an average purity of 98 to 99%, was suspended in 0.9% sodium chloride at 5×10^7 PMN/ml.

Iodination. Iodination was measured by the conversion of radioiodide to a trichloroacetic acid (TCA)-precipitable form as described (14). The components of the reaction mixture, as described in the legends to the figures and tables, were incubated for the periods indicated at 37°C in 12 x 75-mm polystyrene test tubes on a Fisher Rotor-Rack (Fisher Scientific Co., Pittsburgh, PA), and the reaction was stopped by the addition of 1 ml of cold 10% TCA. The precipitate was collected by centrifugation at 2500 × G for 5 min in a refrigerated centrifuge, was washed four times with 2 ml of 10% TCA, and the counts per minute (cpm) were determined in a gamma scintillation counter. A blank containing the standard salt solution, iodide and albumin, was run with each experiment, and the results were subtracted from the experimental values. Less than 0.05% of the total added radioiodide was TCA-precipitable in the blank. A standard containing the total ^{125}I added to each experimental tube was counted and the percent iodination was determined as follows:

$$\frac{\text{cpm experimental} - \text{cpm blank} \times 100}{\text{cpm standard}}$$

The results are expressed as nmol of iodide converted to a TCA-precipitable form per 10^7 PMN per hour. Each experimental value was determined in duplicate and was averaged to give a single value for statistical analysis.

Phagocytosis. The phagocytosis of ZYM by PMN was determined microscopically as described by Jandl et al. (22), using reaction mixtures and incubation conditions identical to those used for measurement of iodination. In this method, the reaction was stopped with *n*-ethylmaleimide; a solution containing sucrose, EDTA, colchicine, and albumin was added; and after vigorous mixing, cytocentrifuge smears were prepared. The smears were fixed with formaldehyde-ethanol, were stained with periodic acid-Schiff, and were counterstained with methyl green. Slides were coded and cell-associated ZYM was determined by counting ZYM particles per 100 PMN in a blinded fashion. The experimental values are the means of quadruplicate counts, each from a different slide.

Degranulation. The components of the reaction mixture (as described in the footnotes to Table IV) were centrifuged at 8730 × G for 1 min in a Beckman Microfuge B centrifuge, and the experimental fluid, kept on ice, was assayed on the same day for MPO, β-glucuronidase, lysozyme, and lactate dehydrogenase (LDH). One hundred percent enzyme activity was determined by the assay of supernatants prepared by treatment of the same number of PMN with 2% Triton X-100 in phosphate-buffered saline. MPO activity of Triton X-100-lysed PMN was determined immediately, because activity declines rapidly. MPO (23), β-glucuronidase (24), lysozyme (25), and LDH (26) were determined as described previously.

H_2O_2 determination. H_2O_2 was determined by scopoletin oxidation as described by Root et al. (27), using a Perkin-Elmer model LS-5 fluorescence spectrophotometer. (The components of the reaction mixture are described in the footnote to Table V.) The reaction mixture was maintained at 37°C with a thermostatted cuvette and was mixed with a magnetic stirrer, and the change in fluorescence intensity (excitation wavelength 350 nm; emission wavelength 460 nm) followed with time. The rate of fall in fluorescence was determined from the linear portion of the curve and was converted to nmol/10^7 PMN/hour by using a H_2O_2 standard curve.

Statistical analysis. Data were analyzed by Student's two-tailed *t*-test (not significant; $p > 0.05$).

RESULTS

Iodination. Figure 1 demonstrates the stimulatory effect of TNF on iodination in the presence of ZYM which had not been opsonized by preincubation with serum. The addition of unopsonized ZYM or TNF alone produced a small but significant increase in iodination. However, when the two were combined, iodination was considerably greater than additive. The iodination was time-dependent, increasing to a maximum at about 40 min under the conditions employed. Iodination by unopsonized ZYM plus TNF developed more slowly and did not reach as high a level as that seen when serum-opsonized ZYM was employed as the stimulus. Iodination increased with both the ZYM and TNF concentrations (Fig. 2). Significant stimulation of iodination was observed at all ZYM concentrations with the addition of either 10 or 100 U/ml of TNF under the conditions employed.

Optimal iodination by PMN is dependent on the formation of H_2O_2 and the release of MPO, and thus the inhibition of TNF-dependent iodination by catalase but not by heated catalase, and by the hemeprotein inhibitor azide (Table I), is compatible with these requirements. Heating TNF at 100°C for 15 min abolishes its effect. TNF-dependent iodination also is inhibited by TNF-D, an Mab directed against TNF, and by two Mab, 60.1 and 60.3, directed against a neutrophil surface antigen required for optimal adherence and C3bi receptor function (18, 19). In contrast, Mab 60.5 and H11, which recognize neutrophil surface antigens unrelated to the adherence-

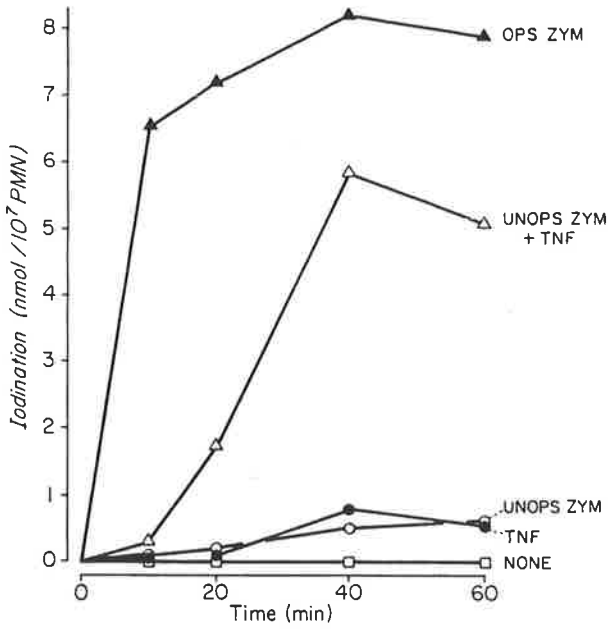

Figure 1. Stimulation of iodination by TNF. The reaction mixture contained 4×10^{-3} M sodium phosphate buffer, pH 7.4, 0.128 M NaCl, 1.2×10^{-2} M KCl, 10^{-3} M $CaCl_2$, 2×10^{-3} M $MgCl_2$, 8×10^{-6} M NaI (4 nmol; 0.05 μCl ^{125}I), 2×10^{-3} M glucose, 0.25 mg albumin, 2.5×10^6 PMN, and, where indicated: no further additions (□), 50 U TNF (●), 0.5 mg ZYM (UNOPS ZYM) (○), 50 U TNF + 0.5 mg ZYM (△), or 0.5 mg opsonized ZYM (OPS ZYM) (▲). The final volume was 0.5 ml, and incubations were for the periods indicated. The data are the mean of three to four experiments, and all values were significantly greater ($p < 0.05$) than the values for no addition (NONE) at each time point, except for UNOPS ZYM + TNF at 20 min ($p > 0.05 < 0.1$).

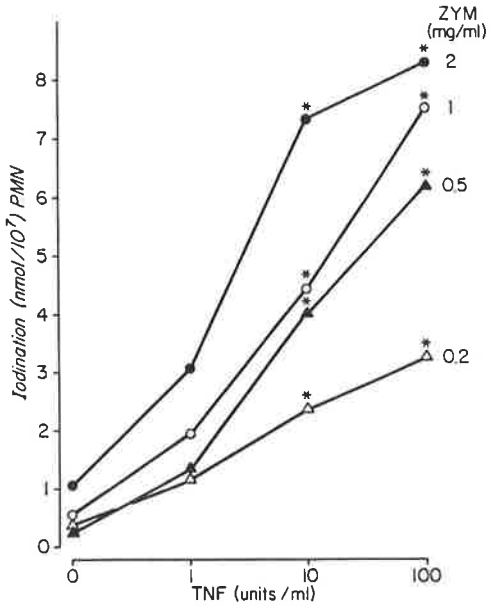

Figure 2. Effect of TNF concentration on iodination at different ZYM concentrations. The reaction mixture was as described for Figure 1 for UNOPS ZYM + TNF, except that the ZYM and TNF were added at the concentrations indicated. The incubation period was 60 min. *Values are significantly different from no TNF, $p < 0.05$.

promoting/C3bi receptor molecule, had no effect on TNF-dependent iodination. The recombinant TNF, which was purified from *E. coli*, contained 0.8 ng/ml of endotoxin in the undiluted preparation as determined by a limulus amebocyte lysate assay performed by the supplier. In Table I, the TNF was diluted 200,000-fold, so that the final endotoxin concentration from that source was approximately 2 fg/ml. Under the conditions employed in Table I, polymyxin B, an inhibitor of endotoxin action (28), did not significantly inhibit TNF-dependent iodination at concentrations of 1 and 10 μg/ml, nor could endotoxin at 100 ng/ml replace TNF as a stimulant of iodination in the presence of unopsonized ZYM. Thus, endotoxin contamination did not appear to contribute to stimulation of iodination by TNF. It is theoretically possible that the level of protein (albumin) added to provide iodine acceptor groups for extracellular iodination is limiting, and that ZYM and TNF are simply additional sources of protein. However, a 10-fold increase in added albumin did not increase iodination either in the presence or absence of TNF and ZYM (data not shown), indicating that the level of extracellular protein was not limiting.

Studies were performed to determine whether TNF acted on the ZYM or on the neutrophils to increase iodination. As shown in Table II, when the ZYM was preincubated with TNF, no increase in iodination was observed. In contrast, when the PMN were preincubated with TNF, the stimulation of iodination was comparable to that observed when TNF was present in the reaction mixture throughout the 60-min incubation period. Thus, TNF did not react with the ZYM, but rather with the PMN to increase iodination.

TNF could be replaced as a stimulus of iodination in the presence of ZYM by other PMN stimulants such as fMLP, A23187, or PMA (Fig. 3). At the concentrations of these stimuli employed, they had little effect on iodination in the absence of ZYM. However, when added in the presence of ZYM, fMLP, A23187, or PMA significantly increased iodination. In contrast to the finding with TNF (Table I), TNF-D had no effect on iodination when fMLP, A23187, or PMA was employed; however, as with TNF, Mab 60.1 and 60.3 were strongly inhibitory (Fig. 3), and the control Mab 60.5 and H11 were without effect (data not shown).

Phagocytosis. Table III demonstrates the effect of TNF on the phagocytosis of ZYM under the conditions employed in the iodination assay. After a 60-min incubation, six ZYM particles were judged to be intracellular per 100 PMN, and the number was increased to 464 by incubation with TNF. When the ZYM was preincubated with the TNF for 15 min and was washed before the 60-min incubation with PMN, no increase in phagocytosis was observed, whereas when TNF was preincubated with PMN, the increase in phagocytosis was comparable to that observed when TNF was present in the incubation mixture. The extent of phagocytosis by TNF-treated PMN over the 60-min incubation period was comparable to that in the absence of TNF, when serum-opsonized ZYM was added. However, when a shorter incubation period was employed (20 min), a greater number of intracellular particles was detected with serum-opsonized ZYM than with unopsonized ZYM in the presence of TNF.

Degranulation. Table IV demonstrates the effect of TNF on degranulation by neutrophils, as measured by the release of MPO, β-glucuronidase, and lysozyme into the extracellular fluid. The release of the cytoplasmic marker LDH was employed as a measure of cell lysis. Release of the azurophil (primary) granule enzymes MPO and β-glucuronidase was significantly increased by a combination of TNF and ZYM, and TNF alone increased the release of β-glucuronidase. However, LDH release was increased by TNF to an equivalent degree. In contrast,

TABLE I

Effect of inhibitors on iodination[a]

Supplements	Iodination		
	nmol/10^7 PMN	p[b]	p[c]
ZYM	0.33 ± 0.15 (8)[d]	<0.001	
ZYM + TNF	6.52 ± 0.65 (7)		<0.001
ZYM + TNF + catalase	2.52 ± 0.72 (3)	<0.01	<0.002
ZYM + TNF + heated catalase	6.97 ± 0.89 (3)	NS[e]	<0.001
ZYM + TNF + azide	0.87 ± 0.11 (3)	<0.001	NS
ZYM + heated TNF	0.57 ± 0.17 (3)	<0.001	NS
ZYM + TNF + Mab TNF-D	1.30 ± 0.63 (3)	<0.002	NS
ZYM + TNF + Mab 60.1	0.14 ± 0.06 (6)	<0.001	NS
ZYM + TNF + Mab 60.3	0.17 ± 0.14 (6)	<0.001	NS
ZYM + TNF + Mab 60.5	5.76 ± 0.70 (3)	NS	<0.001
ZYM + TNF + Mab H11	7.10 ± 1.59 (3)	NS	<0.001
ZYM + TNF + PB (1 μg/ml)	5.42 ± 1.31 (3)	NS	<0.001
ZYM + TNF + PB (10 μg/ml)	4.37 ± 1.16 (3)	NS	<0.001
ZYM + endotoxin	0.77 ± 0.29 (5)	<0.001	NS

[a] The reaction mixture was as described for Figure 1, with the supplements added where indicated as follows: unopsonized ZYM, 1 mg/ml; TNF, 100 U/ml; catalase, 78 μg/ml; sodium azide, 10^{-4} M; Mab TNF-D, 10 μg/ml; Mab 60.1, 1 μg/ml; Mab 60.3, 80 μg/ml; Mab 60.5, 20 μg/ml; Mab H11, 37 μg/ml; Polymyxin B at the concentrations indicated; and endotoxin, 100 ng/ml. The catalase and TNF were heated at 100°C for 15 min where indicated. The incubation period was 60 min.

[b] The value for the difference from ZYM + TNF.

[c] The value for the difference from ZYM.

[d] Mean ± SE of (n) experiments.

[e] NS, not significant.

TABLE II

Effect of preincubation of TNF with ZYM or neutrophils on iodination[a]

Supplements	Iodination (nmol/10^7 PMN)	
PMN + ZYM	0.76 ± 0.21 (3)[b]	
PMN + ZYM + TNF	10.86 ± 0.12 (3)	<0.001[c]
PMN + ZYM preincubated with TNF	0.83 ± 0.36 (3)	NS[d]
PMN preincubated with TNF + ZYM	9.36 ± 0.83 (3)	<0.001

[a] The reaction mixture was as described for Figure 1, except that, where indicated, ZYM (10 mg/ml) or neutrophils (5×10^7/ml) suspended in 1 ml of HBSS containing Ca^{2+} and Mg^{2+} were incubated with 0.5 ml of TNF (2000 U/ml in 0.9% NaCl) for 15 min at 37°C in a shaking water bath. The ZYM or PMN were then washed three times with approximately 5 vol of HBSS, and the ZYM was suspended in water at 10 mg/ml and the PMN in 0.9% NaCl at 5×10^7/ml, before addition to the other components of the reaction mixture. The ZYM and PMN used without preincubation with TNF were treated in an identical fashion, except that TNF was not present during the 15-min preincubation at 37°C. The incubation period was 60 min.

[b] Mean ± SE of (n) experiments.

[c] The values for the difference from PMN + ZYM.

[d] NS, not significant.

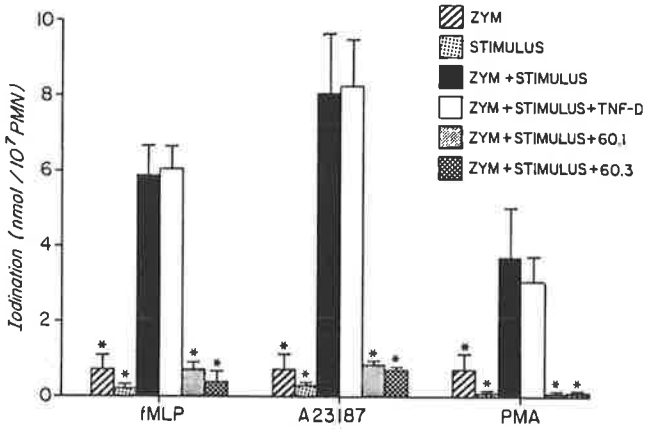

Figure 3. Stimulation of iodination by fMLP, A23187, or PMA when combined with ZYM. The reaction mixture was as described for Table I, except that TNF was replaced with 10^{-7} M fMLP, 1 μg/ml A23187, or 1 ng/ml PMA. The results are the mean ± SE of three (fMLP, A23187) or four (PMA) experiments. *Values are significantly different from ZYM + stimulus, $p < 0.05$.

lysozyme release was increased by TNF to a greater degree than was LDH release, both in the presence and absence of ZYM. The degranulating effect of PMA, shown for comparison, is comparable to that of ZYM plus TNF.

TABLE III

Effect of TNF on the phagocytosis of ZYM[a]

Supplements	No. of ZYM/100 PMN	
	20 min	60 min
ZYM	ND[b]	6
ZYM + TNF	115	464[c,d]
ZYM preincubated with TNF	ND	3
PMN preincubated with TNF	ND	395[c]
Serum-opsonized ZYM	359	534[c,d]

[a] The reaction mixture was as described for Figure 1, except that iodide was deleted. Where indicated, either the ZYM or PMN were preincubated with TNF for 15 min at 37°C and were washed once with HBSS. ZYM opsonized by preincubation with pooled human serum was employed where indicated. After incubation for either 20 or 60 min, the reaction was stopped and phagocytosis was determined microscopically. The results are the mean of two to four experiments.

[b] ND, not done.

[c] Significantly different from ZYM ($p < 0.001$).

[d] Significantly different from 20 min ($p < 0.001$ for ZYM + TNF; $p < 0.02$ for serum-opsonized ZYM).

H_2O_2 production. The effect of TNF on H_2O_2 production by PMN is shown in Table V. TNF alone significantly increased H_2O_2 production, although the effect was considerably less than that observed with opsonized ZYM or PMA as the stimulus. Unopsonized ZYM alone did not significantly increase H_2O_2 production, whereas the combined effect of TNF and unopsonized ZYM was greater than additive.

DISCUSSION

TNF is a weak direct stimulus of the respiratory burst and degranulation of neutrophils. Respiratory burst activity was measured by H_2O_2 production, and degranulation by the release of the granule enzymes MPO, β-glucuronidase, and lysozyme. The small but statistically significant increase in the release of the cytoplasmic marker LDH by TNF suggests a weak lytic effect on neutrophils. MPO and β-glucuronidase release was not greater than that of LDH, suggesting that cell lysis could account for the TNF-dependent release of these azurophil (primary) granule enzymes. However, lysozyme release induced by TNF was significantly greater than that of

TABLE IV

Effect of TNF on degranulation by neutrophils[a]

Supplements	Enzyme Release (% of maximum)			
	MPO	β-glucuronidase	lysozyme	LDH
PMN	0.78 ± 0.22 (6)[b]	1.38 ± 0.28 (6)	1.39 ± 0.62 (6)	1.50 ± 0.41 (6)
PMN + TNF	2.30 ± 0.78 (6) NS[c]	4.73 ± 1.41 (6) <0.05	18.46 ± 4.29 (6) <0.01	7.82 ± 1.39 (6) <0.002
PMN + ZYM	1.30 ± 0.35 (5) NS	2.66 ± 0.62 (5) NS	7.03 ± 1.81 (6) <0.02	1.28 ± 0.53 (6) NS
PMN + ZYM + TNF	9.39 ± 2.78 (6) <0.02	12.13 ± 2.03 (6) <0.001	45.06 ± 4.60 (6) <0.001	8.93 ± 1.33 (6) <0.001
PMN + PMA	17.58 ± 3.37 (6) <0.001	11.31 ± 2.68 (6) <0.01	48.59 ± 7.66 (6) <0.001	5.10 ± 1.37 (3) <0.02

[a] The reaction mixture was as described for Figure 1, except that iodide was deleted and PMA (100 ng/ml) was added where indicated and the volume of the reaction mixture was doubled. The incubation period was 60 min.
[b] Mean ± SE of (n) experiments.
[c] The p value for the difference from PMN alone.

TABLE V

Effect of TNF on H_2O_2 production by neutrophils[a]

Supplements	H_2O_2 Production (nmol/10^7 PMN/hr)	
PMN	6.3 ± 2.6 (3)[b]	
PMN + TNF	52.7 ± 6.7 (3)	<0.01[c]
PMN + ZYM	28.1 ± 11.0 (3)	NS[d]
PMN + ZYM + TNF	109.5 ± 32.4 (3)	<0.05
PMN + opsonized ZYM	686.0 ± 47.3 (3)	<0.001
PMN + PMA	856.0 ± 16.0 (3)	<0.001

[a] The reaction mixture was as described for Figure 1 except that albumin was deleted, PMA (100 ng/ml) was added where indicated, and the volume of the reaction mixture was increased fivefold to 2.5 ml.
[b] Mean ± SE of (n) experiments.
[c] The p value for the difference from PMN alone.

LDH. Because lysozyme is present predominantly in the specific (secondary) granules of neutrophils, this finding suggests that TNF is a secretagogue affecting primarily specific granules. Iodination, which generally requires both H_2O_2 production and MPO release, also is weakly but significantly increased by TNF. The stimulation of these parameters of neutrophil function by TNF is considerably increased by the presence of unopsonized ZYM in the reaction mixture.

The effect of TNF on neutrophil degranulation and respiratory burst activity in the presence of unopsonized ZYM was associated with, and presumably caused at least in part by, the increased ingestion of the ZYM particles. Shalaby et al. (13) have reported that TNF-β also increases the ingestion of latex beads by PMN. TNF does not stimulate phagocytosis by acting as an opsonin, because ZYM preincubated with TNF and then washed free of unbound agent is not ingested to a greater degree than is untreated ZYM. In contrast, neutrophils, preincubated with TNF and then washed, have increased phagocytic activity, suggesting a direct effect of TNF on the cell. The mechanism of the stimulation of phagocytosis by TNF is now known. TNF increases the surface expression of the C3bi receptor/adherence-promoting glycoprotein CD11 (12), possibly due in part to translocation from intracellular stores (29). The inhibition of TNF-dependent iodination by Mab 60.1 and 60.3, which are directed against different epitopes on this glycoprotein and which inhibit both its C3bi receptor and adherence-promoting properties (18, 19, 30), indicates that TNF-dependent phagocytosis of ZYM is dependent on this surface molecule. Although the glycoprotein is required for the ingestion of C3bi-opsonized particles, its C3bi receptor function is not operative here because the particles are not complement-coated. However, the adherence-promoting properties of this molecule may induce attachment of particle to cell and thus favor ingestion.

The property of TNF of increasing iodination by neutrophils in the presence of unopsonized ZYM is shared by a number of other neutrophil stimulants, namely the chemotactic peptide fMLP, the Ca^{2+} ionophore A23187, and PMA at low concentration. These latter stimulants also increase the surface expression of the C3bi receptor/adherence-promoting glycoprotein (29, 31, 32), and their effect on iodination, like that of TNF, is inhibited by Mab 60.1 and 60.3. However, in contrast to that of TNF, stimulation by fMLP, A23187, or PMA is unaffected by TNF-D, the Mab directed against TNF. The stimulation of particle-dependent iodination (and phagocytosis) by TNF is not due to endotoxin contamination, because the endotoxin concentration in the TNF preparation is very low, the effect of TNF was unaffected by an inhibitor of endotoxin action, polymyxin B, and the addition of endotoxin could not duplicate the effect of TNF under our conditions.

The action of TNF on some target cells appears to require initial binding to surface receptors (9, 32, 33), with subsequent modification of specific gene expression. In some instances, e.g., as in the developmental expression of lipoprotein lipase in adipocytes, suppression of gene expression with decreased mRNA levels is the result (34). In other instances, e.g., as in the surface expression of Class 1 major histocompatibility complex antigens on endothelial cells and dermal fibroblasts, TNF increases mRNA levels and surface expression of the antigens (35). In contrast, the effect of TNF on granulocytes is rapid and appears to be independent of protein and nucleic acid synthesis (12). TNF is a weak direct stimulus of neutrophils, causing degranulation and respiratory burst activity. Among the consequences of this stimulation is an increase in surface expression of the C3bi receptor/adherence glycoprotein (12), with associated increased adherence of the granulocytes to endothelial cells (12) as well as to plastic surfaces, and increased phagocytosis of unopsonized particles.

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SCIENCE

Prevention of Activated Neutrophil Adhesion to Endothelium by Soluble Adhesion Protein GMP140

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Prevention of Activated Neutrophil Adhesion to Endothelium by Soluble Adhesion Protein GMP140

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Neutrophils and monocytes, but not lymphocytes, adhered strongly to plastic surfaces coated with GMP140, a protein of endothelial cells and platelets. This adhesion of neutrophils was mediated by GMP140 and not by the CD18 integrin complex. By contrast, GMP140 in solution inhibited the CD18-dependent adhesion of tumor necrosis factor–α–activated neutrophils to plastic surfaces and resting endothelium, but not of resting neutrophils to tumor necrosis factor–α–activated endothelium. Thus, the binding of a soluble form of an adhesion protein selectively inhibited another set of adhesive events. Soluble GMP140 may be important in maintaining the nonadhesiveness of neutrophils in the circulation and may serve to limit inflammatory reactions.

DURING INFLAMMATION, CIRCULATING blood cells adhere to patches of endothelium and migrate into tissues. This process is regulated by cytokines such as tumor necrosis factor–α (TNF-α) and is mediated by adhesion proteins, some of which belong to the LEC-CAM (lectin–epidermal growth factor–complement binding cell adhesion molecule) family (*1*). GMP140, a glycoprotein of 140 kD, is present in the alpha granules of platelets and the Weibel Palade bodies of endothelial cells (ECs) (*2–4*). Analysis of the cDNA suggests three possible forms of GMP140: two transmembrane forms (with complement binding regions of different lengths) and a soluble form, with the transmembrane domain deleted (*2*). Upon platelet activation or treatment of ECs with thrombin or histamine, the secretory granules are rapidly exocytosed, resulting in a redistribution of transmembrane GMP140 into the plasma membrane (*5–7*). Endothelial GMP140 has structural similarity to adhesion molecules ELAM-1 (endothelial leukocyte adhesion molecule–1) and MEL-14, which belong to the LEC-CAM family (*2, 8, 9*). ELAM-1, although not present on resting endothelium, mediates adhesion of neutrophils [polymorphonuclear leukocytes (PMNs)] to endothelium activated by TNF or interleukin-1 (IL-1) (*10*). MEL-14, present on PMNs and lymphocytes, is involved in lymphocyte homing to high endothelial venules (*11*). We used platelet GMP140 that was purified to homogeneity (*12*) to investigate its role in adhesive phenomena, and our data suggest that elaboration or secretion of GMP140 serves to prevent adhesion and the development of inflammatory responses.

GMP140 was coated onto plastic microtiter wells and was adhesive for freshly isolated, nonactivated PMNs and monocytes, but not T lymphocytes (Fig. 1A). Adhesion was concentration-dependent (Fig. 1A), and the

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adhesion of PMNs was inhibited by affinity-purified rabbit Fab fragments of the antibody to GMP140 (Fig. 1B). Thus, immobilized GMP140 can promote the selective adhesion of leukocytes. The adhesion of PMNs to GMP140 was not dependent on the CD18 adhesion complex (Fig. 1B), as antibodies to the α chain of Mac-1 (CD11b) or to the common β chain (CD18) did not inhibit adhesion.

The effect of soluble GMP140 on the adhesion of PMNs to human umbilical vein ECs was investigated. Monolayers of ECs were incubated with PMNs for 30 min either in the presence or absence of TNF-α. Soluble GMP140 was also simultaneously added to some groups (Fig. 2A). GMP140 prevented TNF-α–stimulated PMN adhesion to ECs in a concentration-dependent manner (Fig. 2A). In experiments with significant basal adhesion (that is, PMNs that were not intentionally stimulated), GMP140 was also inhibitory (*13*). The effect of soluble GMP140 on PMN adhesion to TNF-α–stimulated ECs was also investigated. In contrast to the TNF-α–stimulated PMNs' adhesion to resting ECs, the adhesion of resting PMNs to TNF-α–stimulated ECs was not inhibited by GMP140 (Fig. 2B).

The effect of GMP140 on PMN adherence was a result of binding to the PMNs, because pretreatment and washing of the PMNs but not the ECs with GMP140

Table 1. PMN adhesion to ECs is inhibited by GMP140 action on the PMNs. In experiment A, PMNs (15×10^8) were first incubated with GMP140 (8 μg/ml) or medium in 300 μl for 15 min at 37°C. The tubes were then placed on ice, diluted with cold assay medium, and spun for 15 s at 12,000*g*. The cells were resuspended in assay medium and added to ECs either with or without the addition of TNF-α (10 U/ml). Parallel experiments with GMP140 added directly to the assay are also shown. Percent adhesion is expressed as the mean ± SEM of triplicate determinations and is representative of three similar experiments. No decrease in cell number or viability was observed with this protocol between groups receiving GMP140 or those with medium alone. The inhibition seen when PMNs were treated with GMP140 before the assay was consistently significant, but less than the inhibition seen when GMP140 was included in the assay. In experiment B, the ECs were treated with GMP140 or medium before the assay. EC monolayers were washed once and incubated with either GMP140 (8 μg/ml) or medium alone for 15 min at 37°C. The wells were then washed in ice-cold assay medium, and PMNs were added and assayed as in experiment A. No change in cell morphology, monolayer integrity, or uptake of Rose Bengal stain was observed with ECs treated in this way. This is a representative of three similar experiments.

Experiment	Percent adherence to EC monolayers with GMP140 added	
	Before assay	During assay
A PMN only	1.2 ± 1.0	2.4 ± 1.2
PMN + TNF-α	72.9 ± 5.4	72.5 ± 1.8
PMN + TNF-α + GMP140	51.6 ± 0.1*	20.7 ± 1.8†
B PMN only	1.7 ± 0.5	3.9 ± 2.0
PMN + TNF-α	59.8 ± 0.1	61.0 ± 1.1
PMN + TNF-α + GMP140	58.9 ± 0.8	27.6 ± 2.4‡

*$P < 0.05$ compared to group stimulated with TNF-α but not receiving GMP140. †$P < 0.001$ compared to group stimulated with TNF-α but not receiving GMP140. ‡$P < 0.02$ compared to group stimulated with TNF-α but not receiving GMP140.

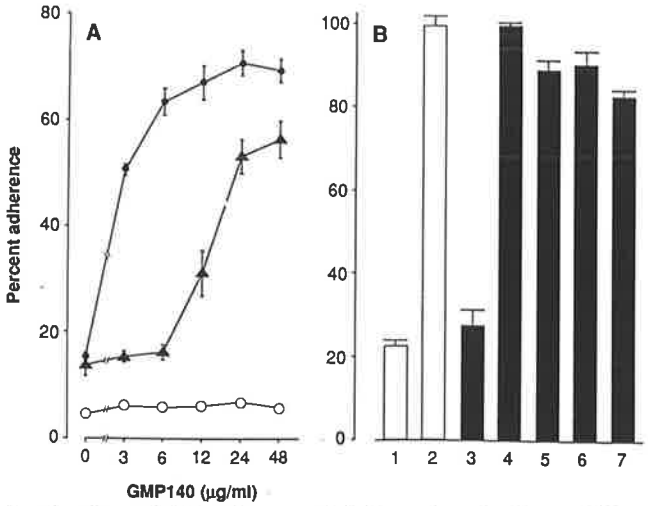

Fig. 1. (**A**) Effect of GMP140 on the adherence of PMNs, lymphocytes, and monocytes to plastic. Various concentrations of GMP140 diluted in phosphate-buffered saline, pH 7.3, were added to microtiter wells at room temperature. After 4 hours the wells were washed in assay medium (RPMI containing 2.5% fetal bovine serum). PMNs (5×10^5) (▲), T lymphocytes (○), or 2×10^5 monocytes (●) were added, incubated for 30 min, and adherence was determined. The results of individual experiments are shown and are representative of three or four experiments performed with each cell type (arithmetic mean of triplicate determinations ± SEM). The relative adhesion of PMNs and monocytes varied among the different donors. In parallel experiments, T lymphocyte adhesion to plastic could be induced by phorbol myristate acetate (10 ng/ml) (*13*). Purified GMP140 was isolated as described (*12*). The glycoprotein was stored at −70°C in 0.02 M tris buffer, 0.15 M NaCl, 0.1% Triton X-100, and 0.001 M $CaCl_2$ (buffer pH 7.4). Before use, the preparation was separated on an Extracti-Gel D column (Pierce Chemicals) to remove the Triton X-100. GMP140 was eluted in the void volume, and ~50% of the loaded protein was recovered and used within 24 hours. Monocytes were purified, and their adherence was assayed as in (*34*), PMNs as in (*35*), and T cells as in (*35, 36*). The absorbance at 570 nm (A_{570}) was determined for known numbers of Rose Bengal–stained ethanol-lysed PMNs and T cells, and a standard curve was obtained. A linear relation exists between A_{570} and number of cells attached, and from this the percent adhesion can be calculated. (**B**) Effect of antibodies on PMN adhesion to GMP140 immobilized on plastic. (Bar 1) Plastic without GMP140 coating. All other wells coated with GMP140; (bar 2) GMP140 only. Additions: (bar 3) affinity-purified rabbit anti-GMP140 Fab fragment and (bar 4) rabbit non-immune Fab fragment were used at a concentration of 20 μg/ml. (Bar 5) Control antibody 60.5 (anti-HLA) and monoclonal antibodies (bar 6) 60.3 (anti-β chain of CD18 complex), and (bar 7) 60.1 (anti-Mac-1) were used at a final concentration of 1:400 of ascites, which gives maximal inhibition in CD18-dependent adhesion assays. Results have been normalized relative to the level of adhesion to GMP140-coated plastic as 100% (bar 2).

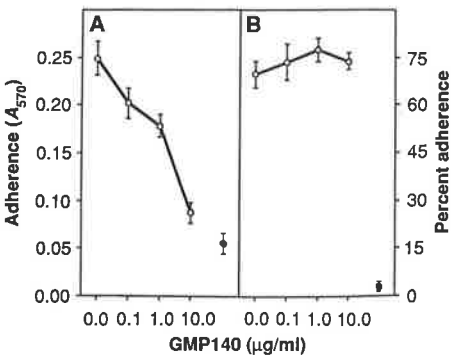

Fig. 2. The effect of GMP140 on PMN adherence to EC monolayers. Endothelial cells were harvested from human umbilical veins by collagenase treatment and grown as described (*37*) without the addition of EC growth factor and heparin. Two to 6 days after establishment of culture, ECs were harvested by trypsin-EDTA and replated into gelatin-coated microtiter wells at 2×10^4 cells per well and grown to confluency overnight. PMNs (5×10^5) were added to EC monolayers, and adhesion was measured after 30 min. (**A**) PMNs were incubated with TNF-α (10 U/ml) and graded loss of GMP140 (○). The basal level of adhesion of unstimulated PMNs to unstimulated endothelium is also shown (●). The results shown are from a single experiment which is representative of eight experiments where the average percent inhibition of TNF-α–stimulated adherence to ECs was 19, 41, and 78% for 0.1, 1.0, and 10 μg of GMP140 per milliliter, respectively. Groups receiving 1.0 and 10 μg of GMP140 per milliliter were significantly different (at $P < 0.001$) from the group not receiving GMP140 (level of significance = 0.025, Bonferroni correction). (**B**) ECs were stimulated with TNF-α (100 U/ml) for 4 hours before washing. PMNs were then added with graded doses of GMP140 (○). The basal level of adhesion of unstimulated PMN to unstimulated endothelium is also shown (●). The results shown are representative of four experiments where average inhibition was 2, 5, and 11%, for 0.1, 1.0, and 10 μg of GMP140 per milliliter, respectively (not significantly different from the group not receiving GMP140, level of significance = 0.016, Bonferroni correction).

resulted in significant inhibition of adhesion (Table 1). The inhibition of adhesion was transient (Table 1) (*13*). GMP140 did not inhibit TNF-α–activated PMN adhesion by interfering with the capacity of TNF-α to bind its receptor: treatment of the PMNs with TNF-α before addition of GMP140 still resulted in significant inhibition of adhesion to EC monolayers (Table 2, experiment A). The inhibition by GMP140 was blocked by a specific antibody and by boiling the protein (Table 2, experiments B and C), suggesting that GMP140, and not a contaminant, was mediating the effect. Inhibition by GMP140 required the presence of GMP140 from the beginning of the assay. Once the PMNs had contacted the ECs GMP140 had no effect on adhesion (Table 2, experiment D), showing that GMP140 inhibits PMN adhesion, but does not, in static adhesion assays, cause PMN detachment from ECs.

The adhesion of stimulated PMNs to endothelium is mediated through the CD18 complex [lymphocyte function–associated antigen-1 (LFA-1), Mac-1, and p150,95] of adhesion glycoproteins on the PMNs, particularly by CD11b/CD18 (Mac-1) and CD11a/CD18 (LFA-1) (*14–18*). The ligand for LFA-1 is intercellular adhesion molecule–1 (ICAM-1) (*19, 20*); the ligand for Mac-1 on the endothelium is unknown, but may also partially involve ICAM-1 (*21*). After activation of PMNs with agents such as TNF-α, C5a, and lipopolysaccharide, the expression or conformational state (*22–24*) of these molecules is altered. PMNs become more adhesive (*14, 22–25*), which is a transient state (*25*) and by itself does not lead to transmigration (*26*). More than 95% of this adhesion to EC is blocked by antibodies to the CD18 complex (*27*) and, as shown here, by the glycoprotein GMP140. Adherence of TNF-α–activated PMNs to plastic is also totally Mac-1–dependent (*27*), and soluble GMP140 also inhibits this adhesion (Table 3). Since adhesion to immobilized GMP140 is not CD18-dependent, this work suggests that GMP140 inhibits at least the Mac-1–dependent process of PMNs.

Table 3. The effect of soluble GMP140 on PMN adhesion to plastic surfaces. Soluble GMP140 was added to PMNs in plastic microtiter wells 2 min before activation with TNF-α (10 U/ml), and adhesion was determined 30 min later. The percent adhesion ± SEM of triplicate determinations of one experiment representative of two such experiments is shown.

GMP140	Activation	Percent adherence
Nil	Nil	5.4 ± 0.6
Nil	TNF-α	26.2 ± 1.0
2.5 μg/ml	TNF-α	18.7 ± 0.7*
5.0 μg/ml	TNF-α	13.8 ± 0.4*

*Significantly different from group that was TNF-activated, but not receiving soluble GMP140; $P < 0.025$, level of significance = 0.025, Bonferroni correction.

Adhesion to endothelium that is activated with agents such as TNF-α and IL-1 (*14, 28*) is qualitatively different from the adhesion of activated PMNs to resting endothelium. Adhesion to activated endothelium is slower to develop, is only partially inhibited by antibodies to CD18, is mediated through the expression of a new protein ELAM-1, and possibly other ligands (*29*), on ECs, results in transmigration (*30*), and is not inhibited by GMP140. Our findings show that soluble GMP140 selectively inhibits the adhesion process of activated neutrophils to endothelium and demonstrate that the mechanism of the two types of adhesion is different.

GMP140 is expressed on endothelial cells after stimulation with thrombin and histamine (*7*) and has been implicated in the rapid adhesion of PMNs to endothelium stimulated by these agents (*31, 32*). In addition, cDNA clones have been identified that lack the transmembrane domain (*2*), suggesting that GMP140 exists in soluble form. The release of soluble GMP140 under appropriate conditions may limit the adhesion of activated PMNs to endothelium. The shedding of MEL-14 from activated PMNs has led to the hypothesis that it also may inhibit adhesion (*33*). Antiadhesive mechanisms could be important in situations in which neutrophils are activated, such as in blood infections, when either the coagulant or complement pathway is activated, or when cytokines are administered in vivo. Therapeutic administration of GMP140 may therefore be useful to limit vascular pathology.

Table 2. Characterization of GMP140 inhibition of PMN adherence to ECs. Experiment A: PMNs were incubated for 10 min at 37°C with TNF-α (10 U/ml) or medium and then added to the EC monolayers with or without GMP140 (3 μg/ml). Experiment B: GMP140 was boiled for 15 min and then added at a concentration of 10 μg/ml to the assay. Experiment C: GMP140 (3 μg/ml) was preincubated with rabbit antibody (Fab fragment, 50 μg/ml) for 30 min at room temperature and then added to the adherence assay. Experiment D: GMP140 (10 μg/ml) was either added at the same time as PMN and TNF to the EC monolayers or added 15 min later. The mean percentage adherences ± SEM of single experiments are given that are representative of at least two similar experiments performed for each.

	TNF-α addition		GMP140		Other addition	Percent adherence
	PMN pretreatment	Directly to assay	Presence	How added		
A						7.0 ± 1.2
	10 min					32.7 ± 2.9
	10 min		+			17.3 ± 1.2*
B						7.8 ± 2.6
		+				82.2 ± 3.7
		+	+			43.7 ± 3.7*
		+	+	Boiled		84.1 ± 5.2†
C						12.0 ± 2.6
		+				80.3 ± 4.7
		+	+			64.1 ± 2.6
		+	+		Anti-GMP140 Fab	84.1 ± 4.4
		+	+		Nonimmune Fab	63.5 ± 0.3‡
D						2.9 ± 1.5
		+				47.1 ± 2.9
		+	+			25.6 ± 2.1*
		+	+	15 min after start of assay		57.6 ± 6.5

$P < 0.05$ compared to group not receiving GMP140. †$P < 0.05$ compared to group receiving unboiled GMP140. ‡$P < 0.05$ compared to group receiving anti-GMP140 Fab.

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Adhesion protein GMP140 inhibits superoxide anion release by human neutrophils

(lectin/epidermal growth factor/complement-binding domain cell adhesion molecule/selectin/tumor necrosis factor α/inflammation/thrombosis)

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ABSTRACT **The respiratory burst of blood neutrophils has a critical role in the destruction of microorganisms and tissue damage in inflammation. Neutrophils adhere in a dose-dependent fashion to granule membrane protein 140 (GMP140), a member of the LEC-CAM (lectin/epidermal growth factor/complement-binding domain cell adhesion molecule) family of adhesion proteins when it is immobilized onto plastic surfaces. Adherence to GMP140 was associated with less superoxide anion generation than adherence to other surfaces, an effect that is especially remarkable after activation of neutrophils with tumor necrosis factor α, an agent that on other surfaces promotes adhesion and spreading. However, on GMP140 the cells fail to spread and instead remain rounded and refractile. Neutrophils adhering to GMP140 were also deficient in superoxide anion generation to formylmethionylleucylphenylalanine. Furthermore, fluid-phase GMP140 also inhibited the superoxide generation by neutrophils stimulated by tumor necrosis factor α. The effect of GMP140 was reversible by washing and was inhibited by anti-GMP140 Fab antibody. GMP140 appears to be a natural antiinflammatory molecule that may prevent the inappropriate activation of neutrophils in the circulation.**

The respiratory burst of blood neutrophils has a critical role in the destruction of microorganisms and tissue damage in inflammation. The prevention of inappropriate respiratory burst is therefore likely to be an important homeostatic mechanism. Granule membrane protein 140 (GMP140) is a 140-kDa integral membrane glycoprotein found in the α granules of platelets and in the Weibel–Palade bodies of endothelial cells (1–4). Upon activation of endothelial cells and platelets, GMP140 is rapidly redistributed to the plasma membrane and has been shown to mediate adhesion of activated platelets to monocytes (5) and neutrophils (5, 6) and adhesion between endothelial cells and neutrophils (7).

The cloning of GMP140 from human umbilical vein endothelial cells predicts a cysteine-rich protein with multiple domains, including calcium-dependent mammalian lectin, epidermal growth factor, and complement-binding domains. These domains are followed by a transmembrane sequence and a short cytoplasmic tail. Analysis of cDNA also suggests a soluble form with the transmembrane domain deleted (8). The structure of GMP140 is similar to two known proteins involved in leukocyte adhesion, the Mel-14 antigen (9) and ELAM-1 (10); these proteins are classified as LEC-CAMs (lectin/epidermal growth factor/complement-binding domain cell adhesion molecules) (11) or selectins (7). GMP140, as another member of the LEC-CAM family, ELAM-1, binds at least in part to carbohydrate structures in the Lewis-x family (12, 13). Lacto-*N*-fucopentaose III appears to be the specific ligand for GMP140 (13). We have shown previously (14) that GMP140 in the fluid phase (which may be similar to the soluble or secreted form) inhibits the binding of activated neutrophils to resting endothelium. Since this adhesion is not mediated by GMP140 but rather the CD18 integrins (15), we hypothesized that the *in vivo* role of soluble GMP140 is to limit the adhesion of circulating neutrophils [polymorphonuclear neutrophils (PMNs)] to endothelium (14).

The possibility that GMP140 has an antiinflammatory effect was investigated by measuring the production of superoxide anions (O_2^-) from PMNs either adherent to immobilized GMP140 or in the presence of fluid-phase GMP140. We show here that immobilized GMP140 and fluid-phase GMP140 inhibit the production of O_2^- generated by PMNs, either unstimulated or when stimulated with the cytokine tumor necrosis factor α (TNF-α) or with the PMN chemoattractant formylmethionylleucylphenylalanine (fMet-Leu-Phe). These results suggest that GMP140 is a protein that inhibits several aspects of neutrophil function associated with inflammation.

MATERIALS AND METHODS

Purification of Human Neutrophils. Neutrophils were purified from normal donors by dextran sedimentation and Ficoll/Hypaque gradient centrifugation as described (16). The cells were resuspended in RPMI 1640 medium (Multisystem, Sydney, Australia) adjusted to pH 7.4 by adding 20 mM Hepes and 2.25% $NaHCO_3$ with antibiotics. Bovine serum albumin (0.1%) (Commonwealth Serum Laboratories, Melbourne, Australia) was added to medium used for superoxide assay and fetal calf serum (2.5%) (Pacific Bioindustries, Sydney, Australia) was used for adhesion assays (adhesion medium). The purity of the PMN preparations were >95% as judged by morphology on cytocentrifuged preparations and >99% viability as judged by trypan blue exclusion.

Stimuli. TNF-α [lot no. S9010AX; specific activity, 6×10^7 units/mg (by TNF-α bioassay)] produced in *Escherichia coli* was supplied by Genentech. A final concentration of 10 units/ml was used in all experiments except where otherwise stated. Endotoxin contamination as judged by the *Limulus* amebocyte lysate assay was <0.72 ng/mg. fMet-Leu-Phe was purchased from Sigma. A stock solution of 1 mM fMet-Leu-Phe in absolute alcohol was stored at −20°C and diluted on the day of each experiment. A final concentration of 0.1 μM was used in all experiments.

Preparation of GMP140. GMP140 was extracted from human platelets and affinity purified; purity was confirmed

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Abbreviations: TNF-α, tumor necrosis factor α; O_2^-, superoxide anion(s); fMet-Leu-Phe, formylmethionylleucylphenylalanine; PMN, polymorphonuclear neutrophil; LEC-CAM, lectin/epidermal growth factor/complement-binding domain cell adhesion molecule.

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by N-terminal sequence analysis as described (17). The purified material was stored in 0.02 M Tris/0.15 M NaCl/0.001 M $CaCl_2$/0.1% Triton X-100 buffer, pH 7.4 (Triton X-100 buffer), and kept at −70°C. For immobilization of GMP140 it was found that removal of Triton X-100 from the preparation was not required provided the wells were washed four times prior to addition of cells. Control wells were coated in Triton X-100 buffer. No change in cell viability was evident with either GMP140- or Triton X-100 buffer-coated wells. For use of GMP140 in the fluid phase, the Triton X-100 was removed by extracti-Gel D (Pierce) on the day of each experiment. The gel was washed in the above buffer without Triton X-100. Two volumes of packed gel to 1 volume of GMP140 was found to be adequate for complete Triton X-100 removal. For controls, the buffer with Triton X-100 removed by extracti-Gel D was used. The concentration of GMP140 was determined by the Bio-Rad (Bio-Rad) protein assay.

Antibodies. Polyclonal antibody to GMP140 was raised in rabbits and Fab fragments were prepared (14). The antibody has been shown to bind specifically to GMP140. Control Fab fragments were made from nonimmune rabbits.

Superoxide Assay. O_2^- release by PMN was measured by the reduction of cytochrome *c* at 550 nm (18). The PMNs were either applied to microtiter wells or incubated in suspensions in Eppendorf tubes. For determination of O_2^- in microtiter wells, flat-bottomed microtiter wells (Nunclon-Delta, Roskilde, Denmark) were either left uncoated or precoated for 2 hr with 30 μl of either fibrinogen (KabiVitrum, Stockholm) or fibronectin (Collaborative Research) at 100 μg/ml or GMP140 at various concentrations. These concentrations of fibronectin and fibrinogen in preliminary assays were found to be saturating. The wells were washed with assay medium four times prior to assay. Human neutrophils (1.5×10^5) in 50 μl were added together with cytochrome *c* (Sigma type VI; final concentration, 110 μM) and stimuli to give a final volume of 100 μl. For determination of O_2^- production in Eppendorf tubes, the same mixture was added to Eppendorf tubes and the cells were incubated in a shaking water bath at 37°C. One hundred microliters of the contents was then transferred to a microtiter plate and the absorbance at 550 nm was determined. For calculation of superoxide dismutase-inhibitable O_2^- generation, a mixture of PMNs, cytochrome *c*, and superoxide dismutase (final concentration, 1 mg/ml) (Boehringer Mannheim) was incubated either in microtiter wells or in Eppendorf tubes. Using the correction factor of Pick and Mizel (19) for estimation of O_2^- production in microtiter wells, the amount of O_2^- produced was determined by reduction of cytochrome *c* measured at 550 nm (using a Dynatech microplate reader). The results are expressed as the amount (nmol) of superoxide dismutase-inhibitable O_2^-.

Hypoxanthine-Xanthine Oxidase System. Hypoxanthine (Sigma) and xanthine oxidase (Sigma) were used to generate O_2^- anion in a cell-free system (20). A 1-ml reaction mixture of 1 mM hypoxanthine and 0.03 unit of xanthine oxidase with or without GMP140 (10 μg/ml) and 620 μM cytochrome *c* was incubated at 37°C and the OD_{550} was determined at 15-min intervals over a 60-min period.

Adhesion Assay. Neutrophils (5×10^5), resuspended in 100 μl of adhesion medium either with or without TNF-α (10 units/ml), were allowed to adhere to flat-bottomed plastic or GMP140-coated microtiter wells for 30 min at 37°C. The medium was then aspirated off and the cells were stained with 0.25% rose bengal for 8 min as described (16, 21). Excess stain and nonadherent cells were removed by washing. The OD was read at 570 nm and the percentage of cells adhering was calculated from a standard curve (21).

Photography of Adherent Neutrophils. Glass slides (Lab-Tek, Nunc) were either uncoated or precoated with 150 μl of GMP140 (10 μg/ml) at 20°C for 2 hr and then washed three times with adhesion medium. Neutrophils (2×10^5) with TNF-α (10 units/ml) in 200 μl of adhesion medium were incubated on the slides at 37°C for 30 min. Nonadherent cells were removed by washing three times in adhesion medium before photographs were taken using an Olympus microscope under 400× magnification. Similar morphology was observed using plastic slides; however glass slides provided better optics for photography.

Endotoxin Assay. A chromogenic *Limulus* amebocyte lysate assay (Coatest, KabiVitrum, Stockholm) with a sensitivity of 0.8 pg/ml was used to measure endotoxin contamination in preparations of fibrinogen, fibronectin, and GMP140.

Statistics. All data were analyzed using unpaired Student's *t* tests.

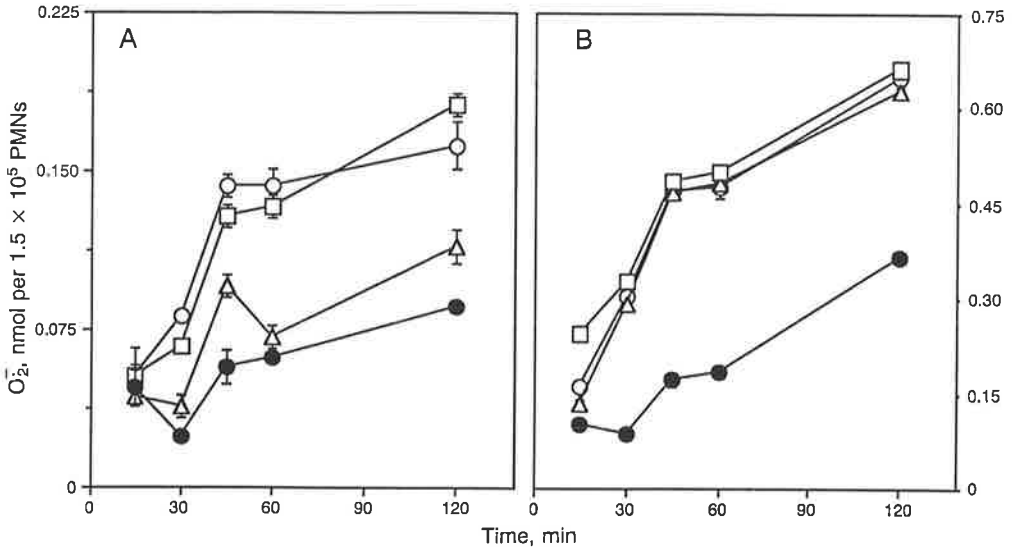

FIG. 1. Time course of O_2^- release on different matrices. Microtiter wells were coated with Triton X-100 buffer (○), fibronectin (100 μg/ml) (△), fibrinogen (100 μg/ml) (□), or GMP140 (10 μg/ml) (●) for 2 hr at room temperature. The wells were then washed four times prior to use. PMNs in medium alone (*A*) or with 10 units of TNF-α per ml (*B*) were then added to the wells and incubated at 37°C. The OD_{550} was read at the indicated times, and the nmol of O_2^- release per 1.5×10^5 PMNs was calculated. Each point represents the arithmetic mean (±SEM) of triplicate determinations from one experiment that is representative of three similar ones performed.

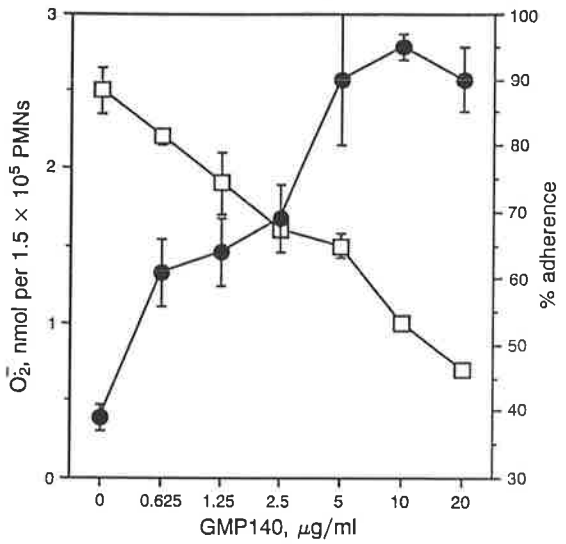

FIG. 2. O_2^- release and percentage of adherent TNF-α-activated PMNs in response to increasing dose of immobilized GMP140. Microtiter wells were coated with increasing concentrations of GMP140 for 2 hr at room temperature followed by four washes in medium. The O_2^- release (□) of TNF-α-activated PMNs was determined after 30 min. In parallel wells the extent of adhesion (●) of TNF-α-activated PMNs was also determined. Each point is the arithmetic mean (±SEM) of triplicate results from one experiment representative of three such experiments.

RESULTS

Effect of Solid-Phase GMP140 on O_2^- Generation by Human Neutrophils. Neutrophils were added to microtiter plates previously coated with GMP140, fibrinogen, or fibronectin in Triton X-100 buffer or control plates of Triton X-100 buffer alone for 2 hr and the plates were washed four times in medium containing 0.1% bovine serum albumin. The O_2^- generation during a 2-hr incubation is shown in Fig. 1. Neutrophils added to wells coated with medium or fibrinogen generated greater amounts of O_2^- than those on GMP140 or fibronectin. No difference in cell viability was observed in PMN adherence to the different matrices. In parallel incubations 10 units of TNF per ml was included. This dose was chosen as it was found to give consistently high levels of adhesion and O_2^- generation of PMNs on plastic (data not shown). As previously described (22), there was a strong O_2^- generation by cytokine-activated PMNs on fibronectin, fibrinogen, and medium. By contrast, O_2^- generation by TNF-α-activated PMNs on GMP140 was absent until 60 min and remained significantly less than on other matrices.

Table 1. Reversal of GMP140 effect by anti-GMP140 antibody

GMP140, μg/ml	Antibody (Fab fragment)	O_2^-, nmol per 1.5 × 10^5 PMNs per 30 min
0	Nil	0.38 ± 0.04
0	Anti-GMP140	0.28 ± 0.02
0	Control	0.32 ± 0.05
3	Nil	0.06 ± 0.03
3	Anti-GMP140	0.34 ± 0.07*
3	Control	0.04 ± 0.01†

Three micrograms of GMP140 was bound to plastic wells for 2 hr at 20°C and excess was removed by four washes with medium. Anti-GMP140 Fab fragment or nonimmune Fab fragment (final concentration of each, 10 μg/ml) was added to the appropriate wells of immobilized GMP140 for 30 min at 20°C; this was followed by washing prior to addition of unstimulated PMNs and cytochrome *c* mix. The amount of O_2^- generated was determined after a 30-min incubation at 37°C. The results show the mean ± SEM of 12 determinations from a pool of four experiments.
*P = 0.02 compared to group receiving no antibody.
†P = 0.004 compared to group receiving anti-GMP140 Fab antibody.

The difference between GMP140 and plastic with unstimulated PMNs at 30 min was observed on 21 occasions with five different preparations of GMP140 (mean inhibition ± SD = 72% ± 16%) and with TNF-α-activated cells on 17 occasions (mean inhibition = 81% ± 13%). At 60 min the differences were 83% ± 4% and 60% ± 20% for unstimulated and TNF-α-stimulated cells, respectively (n = 3 and n = 4). The reduction on GMP140-coated wells in comparison with fibrinogen-coated plates was 74% ± 13% and 74% ± 12%, respectively, at the 30-min time point (n = 7). GMP140 did not alter O_2^- generation in a cell-free system generating O_2^- by hypoxanthine and xanthine oxidase (data not shown).

Relationship of Adhesion to GMP to O_2^- Generation. We have shown previously that unactivated PMNs adhere strongly to immobilized GMP140 (14). TNF-α-activated PMNs also adhere to immobilized GMP140 as is shown in Fig. 2. In parallel wells the O_2^- generation was measured. With increasing amounts of GMP140 immobilized onto microtiter wells there is a dose-dependent increase in adhesion but a dose-dependent decrease in O_2^- generated. Using unactivated PMNs a similar inverse correlation between dose of GMP140 and generation of O_2^- is seen, but the overall level of O_2^- generated is lower than that seen using TNF-α-activated PMNs (data not shown).

The inhibitory effect of GMP140 on O_2^- release could be reversed by rabbit polyclonal anti-GMP140 Fab fragments but not by control Fab fragments, as shown in Table 1. This reversibility also applied to PMN adhesion to GMP140 as shown previously (14).

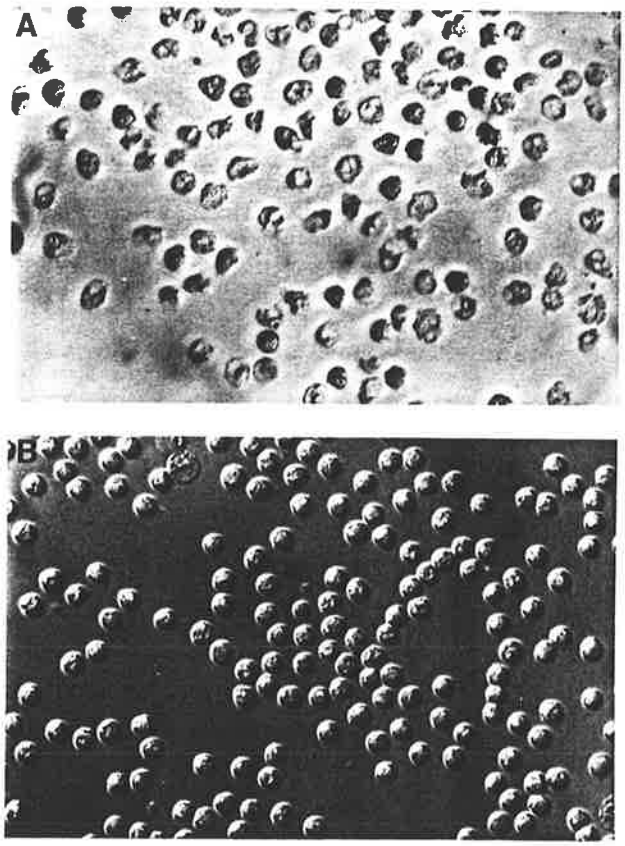

FIG. 3. Morphology of TNF (10 units/ml) activated PMNs on medium-coated glass (*A*) and on GMP140-coated glass (*B*). PMNs (2 × 10^5) were incubated on either medium-coated (*A*) or GMP140-coated (*B*) slides for 30 min at 37°C. Slides were then washed three times to remove nonadherent cells. The cells were maintained under medium at 37°C while photographs were taken. (×456.)

Table 2. Inhibition of O_2^- production by fMet-Leu-Phe-stimulated PMNs adherent to immobilized GMP140

Stimulus	O_2^-, nmol per 1.5×10^5 PMNs per 30 min		
	Medium only	Fibrinogen	GMP140
Nil	0.58 ± 0.1	0.86 ± 0.1	0.23 ± 0.07*
fMet-Leu-Phe	1.4 ± 0.1	2.1 ± 0.2	0.64 ± 0.1*
TNF-α	2.1 ± 0.2	1.7 ± 0.1	0.35 ± 0.06*

PMNs with cytochrome *c* were added to microtiter wells coated with medium, 30 μl of GMP140 at 10 μg/ml, or 30 μl of fibrinogen at 100 μg/ml. fMet-Leu-Phe (0.1 μM) or TNF-α (10 units/ml) was then added. O_2^- was measured after 30 min of incubation at 37°C. Results show the mean ± SEM of 12 determinations performed in four independent experiments.

*$P < 0.001$ for unstimulated and fMet-Leu-Phe- or TNF-α-stimulated PMNs applied to GMP140-coated wells compared to groups incubated on medium or fibrinogen-coated plastic.

Microscopic Appearance of PMNs Adherent to GMP140 and Other Matrices. It has been well described that PMNs adhering to plastic or glass become flattened and polarized (23). We found this process could be exaggerated by TNF-α treatment (Fig. 3*A*). By contrast, PMNs adhering to GMP140 remain rounded and refractile and failed to polarize even in response to TNF-α treatment (Fig. 3*B*).

Effect of fMet-Leu-Phe on O_2^- Generation by PMNs Adherent to GMP140. Table 2 shows the results of four experiments in which O_2^- generation by PMNs was measured on plastic, fibrinogen, and GMP140-coated wells in response to 0.1 μM fMet-Leu-Phe (a dose previously determined to give maximal O_2^- generation) or TNF-α. The O_2^- generated in response to both stimuli was reduced on GMP140.

Effect of Fluid-Phase GMP on O_2^- Generation. Isolation of mRNA for GMP140 suggests the existence of a secreted or soluble form (8). Our previous data (14) showed that GMP140 in the fluid phase (which may mimic soluble GMP140) inhibits TNF-α-activated PMN adhesion to endothelium. We therefore tested the effects of fluid-phase GMP140 on generation of O_2^- by PMNs.

Neutrophils were mixed with GMP140 before placement in microtiter wells with or without TNF-α. As shown in Fig. 4, there was a dose-dependent decrease in the capacity of cells incubated with GMP140 to generate O_2^-.

Antibodies to GMP140 inhibited the effects of fluid-phase GMP140 on the O_2^- production by PMNs although the results were inconsistent for reasons not known at present. The most consistent effects for the reversal of the GMP140-mediated inhibition of O_2^- were observed when a suboptimal dose of GMP140 was preincubated with anti-GMP140 Fab antibodies in Eppendorf tubes prior to addition of PMNs, cytochrome *c*, and TNF-α (Fig. 4*B*).

Reversibility of GMP140. To test whether the effect of GMP140 was reversible, PMNs were incubated with GMP140 or with medium for 10 min followed by two washes in medium. Parallel tubes were centrifuged twice and either resuspended in existing medium or washed with fresh medium. The O_2^- generation in response to TNF-α was measured in microtiter plates. As seen in Fig. 5, PMNs in the presence of GMP140 failed to generate significant amounts of O_2^- (group a compared to group b). This inhibition was reversed by washing (group d) but was restored by adding GMP140 back to the cells at the end of the washing process (group e). These effects were evident over 120 min, at which time there was <10% recovery of O_2^- generation by PMNs in the presence of GMP140. The PMNs that were incubated in GMP140 for this 120 min were still viable as judged by trypan blue exclusion (data not shown).

DISCUSSION

The central finding of this paper is that GMP140 interacts with human neutrophils in a way that prevents or does not allow the generation of O_2^-. We have previously shown that GMP140 appears to have a signaling role to PMNs in inhibiting the CD18-dependent adhesion of activated cells to

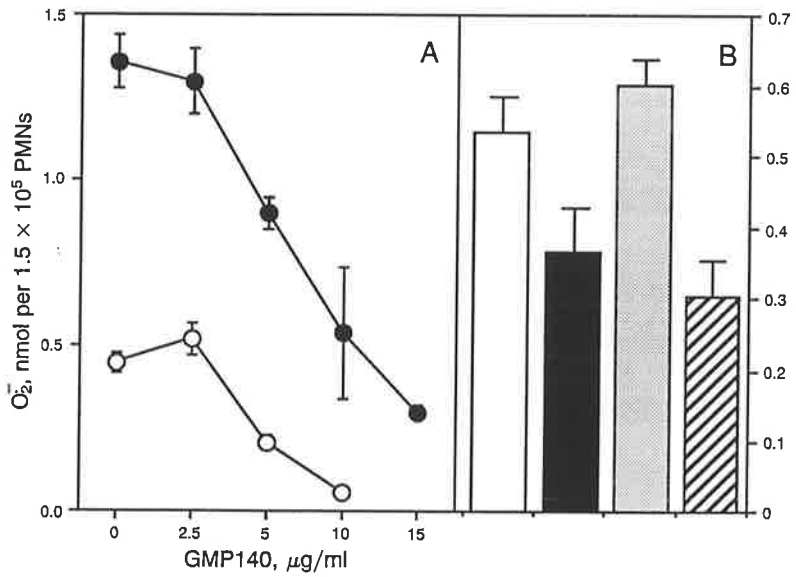

FIG. 4. (*A*) Inhibition of O_2^- production by fluid-phase GMP140. Neutrophils were incubated with control buffer or graded doses of GMP140 for 10 min at 37°C and then added to plastic microtiter plates either with (●) or without (○) TNF-α. O_2^- generation was measured at 30 min. Results show the mean ± SEM of 8–12 determinations pooled from four experiments. (*B*) Anti-GMP140 antibody reverses the GMP140-mediated inhibition of O_2^- generation of TNF-α-activated PMNs. PMNs and cytochrome *c* with TNF-α (5 units/ml) and with or without GMP140 (3 μg/ml) were incubated in Eppendorf tubes in a shaking water bath at 37°C for 30 min. The contents of the tube were then resuspended and transferred to microtiter wells and the OD_{550} was determined. In groups containing anti-GMP140 Fab or control Fab fragments, GMP140 had been incubated with these antibodies for 30 min before addition to the PMNs. Results are the mean ± SEM of six determinations performed in two experiments. Open bar, no GMP140; solid bar, GMP140 (3 μg/ml); stippled bar, GMP140 (3 μg/ml) with anti-GMP140 Fab fragments (40 μg/ml); striped bar, GMP140 (3 μg/ml) with nonimmune Fab fragments (40 μg/ml). $P = 0.035$ for the group incubated with GMP140 compared with no GMP140 (solid vs. open bars); $P = 0.003$ for the group incubated with anti-GMP140 antibody and GMP140 compared with GMP140 alone (stippled vs. solid bar); $P = 0.37$ for the group incubated with control Fab antibody and GMP140 compared with GMP140 alone (striped vs. solid bar).

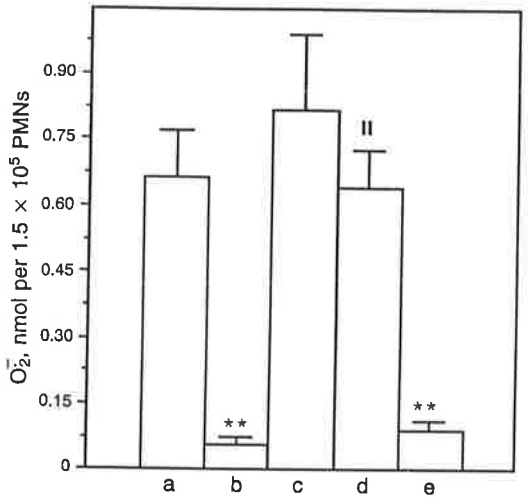

FIG. 5. Inhibition of O_2^- production by GMP140 is reversible. PMNs were incubated with GMP140 (20 μg/ml) (b, d, and e) or buffer (a and c) for 10 min at 37°C. Groups a and b were centrifuged twice but resuspended each time in the existing medium. A solution containing cytochrome *c* and TNF-α (10 units/ml) was then added to the tubes. Groups c–e were washed twice in fresh RPMI medium with bovine serum albumin and then resuspended in the cytochrome *c*/TNF-α mixture. To group e, a further 20 μg of GMP140 per ml was also added. All groups of cells were then transferred to microtiter wells and incubated for 60 min at 37°C, at which time the level of O_2^- production was calculated. The results show the mean ± SEM of nine determinations from three experiments (a–d) and six determinations from two experiments (e). **, $P \leq 0.001$ compared to group a; ||, $P > 0.1$ compared to group c.

endothelium (14). We now extend these findings and show that PMNs that adhere to GMP140 generate little or no O_2^-.

The adhesion of TNF-α-activated neutrophils to GMP140 is qualitatively different from their adhesion to other matrices, such as the extracellular proteins, fibrinogen, and fibronectin, and to plastic surfaces. First, activated PMNs adhering to GMP140 remain rounded and refractile (Fig. 3*B*) rather than spread and polarized as is seen on plastic (Fig. 3*A*) or on fibrinogen and fibronectin (data not shown). Second, adhesion of TNF-α-activated PMNs to GMP140 results in only low levels of O_2^- generation in contrast to their adhesion to fibronectin, fibrinogen, and plastic surfaces, which result in strong O_2^- generation (Fig. 1*B*). The effect of GMP140 on PMN function is best seen in Fig. 2, where increasing amounts of immobilized GMP140 on plastic wells, although leading to an increase in PMN adhesion, result in a decrease in the generation of O_2^-. Interestingly, the morphology of PMNs adhering to GMP140 is similar to that seen for PMNs adhering to endothelium, a situation in which O_2^- generation is also limited (23).

The capacity of GMP140 to inhibit O_2^- generation from activated PMNs (rather than a failure to stimulate it) is seen in two types of experiments. First, PMNs adhering to immobilized GMP140 produce less O_2^- in response to soluble stimuli such as the bacterial chemotactic peptide fMet-Leu-Phe. Second, fluid-phase GMP140 inhibits O_2^- generation by TNF-α-activated PMNs that adhere to plastic (Fig. 4) or other surfaces (not shown). Thus it would appear that GMP140, whether in the fluid phase or bound to plastic, significantly alters the capacity of PMNs to respond to agents that stimulate O_2^-. The PMNs are viable as judged by the vital dye trypan blue exclusion and return to full functional status after the removal of GMP140 by washing (Fig. 5).

Using unactivated PMNs, the effect of GMP140 on O_2^- production is not dissimilar to the situation in which PMNs adhere to fibronectin-coated surfaces where little O_2^- is generated (22). Our findings with fibrinogen-coated plates are somewhat different from published results (22) that show little O_2^- generation on this matrix. Since our preparation of fibrinogen contains 1 ng of endotoxin per ml, this may be responsible for the conflicting results.

Our experiments suggest a new function for GMP140, an adhesion protein of the LEC-CAM family that is only found in the Weibel–Palade bodies of endothelial cells and the α granules of platelets. GMP140 is normally elaborated after activation of endothelium by agents such as thrombin or histamine or by thrombin activation of platelets. GMP140, perhaps in the membrane-bound form, may have a role in maintaining neutrophils in an unactivated state at a time when thrombotic or allergic reactions are taking place. Our observations could also explain the observation that PMNs adhering to endothelial cells *in vitro* generate diminished O_2^- (23) and the lack of damage to the endothelium in thrombotic conditions. In addition, detection of ≈0.2–0.3 μg of GMP140 per ml in ultracentrifuged plasma (100,000 × *g*) (M.C.B., L. Dunlop, and M.P.S., unpublished data) suggests that soluble GMP140 may be a normal blood constituent and may tonically prevent release of O_2^- by blood-borne neutrophils until adhesion and migration to sites of inflammation occur. The reversibility of the effects of GMP140 (Fig. 5) would suggest that rapid reversal of the functional state of neutrophils will take place. Our results have implications for prevention of activation of other blood cells, such as eosinophils and basophils, which also adhere to GMP140 (M.A.V., C.M.L., A. Lopez, and J.R.G., unpublished data), and suggest that this natural protein or its derivatives may constitute a powerful therapeutic antiinflammatory agent.

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Residue 21 of human granulocyte-macrophage colony-stimulating factor is critical for biological activity and for high but not low affinity binding

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The functional role of the predicted first α-helix of human granulocyte-macrophage colony-stimulating factor (GM-CSF) was analysed by site-directed mutagenesis and multiple biological and receptor binding assays. Initial deletion mutagenesis pointed to residues 20 and 21 being critical. Substitution mutagenesis showed that by altering Gln20 to Ala full GM-CSF activity was retained but that by altering Glu21 for Ala GM-CSF activity and high affinity receptor binding were decreased. Substitution of different amino acids for Glu21 showed that there was a hierarchy in the ability to stimulate the various biological activities of GM-CSF with the order of potency being Asp21 > Ser21 > Ala21 > Gln21 > Lys21 = Arg21. To distinguish whether position 21 was important for GM-CSF binding to high or low affinity receptors, GM-CSF (Arg21) was used as a competitor for [^{125}I]GM-CSF binding to monocytes that express both types of receptor. GM-CSF (Arg21) exhibited a greatly reduced capacity to compete for binding to high affinity receptors, however, it competed fully for [^{125}I]GM-CSF binding to low affinity receptors. Furthermore, GM-CSF (Arg21) was equipotent with wild-type GM-CSF in binding to the cloned low affinity α-chain of the GM-CSF receptor. These results show that (i) this position is critical for high affinity but not for low affinity GM-CSF receptor binding thus defining two functional parts of the GM-CSF molecule; (ii) position 21 of GM-CSF is critical for multiple functions of GM-CSF; and (iii) stimulation of proliferation and mature cell function by GM-CSF are mediated through high affinity receptors.

Key words: growth factors/haemopoiesis/mutagenesis/structure-function/receptors

Introduction

Human (h) granulocyte-macrophage (GM) colony-stimulating factor (CSF) is a multi-potential growth factor capable of stimulating several haemopoietic cell lineages such as the neutrophilic, eosinophilic, monocytic and megakaryocytic series (Sieff *et al.*, 1985; Metcalf *et al.*, 1986). In addition GM-CSF is also able to stimulate the function of the differentiated progeny enhancing the effector functions of neutrophils and eosinophils (Vadas *et al.*, 1983; Gasson *et al.*, 1984; Lopez *et al.*, 1986) and the capacity of monocytes to kill tumour cells (Grabstein *et al.*, 1986) and adhere to various surfaces (Gamble *et al.*, 1989; Elliott *et al.*, 1990). Because of this pleiotropic effect GM-CSF has been used *in vivo* where it has been shown to increase the granulocyte counts in AIDS patients (Groopman *et al.*, 1987), accelerate bone marrow reconstitution following chemotherapy (Antman *et al.*, 1988) and enhance the effector function of circulating neutrophils (Baldwin *et al.*, 1988). In addition to normal haemopoietic cells, certain tumour cell lines have also been shown to respond to GM-CSF by proliferating *in vitro* (Dedhar *et al.*, 1988; Berdel *et al.*, 1989).

The human GM-CSF receptor has now been cloned and shown to comprise at least a binding (α) chain that binds GM-CSF with low affinity (Gearing *et al.*, 1989) and a second (β) chain that does not seem to bind GM-CSF by itself but which allows the formation of a high affinity receptor when co-expressed with the α-chain (Hayashida *et al.*, 1990). The functions mediated by each chain of the GM-CSF receptor are not yet known.

Despite the multiple *in vitro* and *in vivo* studies with GM-CSF, little is known about regions of the molecule essential for activity and in particular whether different regions participate in binding to high and low affinity receptors and their relationship to function. Using a chemical-synthesis approach we have previously shown that the 14 most N-terminal and the six most C-terminal residues of GM-CSF are not required for function, and importantly that the 14-24 region in the first predicted α-helix of GM-CSF is essential for bioactivity (Clark-Lewis *et al.*, 1988). Similar results were obtained using human-mouse GM-CSF chimeric molecules (Kaushansky *et al.*, 1989; Shanafelt *et al.*, 1991) and monoclonal anti-GM-CSF antibodies which blocked activity (Brown *et al.*, 1990). The latter two approaches identified, in addition, a second region in GM-CSF between residues 88-96 important for activity.

We have now used site-directed mutagenesis to study in more detail the predicted first α-helix of GM-CSF and, in particular, focussed on the hydrophilic residues Gln at position 20 and Glu at position 21. Our results show that residue Glu21 is critical for the full biological activity of GM-CSF. Significantly, substitution of Glu21 with Arg impaired the binding to high affinity but not to low affinity GM-CSF receptors, thus linking high affinity binding to the various functions examined and suggesting that Glu21 may be involved in binding to the β-subunit of the GM-CSF receptor.

Results

Mutagenesis of Gln20 and Glu21 of human GM-CSF

Initial experiments designed to examine the effect of N-terminal deletions on GM-CSF activity revealed that deletion of residues 1-24, 7-24 and 14-24 caused loss of GM-CSF activity (data not shown). We then focused on residues

Gln20 and Glu21 predicted to constitute a hydrophilic face of the GM-CSF molecule (Parry *et al.*, 1991). Deletion of residues 20–21 resulted in the complete loss of GM-CSF ability to stimulate bone marrow colony formation, the neutrophil respiratory burst and antibody-dependent cytotoxicity (ADCC), eosinophil-mediated antibody-dependent cytotoxicity (ADCC), and in the ability to compete for [^{125}I]GM-CSF high affinity binding to human neutrophils. In order to ascertain the importance of positions 20 and 21 for the biological activities of GM-CSF less severe modifications were carried out by introducing the non-polar residue alanine at both these positions. Experiments using transiently transfected COS cells showed that GM-CSF(Ala20) had the same potency as wild-type (WT) GM-CSF at stimulating day 14 GM bone marrow colonies (100.3% potency, not different to WT GM-CSF $P = 0.9$, $n = 6$), neutrophil ADCC (116.9%, $n = 6$, $P = 0.45$) and superoxide anion (O_2^-) generation (104.3%, $n = 12$, $P = 0.65$) and eosinophil ADCC (94.7%, $n = 4$, $P = 0.76$). In contrast GM-CSF(Ala21) was less potent than WT GM-CSF at stimulating neutrophil ADCC (24.2%, $n = 8$, $P < 0.001$) and O_2^- generation (28.9%, $n = 12$, $P < 0.001$), and eosinophil ADCC (21.0%, $n = 4$, $P < 0.001$). The double substitution GM-CSF(Ala20 Ala21) was also less potent than WT GM-CSF at stimulating neutrophil ADCC (17.0%, $n = 6$, $P < 0.01$) and O_2^- generation (7.5%, $n = 11$, $P < 0.001$). In addition, GM-CSF(Ala21) and GM-CSF(Ala20 Ala21) failed to stimulate 50% of WT GM-CSF day 14 GM colonies at the highest concentrations tested (100 ng/ml).

Single amino acid substitutions of Glu21

Having identified residue 21 as important for several biological properties of GM-CSF, a series of single substitutions were carried out to replace Glu21 with amino acids of different hydrophilicity and polarity. The amino acids introduced were aspartic acid (hydrophilic, acidic), glutamine (hydrophobic, neutral), serine (hydrophilic, neutral), alanine (hydrophobic, neutral), arginine (hydrophilic, basic) and lysine (hydrophilic, basic).

Examination of these GM-CSF mutants for their ability to stimulate the proliferation of leukaemic cells showed that the substitution of Glu for Asp reduced activity by 4-fold, for Ser by 30-fold, for Ala by 80-fold and for Gln by 100-fold. Substitutions with Lys and Arg yielded GM-CSF mutants with no activity up to a concentration of 10 ng/ml (Figure 1A). A titration of these GM-CSF mutants on stimulation of monocyte adherence showed similar results, with the acidic residue Asp affecting GM-CSF activity the least and the basic residues Arg and Lys virtually abolishing GM-CSF activity up to a concentration of 10 ng/ml (Figure 1B). Essentially the same pattern was observed in the stimulation of neutrophil O_2^- generation (data not shown).

Competition binding experiments on neutrophil high affinity GM-CSF receptors mirrored the hierarchy of the biological data. Using 70 pM [^{125}I]GM-CSF and the different GM-CSF proteins at 30-fold excess the levels of competition were 87.9% for WT GM-CSF, 26.1% for Asp21, 23.0% for Ser21, 18.3% for Ala21, 16.6% for Gln21, 14.6% for Arg21 and 7.4% for Lys21.

Purification and biological activities of GM-CSF with substitutions at positions 20 and 21

To study in more detail the relevance of positions 20 and 21, GM-CSF(Ala20), GM-CSF(Ala21), GM-CSF(Ala20, Ala21), GM-CSF(Arg21) and GM-CSF(Ala20, Arg21) were purified by affinity chromatography and reversed phase HPLC from supernatants of transfected CHO cells. After quantitation by amino acid analysis the GM-CSF mutants were visualized by Western blot analysis (Figure 2A) and silver staining (Figure 2B) to confirm purity and integrity. The purified GM-CSF mutants showed similar molecular weight heterogeneity, hence degree of glycosylation, to the wild-type GM-CSF (Figure 2A and B).

These purified mutants stimulated the [^{3}H]thymidine incorporation of leukaemic cells with different potencies. While GM-CSF(Ala20) was equipotent to the wild-type GM-CSF, GM-CSF(Ala21) was 30-fold, GM-CSF(Ala20, Ala21) 100-fold and GM-CSF(Arg21) and GM-CSF(Ala20,

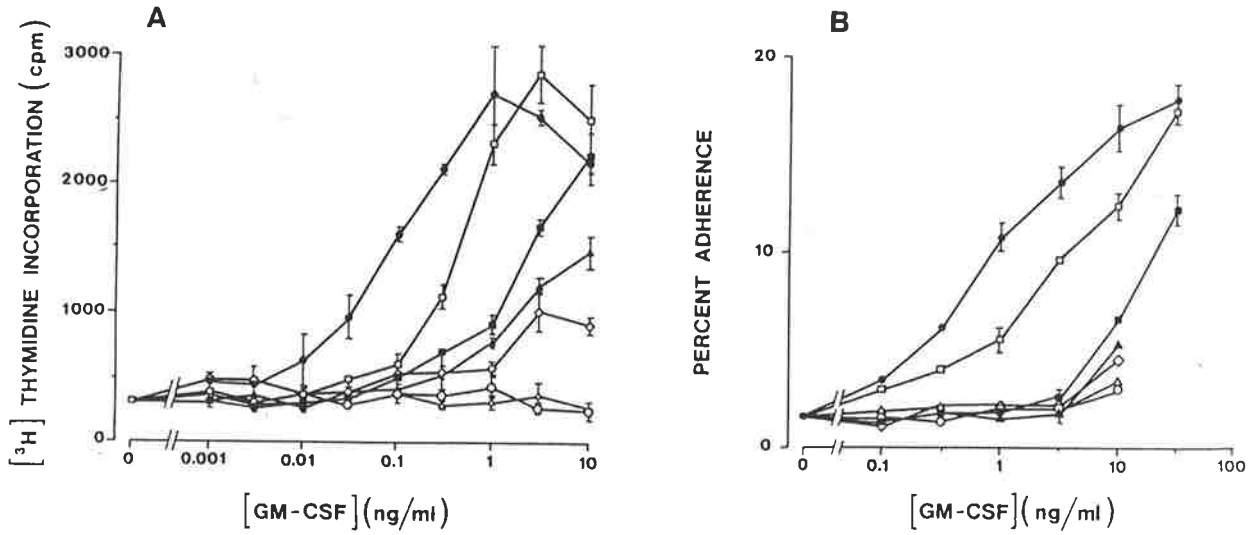

Fig. 1. Titration of GM-CSF mutants containing different residues at position 21 for their ability to stimulate the incorporation of [^{3}H]thymidine into chronic myeloid leukaemia cells (**A**) and to stimulate monocyte adherence (**B**). Wild-type (WT) GM-CSF (●) as well as GM-CSF(Ala21) (▲), GM-CSF(Arg21) (△), GM-CSF(Lys21) (○), GM-CSF(Gln21) (◇), GM-CSF(Ser21) (■) and GM-CSF(Asp21) (□) were tested at the concentrations shown. Representative experiments are shown with the bars spanning the SEM.

Arg21) 200-fold less potent than wild-type GM-CSF respectively (Figure 3A).

Stimulation of mature cell function showed a similar hierarchy and relative potencies in the stimulation of monocyte adherence and neutrophil O_2^- production. On monocyte adherence GM-CSF(Ala21) was 30-fold less potent than wild-type GM-CSF and GM-CSF(Ala20), while GM-CSF(Ala20, Ala21), GM-CSF(Arg21) and GM-CSF(Ala20, Arg21) were ~100-, 200- and 200-fold less potent respectively (Figure 3B). On neutrophil O_2^- production GM-CSF(Ala20), GM-CSF(Ala21), GM-CSF(Ala20, Ala21), GM-CSF(Arg21), GM-CSF(Ala20, Arg21) were 10-, 100-, 200- and 200-fold less potent than wild-type GM-CSF respectively (Figure 4A).

In competition binding experiments on the neutrophil high affinity GM-CSF receptor, GM-CSF(Ala20) was 3-fold, GM-CSF(Ala21) 40-fold, and GM-CSF(Ala20, Ala21), GM-CSF(Arg21) and GM-CSF(Ala20, Arg21) >100-fold less effective than wild-type GM-CSF at competing for [^{125}I]GM-CSF binding (Figure 4B).

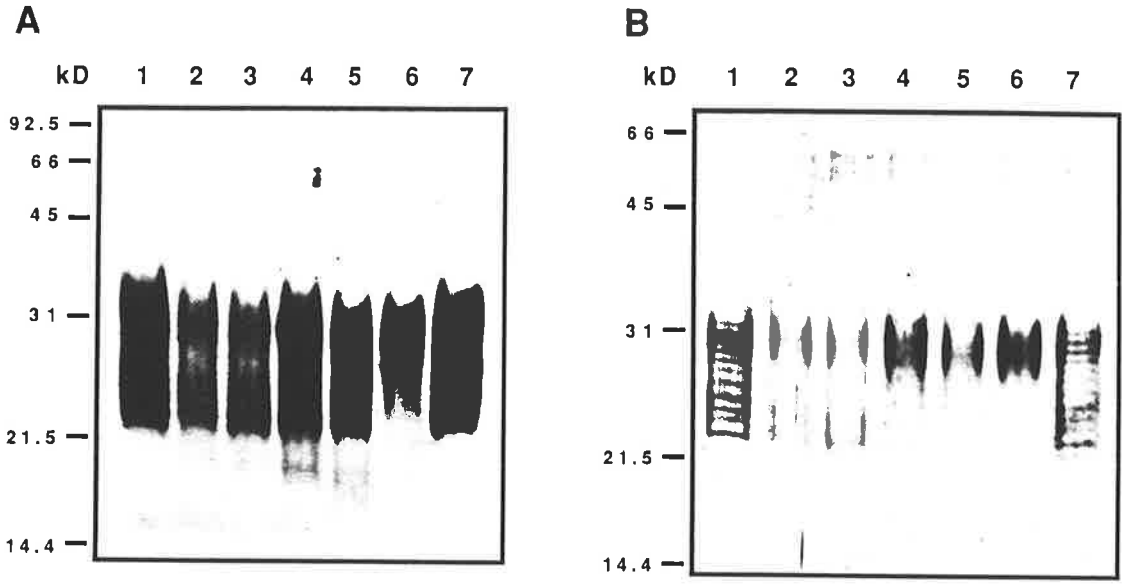

Fig. 2. Western blot analysis (**A**) and silver staining (**B**) of GM-CSF mutants after purification by affinity chromatography and HPLC. After quantitation by amino acid analysis 200 ng of protein was loaded per track and electrophoresed on a 12.5% polyacrylamide–SDS gel. In (A) GM-CSF protein was visualized by using a sheep antibody to human GM-CSF followed by a biotinylated rabbit anti-sheep antibody and developing the reaction with diaminobenzidine. In (B) the same amount of purified GM-CSF protein was visualized by silver staining. The different lanes contain WT GM-CSF (lane 1), GM-CSF(Arg21) (lane 2), GM-CSF(Ala20, Arg21) (lane 3), GM-CSF(Ala20) (lane 4), GM-CSF(Ala21) (lane 5), GM-CSF(Ala20, Ala21) (lane 6) and WT GM-CSF (lane 7).

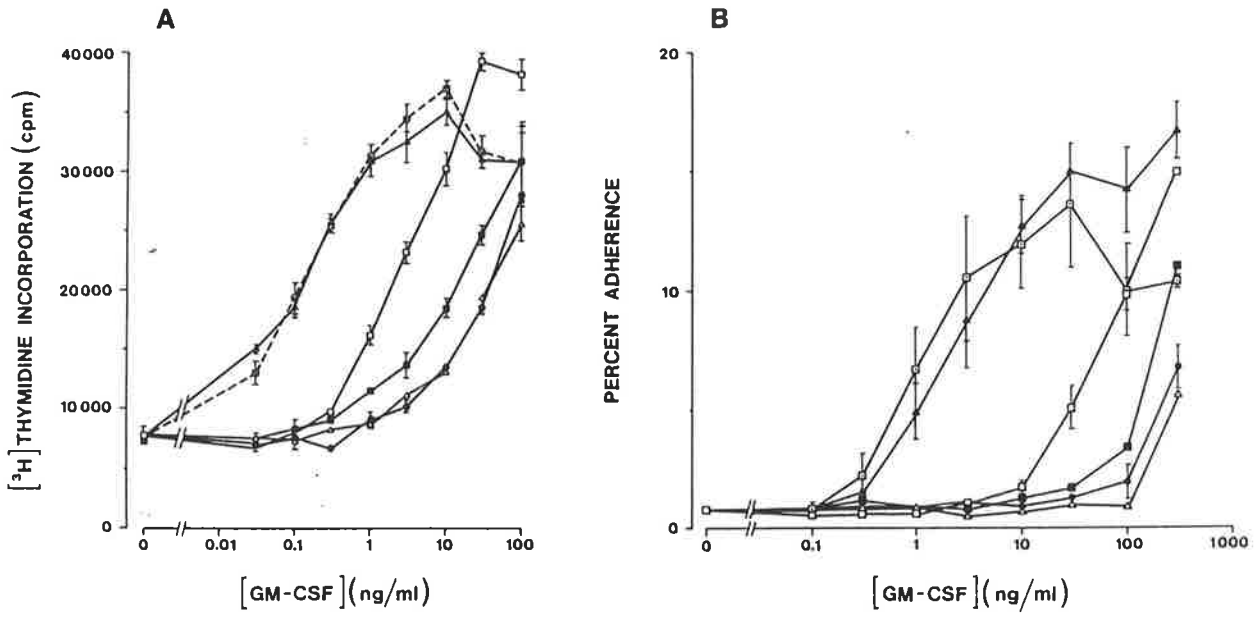

Fig. 3. Titration of purified GM-CSF proteins, WT GM-CSF (□), GM-CSF(Ala20) (▲), GM-CSF(Ala21) (□), GM-CSF(Arg21) (●), GM-CSF(Ala20 Ala21) (■) and GM-CSF(Ala20, Arg21) (△), for their ability to stimulate the [^{3}H]thymidine incorporation in chronic myeloid leukaemic cells (**A**) and to stimulate monocyte adherence (**B**). Representative experiments are shown with the bars spanning the SEM.

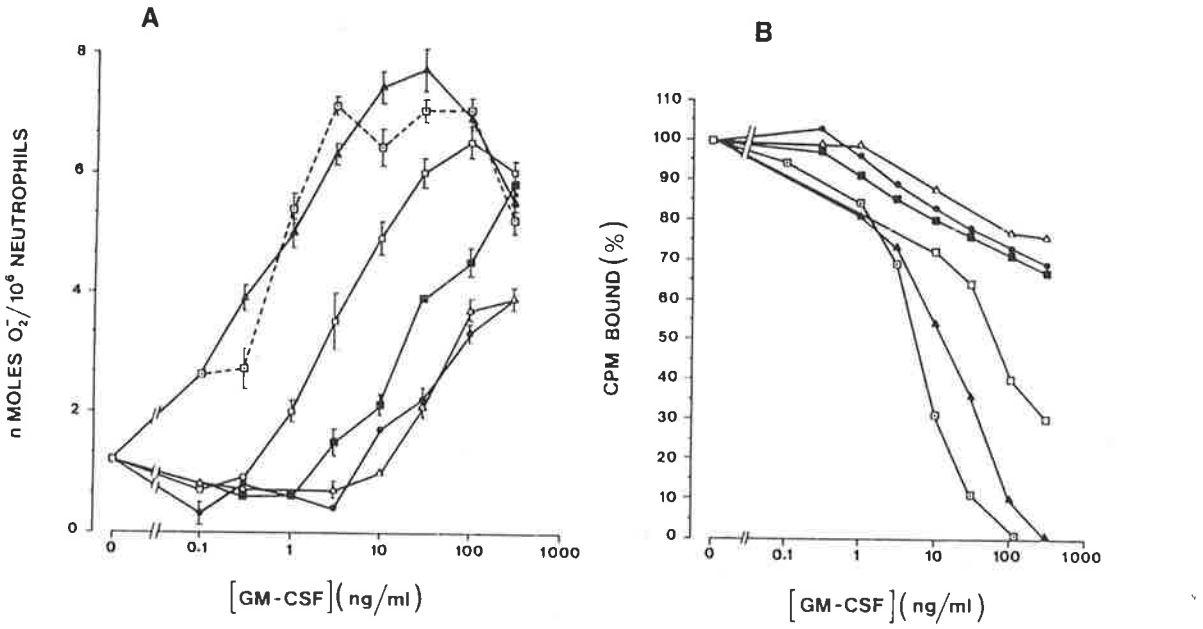

Fig. 4. Comparison of purified GM-CSF mutants for their ability to stimulate neutrophil superoxide production (**A**), and to compete for the binding of yeast-derived ^{125}I-labelled GM-CSF to the high affinity GM-CSF receptors of neutrophils (**B**). The GM-CSF mutants used were as described for Figure 3. Representative experiments are shown with the bars spanning the SEM.

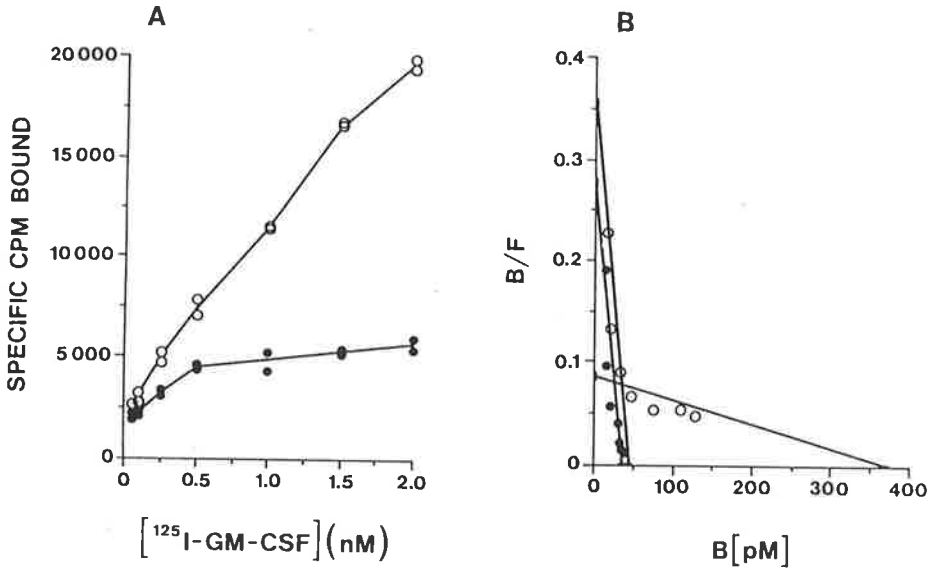

Fig. 5. Competition of yeast-derived [^{125}I]GM-CSF binding to monocytes by GM-CSF(Arg21). (**A**) Binding curve with different concentrations of [^{125}I]GM-CSF in the presence of 100-fold excess GM-CSF (○) or 35-fold excess purified GM-CSF(Arg21) (●). Specific counts for each duplicate determination are shown. (**B**) Scatchard analyses of the experiment in (A) showing the competition in the presence of GM-CSF (○) or GM-CSF(Arg21) (●). The mean values are plotted. Incubation was for 16 h at 4°C.

Differential binding of GM-CSF(Arg21) to high and low affinity GM-CSF receptors

Although the experiments described above showed that Glu21 was important for several biological activities of GM-CSF and for high affinity binding, they did not distinguish whether binding to the low affinity receptor was also affected and as such could not discriminate which type of receptor was responsible for function. To address this question we selected the GM-CSF(Arg21) mutant which had been shown to be one of the weakest GM-CSF analogues. Purified GM-CSF(Arg21) was tested for its ability to inhibit GM-CSF binding to high and low affinity receptors by using human monocytes which express both types of receptor (Elliott *et al.*, 1989). A binding curve using increasing concentrations of [^{125}I]GM-CSF was performed in the presence of 35-fold excess purified GM-CSF(Arg21). This experiment revealed that GM-CSF(Arg21) slightly inhibited the binding of low concentrations of [^{125}I]GM-CSF (high affinity binding) and strongly inhibited the binding of high concentrations of [^{125}I]GM-CSF (low affinity binding) (Figure 5A). The percentage levels of inhibition by GM-CSF(Arg21) for each concentration of [^{125}I]GM-CSF were 13.7% (50 pM), 26.2% (100 pM), 35.6% (250 pM), 37.2% (500 pM), 58.4% (1 nM), 68.7% (1.5 nM) and 71.2% (2 nM). Scatchard transformation of these data showed that GM-CSF(Arg21) eliminated GM-CSF low affinity binding

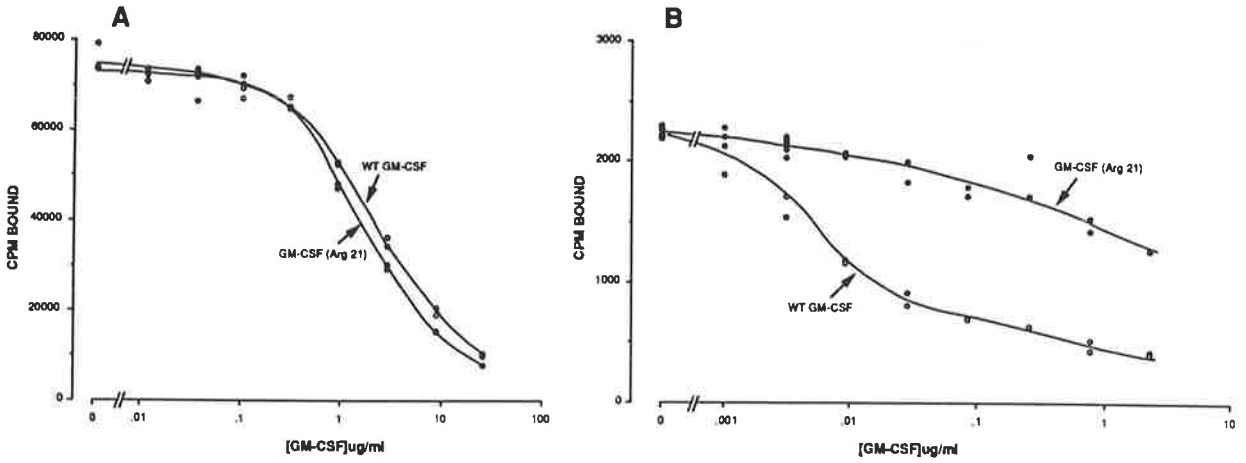

Fig. 6. Competition of *E.coli*-derived [^{125}I]GM-CSF binding to (**A**) COS cells transfected with the low affinity α-chain of the human GM-CSF receptor, and to (**B**) purified human neutrophils by increasing concentrations of (○) WT GM-CSF or (●) GM-CSF(Arg21). Binding was for 3 h at 4°C to (A) 1 × 10^6 transfected COS cells with 6 nM [^{125}I]GM-CSF or (B) 1.5 × 10^6 neutrophils with 2 nM [^{125}I]GM-CSF.

while slightly decreasing the number of high affinity sites (Figure 5B).

To confirm the ability of GM-CSF(Arg21) to distinguish between high and low affinity receptors experiments were carried out using COS cells transfected with the GM-CSF low affinity α-chain of the GM-CSF receptor (Gearing *et al.*, 1989) and neutrophils as controls. The results showed that the binding of GM-CSF(Arg21) to low affinity GM-CSF receptors was indistinguishable from that of wild-type GM-CSF (Figure 6A) and that GM-CSF(Arg21) was 300-fold less potent than wild-type GM-CSF at competing for neutrophil high affinity receptors (Figure 6B).

Discussion

We have performed a structure−function analysis of human GM-CSF by using site-directed mutagenesis and multiple biological and binding assays. Our results show that residue 21 of GM-CSF is important for GM-CSF function including proliferation, differentiation and mature cell function. We also report that residue 21 is implicated in binding to high affinity as opposed to low affinity GM-CSF receptors thus linking high affinity binding with the multiple functions examined. Significantly, substitution of Glu21 with Arg greatly decreased binding to high affinity receptors but retained the capacity to bind to low affinity receptors strongly suggesting that residue 21 is involved in binding to the β-chain of the GM-CSF receptor.

Residue Gln20 appeared not to be critical in that GM-CSF(Ala20) exhibited the same biological activity as the native GM-CSF. This is in agreement with other experiments in which a mouse−human hybrid GM-CSF molecule containing a mouse sequence between amino acids 1−20 was found to be fully active (Kaushansky *et al.*, 1989; Shanafelt *et al.*, 1991), thus showing that a change from Gln20 to the mouse equivalent Lys20 does not alter GM-CSF activity. However, the finding that GM-CSF(Ala20) had a slightly impaired capacity to compete for high affinity binding (Figure 4B) and the fact that GM-CSF(Ala20, Ala21) was less active than either GM-CSF(Ala20) or GM-CSF(Ala21) suggest some contribution by Gln20 to the activities of GM-CSF.

In contrast to Gln20, substitution of Glu21 by amino acids of different hydrophilicity and polarity significantly affected the potency of GM-CSF. Biological analysis of these mutants showed that the substitution of an acidic (Glu) for another acidic (Asp) residue caused the least change of activity while substitution by basic residues (Arg, Lys) caused the biggest reduction in GM-CSF activities. The other substitutions, either hydrophilic uncharged or hydrophobic, exhibited intermediate potency.

These series of substitutions suggest that Glu at position 21 is critical for GM-CSF activity and its acidic nature is important for binding to the high affinity GM-CSF receptor or in maintaining an appropriate tertiary configuration. In view of these results it is tempting to speculate that Glu21 is part of a GM-CSF binding site or close to residues involved in it, a notion supported by predictive conformational studies suggesting that Glu21 lies in the external face of the first α-helix (Parry *et al.*, 1991). However, despite the fact that single point mutations can be more revealing than antibody molecules (which are six times the size of GM-CSF) or deletion mutants, the possibility cannot be ruled out that the mutations we made have induced a conformational change in GM-CSF. Nevertheless the fact that GM-CSF(Arg21) was equipotent to WT GM-CSF in competing for low affinity binding would argue against the latter possibility.

An important finding with these GM-CSF mutants was the demonstration that all the activities of GM-CSF tested were affected. Examination of GM-CSF mutant-mediated stimulation of cell proliferation showed the same hierarchy as for stimulation of neutrophil and monocyte function. In competition binding experiments the order in which these mutants inhibited the binding of [^{125}I]GM-CSF to the high affinity receptor of neutrophils paralleled their bioactivity.

Whilst these studies focused on the 14−24 region of GM-CSF there seem to be other regions in the GM-CSF molecule also important for activity. The fact that a chemically synthesized GM-CSF peptide spanning residues 1−53 was not sufficient for activity (Clark-Lewis *et al.*, 1988) suggests a second region of importance in the C-terminus. This region appears to extend between residues 77 and 94 as shown with human−mouse GM-CSF chimeric molecules (Kaushansky *et al.*, 1989; Shanafelt *et al.*, 1991), and inhibitory monoclonal antibodies that recognize this area (Brown *et al.*, 1990; Nice *et al.*, 1990). In addition the involvement of regions

40−77 and 110−127 has also been suggested based on inhibitory studies using monoclonal antibodies (Nice *et al.*, 1990).

Although mainly two regions of GM-CSF, encompassing residues 14−24 and 77−94, appear to be involved in binding and bioactivity they both need to be preserved for full activity. Neither the peptides 1−53 (Clark-Lewis *et al.*, 1988; Gamble *et al.*, 1990), 54−127 (Clark-Lewis *et al.*, 1988; Gamble *et al.*, 1990) nor 86−93 (Nice *et al.*, 1990) expressed agonistic or antagonistic activity when used separately. These results suggest that either both regions are adjacent in the tertiary structure, forming a single binding site, or that they bind separately to distinct binding sites of the GM-CSF receptor. Our results with GM-CSF(Arg21) showing impairment of binding to high but not low affinity receptors (Figures 5 and 6) argues for the presence of two binding sites and suggests that Glu21 is involved in binding to the β-chain (or a third member of the GM-CSF−receptor complex) but not to the α-chain (Figure 7).

We note that GM-CSF belongs to a family of related cytokines with similar predicted tertiary configuration (Parry *et al.*, 1988) and whose receptors also belong to a common family (Gearing *et al.*, 1989; Bazan 1990). Our model with GM-CSF (Figure 7) may apply to other molecule members of this family, in particular IL-3 and IL-5, both of which have a glutamic acid at positions 22 and 10 respectively which are predicted to lie in the external face of the first α-helix (Parry *et al.*, 1991). Appropriate changes in this region may alter the binding and function of these molecules in a similar way, particularly as the β-chain of the GM-CSF receptor appears to be part of the high affinity IL-3 and IL-5 receptors (Lopez *et al.*, 1990, 1991; Kitamura *et al.*, 1991b; Tavernier *et al.*, 1991).

An important implication of the differential binding to high and low affinity GM-CSF receptors by GM-CSF(Arg21) is that such a mutant, despite having greatly reduced potency at stimulating high affinity receptor-mediated functions, would be expected to fully occupy and activate low affinity receptor-mediated functions. The low affinity receptor may mediate proliferation signals in some cases as suggested by the relatively high concentrations of GM-CSF required for stimulation of [^{3}H]thymidine incorporation in monocytes (Elliott *et al.*, 1989) and in particular by the ability of nM but not pM concentrations of human GM-CSF to stimulate the proliferation of mouse FDCP1 cells transfected with the low affinity human GM-CSF receptor α-chain (Metcalf *et al.*, 1990). However, in a similar system but which used instead transfected CTLL cells, the α-chain alone was insufficient to allow proliferation (Kitamura *et al.*, 1991a) and evidence has been presented that interaction with the β-chain across species is required for signalling even though high affinity binding is not observed.

The latter experiment emphasizes a potential therapeutic use for molecules similar to GM-CSF(Arg21), namely their capacity to act as antagonists in situations where the low affinity α-chain provides binding but is incapable of generating cellular signals. Since binding to the α-chain as well as the β-chain is required for high affinity binding and signalling, GM-CSF(Arg21) may pave the way for the engineering of mutants that have totally lost their ability to interact with the high affinity receptor but which by retaining their ability to bind to the α-chain can antagonize the effect of native GM-CSF. Such antagonists, either as monomers or dimers, could have useful clinical applications in situations where the presence of GM-CSF can exacerbate inflammation (Koyanagi *et al.*, 1988) or lead to tumour cell growth (Baldwin *et al.*, 1989; Dedhar *et al.*, 1988; Berdel *et al.*, 1989).

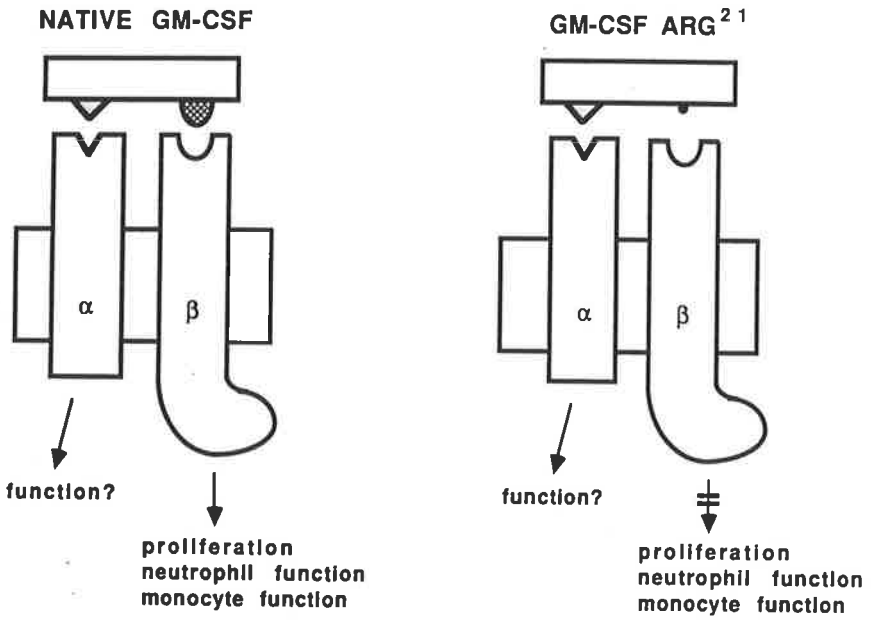

Fig. 7. Proposed model of GM-CSF interaction with the α- and β-chains of the GM-CSF receptor to accommodate the biological and binding data. On the left, binding to both the α- and β-chain of the GM-CSF receptor is shown. The triangular symbol in GM-CSF signifies a distinct structure (probably contributed to by helix D and the loop joining helices C and D) binding the α-chain, and the semi-circular structure, the critical contribution of Glu21 to the β-chain binding. The complex gives high affinity binding and full function. On the right, GM-CSF(Arg21) mutant is shown with an intact interaction with the α-chain (thus giving low affinity binding) but impaired interaction with the β-chain. The possibility that the α-chain by itself is able to stimulate some function in some cells is also indicated on this diagram.

Materials and methods

Site-directed mutagenesis and expression vector constructs

Site-directed mutagenesis of a human GM-CSF cDNA clone kindly provided by Dr S.Clark (Genetics Institute, Cambridge, MA), was performed in phage M13 as described (Zoller and Smith, 1984) with mutant plaques being screened by the 3 M tetramethyl ammonium chloride procedure (Wood *et al.*, 1985). The presence of the correct mutation was confirmed by chain termination sequencing (Sanger *et al.*, 1977) using a Sequenase Kit (United States Biochemical, Cleveland, OH). The mutant GM-CSF cDNAs were then excised from the M13 clones RF DNA and subcloned into the transient mammalian expression vector pJL4 (Gough *et al.*, 1985). Several mutant GM-CSF cDNAs were subsequently subcloned from the pJGM constructs into the neomycin selectable, mammalian expression vector pRSVN.07 (gift from Dr A.Robbins, Department of Biochemistry, University of Adelaide). All plasmid constructs were sequenced at the site of the mutation prior to transfection (Chen and Seeburg, 1985).

Transfection of GM-CSF and its analogues

Transient transfections in COS cells. COS cells were grown to 50–70% confluence in Dulbecco's Modified Eagle's medium (DMEM) containing 20 mM HEPES, penicillin, gentamicin and supplemented with 10% fetal calf serum (FCS). DNA constructs were introduced into COS cells by electroporation using a Bio-Rad Gene Pulser (Chu *et al.*, 1987). For each transfection 20 μg of DNA, 25 μg sonicated salmon sperm DNA and 50 μl FCS were mixed with 5×10^6 COS cells in 0.5 ml of 20 mM HEPES-buffered saline containing 6 mM glucose. After a 24 h incubation the medium was replaced with FCS-free DMEM and incubated for a further 72 h before the conditioned medium was harvested and assayed for GM-CSF protein.

Permanent transfections in CHO cells. CHO cells were grown to ~80% confluence in Hams F12 nutrient mixture containing penicillin, gentamicin and supplemented with 10% FCS. DNA constructs were introduced into CHO cells by electroporation after mixing 10 μg of pRSVNGM plasmid DNA with 5×10^4 CHO cells. 24–48 h after transfection selective F12 media containing geneticin (Gibco Laboratories) was added to the cells. Maximally expressing individual geneticin-resistant CHO colonies were selected and used to produce GM-CSF or mutant analogues.

Visualization of mutant GM-CSF protein

GM-CSF-containing COS cell supernatants and purified GM-CSF protein was size-fractionated by SDS–12.5% PAGE (Laemmli, 1970). For Western blot analysis, protein was transferred to nitrocellulose as described (Towbin *et al.*, 1979). Filters were probed with a sheep anti-GM-CSF (gift from Dr S.Clark, Genetics Institute, Cambridge, MA) followed by a second layer of biotinylated rabbit anti-sheep IgG. After a further incubation with an avidin-biotinylated horseradish peroxidase conjugate, the complex was visualized using a diaminobenzidine substrate solution. For silver staining, the method of Morrissey (1981) was used.

Quantitation and purification of GM-CSF protein

The amount of GM-CSF protein present in COS cell supernatants was quantitated by a radioimmunoassay (RIA). Some mutants were selected for purification and quantitation by amino acid analysis.

RIA. A competitive RIA was developed using ^{125}I-labelled human GM-CSF and a polyclonal sheep anti-GM-CSF serum (gift from Dr S.Clark, Genetics Institute, Cambridge, MA). GM-CSF modified by the addition of an extra tyrosine in the N-terminus (gift from Dr L.S.Park, Immunex Corp., Seattle, WA) was labelled as described below. COS cell supernatants (50 μl) were incubated with sheep anti-GM-CSF serum (50 μl of 1:40,000 dilution). After 4 h incubation at 4°C, 0.1 ng of [^{125}I]GM-CSF was added for a further 16 h before adding 100 μl of reconstituted anti-sheep Immunobead reagent (Bio-Rad Laboratories, Richmond, CA) for 4 h. The mixtures were then washed twice with phosphate-buffered saline (PBS), the pellet resuspended in 200 μl of PBS and transferred to 3DT tubes for counting in a gamma-counter (Packard Instrument Company, Meriden, CT). The amount of GM-CSF protein was calculated from a standard curve constructed with known amounts of GM-CSF.

Protein purification and amino acid analysis. GM-CSF protein in the supernatants of CHO cell lines permanently transfected with selected GM-CSF mutant cDNA was purified using an affinity column containing the monoclonal antibody LMM111 attached to Sepharose beads (Cebon *et al.*, 1988). Further purification was achieved by reversed phase HPLC and the resulting GM-CSF protein quantitated by amino acid analysis as described (Cebon *et al.*, 1990). This procedure also calculated the GM-CSF mutant preparations to be >99% pure.

Stimulation of haemopoietic cell proliferation

Two types of assay were performed.

Colony assay. This assay measured the clonal proliferation and differentiation of bone marrow progenitor cells in semi-solid agar and was carried out as described (Lopez *et al.*, 1988).

Proliferation of chronic myeloid leukaemic (CML) cells. Primary CML cells from one patient were selected for their ability to incorporate [^{3}H]thymidine in response to GM-CSF. This assay was performed as described (Lopez *et al.*, 1988).

Functional activation of human granulocytes and monocytes

Antibody-dependent cell-mediated cytotoxicity (ADCC) assay. Neutrophils and eosinophils were purified on Metrizamide (Nyegaard, Oslo) and tested against antibody-coated, ^{51}Cr-labelled P815 cells coupled with trinitrophenyl as previously described (Vadas *et al.*, 1983).

Superoxide anion production assay. This was carried out as described previously (Lopez *et al.*, 1986).

Monocyte adherence. Monocytes were purified from the peripheral blood of normal donors obtained from the Adelaide Red Cross Transfusion Service, as previously described (Elliott *et al.*, 1990). Stimulation of monocyte adhesion by GM-CSF was measured by an isotopic method essentially as described (Elliott *et al.*, 1990). In each assay the concentration of GM-CSF protein giving 50% maximal response was determined and the relative potency calculated by dividing the concentration of WT GM-CSF giving 50% stimulation by the concentration of mutant GM-CSF protein giving 50% stimulation and multiplying by 100. In some experiments the geometric mean from several experiments was calculated and statistical significance between values from different mutants determined by a paired *t* test.

Radioreceptor assay

Radioiodination of GM-CSF. Yeast-derived human GM-CSF (gift from Dr L.Park, Immunex Corporation, Seattle, WA), or *Escherichia coli*-derived human GM-CSF was radioiodinated by the ICl method (Contreras *et al.*, 1983). Iodinated protein was separated from free ^{125}I by chromatography on a Sephadex G-25 PD 10 column (Pharmacia, Uppsala, Sweden) equilibrated in PBS containing 0.02% Tween 20, and stored at 4°C for up to 4 weeks. Before use, the iodinated protein was purified from Tween and non-protein-associated radioactivity by cation exchange chromatography on a 0.3 ml CM-Sepharose CL-6B column (Pharmacia) and stored at 4°C for up to 5 days. The radiolabelled GM-CSF retained >90% biological activity as judged from titration curves using non-iodinated GM-CSF as controls.

Competition binding assays. Competition for binding to high affinity receptors used freshly purified neutrophils which express only this type of receptor (Gasson *et al.*, 1986). The cells were suspended in binding medium consisting of RPMI 1640 supplemented with 20 mmol/l HEPES, 0.5% bovine serum albumin (BSA) and 0.1% sodium azide. Typically, equal volumes (50 μl) of 4×10^6 neutrophils, 70 pM iodinated GM-CSF, and different concentrations of GM-CSF and GM-CSF analogues were mixed in siliconized glass tubes for 3 h at 4°C. Competition for binding to high and low affinity GM-CSF receptors used human monocytes which express both types of receptor (Elliott *et al.*, 1989). Equal volumes (50 μl) of cells (2×10^6), iodinated GM-CSF at different concentrations and 100-fold excess wild-type GM-CSF to establish non-specific binding, or 35-fold excess GM-CSF(Arg21) were mixed together for 16 h at 4°C before centrifugation of the cells over a cushion of FCS as above. Specific counts were determined by first subtracting the counts obtained in the presence of excess wild-type GM-CSF. Competition for binding to the low affinity GM-CSF receptor was performed using COS cells transiently transfected with a GM-CSF receptor cDNA clone as described (Gearing *et al.*, 1989). To 10^6 COS cells 6 nM of *E.coli*-derived [^{125}I]GM-CSF were added in the presence of different concentrations of wild-type GM-CSF or GM-CSF(Arg21) for 3 h at 4°C. In each case cell suspensions were overlaid on 0.2 ml FCS at 4°C, centrifuged in a Beckman Microfuge 12, and the tip of each tube containing the visible cell pellet cut off and counted in a gamma counter. In the case of monocytes the results are expressed in the form of equilibrium binding data and Scatchard transformation of these data as described (Scatchard, 1949).

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Shanafelt *et al.* [*EMBO J.*, **10**, 4105–4112 (1991)] have recently shown that the amino terminal helix of GM-CSF governs high affinity binding.

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Specific human granulocyte–macrophage colony-stimulating factor antagonists

(cytokines/inflammation/leukemia/receptors)

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ABSTRACT **Human granulocyte–macrophage colony-stimulating factor (GM-CSF) is a pleiotropic hemopoietic growth factor and activator of mature myeloid cell function. We have previously shown that residue 21 in the first helix of GM-CSF plays a critical role in both biological activity and high-affinity receptor binding. We have now generated analogues of GM-CSF mutated at residue 21, expressed them in *Escherichia coli*, and examined them for binding, agonistic, and antagonistic activities. Binding experiments showed that GM E21A, E21Q, E21F, E21H, E21R, and E21K bound to the GM-CSF receptor α chain with a similar affinity to wild-type GM-CSF and had lost high-affinity binding to the GM-CSF receptor α-chain–common β-chain complex. From these mutants, only the charge reversal mutants E21R and E21K were completely devoid of agonistic activity. Significantly we found that E21R and E21K antagonized the proliferative effect of GM-CSF on the erythroleukemic cell line TF-1 and primary acute myeloid leukemias, as well as GM-CSF-mediated stimulation of neutrophil superoxide production. This antagonism was specific for GM-CSF in that no antagonism of interleukin 3-mediated TF-1 cell proliferation or tumor necrosis factor α-mediated stimulation of neutrophil superoxide production was observed. *E. coli*-derived GM E21R and E21K were effective antagonists of both nonglycosylated and glycosylated wild-type GM-CSF. These results show that low-affinity GM-CSF binding can be dissociated from receptor activation and have potential clinical significance for the management of inflammatory diseases and certain leukemias where GM-CSF plays a pathogenic role.**

Granulocyte–macrophage colony-stimulating factor (GM-CSF) is a glycoprotein with an apparent molecular weight of 23,000–28,000 and is produced by a variety of cells including activated T cells, macrophages, and endothelial cells (1). *In vitro* and *in vivo* studies have demonstrated that GM-CSF is a pleiotropic cytokine that stimulates both the production of different hemopoietic cell lineages and the effector function of mature myeloid cells (1–7). These properties have led to the *in vivo* use of GM-CSF to stimulate hemopoiesis and the effector function of neutrophils and monocytes (8, 9).

GM-CSF may also play a role in the pathogenesis of several disease conditions, in particular chronic inflammation and leukemia. Transgenic mice carrying the murine GM-CSF gene display elevated levels of GM-CSF and an accumulation of macrophages in the eyes and striated muscles, leading to blindness, muscle damage, and premature death, possibly as a result of GM-CSF-mediated macrophage activation (10). In humans the presence of elevated levels of GM-CSF in the bronchoalveolar lavage of atopic patients (11) and in the synovial fluid of patients with rheumatoid arthritis (12), along with the activated phenotype of neutrophils in this fluid (13), suggests that GM-CSF plays a pathological role in some inflammatory diseases. GM-CSF also stimulates the production of the neutrophil chemoattractant interleukin (IL) 8 (14) and, in conjunction with lipopolysaccharide, the monocyte chemoattractant macrophage inflammatory protein 1α (15), thus regulating the localization of leukocytes at the site of inflammation. In terms of leukemia, GM-CSF has been shown to be necessary for the survival and continued proliferation of some leukemic cells *in vitro* (1). Certain acute myeloid leukemias (AMLs) (16, 17) and lymphoblastic (18) leukemias have been shown to exhibit disregulated growth in response to autocrine (16) or paracrine (19) GM-CSF.

GM-CSF exerts its effects through binding to its high-affinity receptor (20). This is made up of a GM-CSF-specific, low-affinity receptor α chain (GMRα) (21) and a receptor β chain, which is shared with the IL-3 and IL-5 receptors. This common β chain (β_c) does not by itself detectably bind GM-CSF but confers high-affinity binding when coexpressed with the α chain and is required for signal transduction (22). The exact mechanism of receptor activation is not known, but it is thought to require binding of GM-CSF to GMRα followed by association with β_c, leading to the formation of a high-affinity receptor complex (23, 24). However, signaling can also be observed following low-affinity binding of GM-CSF (25–28), implying that GM-CSF binding to GMRα is sufficient for receptor activation.

The three-dimensional structure of human GM-CSF determined by x-ray crystallography (29) revealed the presence of a four-α-helix bundle with a fold common to many of the cytokines including IL-2 (30), IL-4 (31), IL-5 (32), and growth hormone (33). GM-CSF, IL-5, and IL-3 have been demonstrated to interact with their β_c through residues in their first helix (34) and more specifically with the conserved glutamate residue in this helix (27, 28, 35, 36). Using a CHO cell expression system, we previously showed that a GM-CSF mutant, E21R, had lost high-affinity binding while retaining wild-type affinity for GMRα and exhibited a 100- to 300-fold loss of biological activity (27, 36). We have now expressed several analogues of human GM-CSF mutated at residue 21 in an *Escherichia coli* secretion system and found that while all mutants bound the GM-CSF receptor with similar low affinities, they exhibited differences in their agonistic activities. Two mutants, E21R and E21K, were devoid of measurable agonistic activity and significantly were able to specifically antagonize GM-CSF-dependent proliferation of leukemic cells as well as GM-CSF activation of neutrophils.

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Abbreviations: AML, acute myeloid leukemia; GM-CSF, granulocyte–macrophage colony-stimulating factor; GMRα, GM-CSF receptor α chain(s); β_c, common β chain; IL, interleukin; TNF-α, tumor necrosis factor α; rh, recombinant human.

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The development of specific GM-CSF antagonists has important clinical implications and suggests that similar antagonists may be constructed for GM-CSF-related cytokines such as IL-3 and IL-5.

MATERIALS AND METHODS

Site-Directed Mutagenesis of Human GM-CSF. The plasmid pshGM-CSF containing a synthetic human GM-CSF cDNA cloned into the *E. coli* expression vector pIN-III-OmpH3, a derivative of pIN-III-OmpA2 (37), was kindly provided by A. B. Shanafelt and R. A. Kastelein (DNAX Research Institute, Palo Alto, CA). Oligonucleotide cassette mutagenesis was used to introduce residue 21 substitutions between unique *Nco* I and *Sac* II sites (64 bp). Plasmid constructs were sequenced through the entire oligonucleotide cassette (38) using a Sequenase kit (United States Biochemical).

Expression and Purification of *E. coli*-Derived GM-CSF and Analogues. *GM-CSF expression.* GM-CSF was expressed in logarithmic phase *E. coli* cultures after a 3-hr induction by 0.1 mM isopropyl β-D-thiogalactoside. Soluble GM-CSF was recovered from the periplasmic space by osmotic shock (39) with a typical yield of GM-CSF for wild type and most analogues of 1 mg of GM-CSF per liter of cultured cells as assessed by RIA (27). Certain of the residue-21 analogues proved difficult to express, and a number of *E. coli* strains were screened for their ability to express these analogues. Eventually the strain BL21 (40) (a gift from R. Morona, Department of Microbiology and Immunology, University of Adelaide) was found to be the most suitable, but expression was still poor, notably for E21R, with an expression level of only 1% of wild-type GM-CSF.

GM-CSF purification. Wild-type GM-CSF and analogues were purified from crude osmotic shock supernatants using the 4D4 anti-GM-CSF monoclonal antibody affinity column (36). Affinity-purified GM-CSF was then loaded onto a Brownlee Aquapore RP-300 reverse-phase column (4.6 × 100 mm) and was eluted using a 30–50% gradient of acetonitrile containing 0.1% trifluoroacetic acid. The resulting GM-CSF, typically at >95% purity, was lyophilized and resuspended in phosphate-buffered saline containing 0.02% (vol/vol) Tween 20.

GM-CSF quantitation. Purified GM-CSF and analogues were quantified by high-performance size-exclusion chromatography. Samples were chromatographed on a Beckman Ultraspherogel SEC3000 (7.5 × 300 mm) using a 0.1 M sodium phosphate, pH 7.0/0.1 M sodium sulfate mobile phase. The area under the GM-CSF peak was integrated by using the calculated extinction coefficient of 0.95 absorbance units·ml^{-1}·mg^{-1}. The yield of purified wild-type GM-CSF was typically 500 μg/liter of culture but was as little as 5–10 μg/liter of culture for purified E21R.

Radioiodination of GM-CSF. An *E. coli*-derived GM-CSF analogue containing two tyrosine residues inserted in place of a proline at residue six (GM P6YY) was radioiodinated by the iodine monochloride method (41) to a specific activity of ≈60 μCi/μg (1 Ci = 37 GBq). Iodinated protein was processed as described (27).

Recombinant Human (rh) Cytokines. Wild-type GM-CSF derived from CHO cells and *E. coli*-derived IL-3 were kindly provided by S. Clark (Genetics Institute, Cambridge, MA). *E. coli*-derived tumor necrosis factor α (TNF-α) was obtained from Genentech.

GM-CSF Functional Assays. *Hemopoietic cell proliferation assay.* The proliferative activity of GM-CSF mutants was assayed using the GM-CSF-dependent erythroleukemia cell line TF-1 (42) or primary AML cells selected for the ability to incorporate [^{3}H]thymidine in response to GM-CSF. Proliferation assays were performed essentially as described (42). Briefly cells (5 × 10^4 cells per well) were incubated with growth factors in 96-well plates for 48 hr (TF-1) or 72 hr (AML) before being pulsed with [^{3}H]thymidine (ICN) at 1 μCi per well for 5 hr. The cells were then harvested, and cell-associated radioactivity was determined in a Packard TriCarb liquid scintillation counter.

Superoxide anion production assay. This assay was carried out using purified human neutrophils as described (6).

Receptor Binding Studies. Competition for binding to high-affinity receptors (GMR$\alpha\beta_c$) used freshly purified human neutrophils, which express only high-affinity receptors (20). Competition for binding to low-affinity receptors (GMRα) used the stably transfected A9/C7 CHO cell line, which expresses 2–5 × 10^5 GMRα per cell (36). Binding experiments were performed as described (27). Data from receptor binding experiments were analyzed using the LIGAND program (43) obtained from Biosoft (Cambridge, U.K.).

RESULTS

Differential Biological Activity and Receptor Binding Properties of GM-CSF Analogues Mutated at Residue 21. Wild-type human GM-CSF and analogues with substitutions of the Glu-21 residue were produced by using an *E. coli* expression system and purified by immunoaffinity chromatography and reversed-phase HPLC. Dose-dependent stimulation of TF-1 cell proliferation was observed with the GM-CSF analogues E21A, E21Q, E21F, and E21H, although they exhibited ED_{50} values increased by 40- to 3000-fold compared with wild-type GM-CSF (Table 1). In contrast, the E21R and E21K analogues were unable to stimulate TF-1 cell proliferation even at concentrations 100,000-fold higher than the concentration of wild-type GM-CSF required to stimulate half-maximal proliferation of TF-1 cells (Table 1).

To study the relationship between biological activity and receptor binding, we tested the GM-CSF residue-21 analogues for their ability to compete for the binding of wild-type GM-CSF to the high-affinity (GMR$\alpha\beta_c$) or low affinity (GMRα) GM-CSF receptor. We found that using human neutrophils, which express only the high-affinity receptor (20), all mutants showed a 100- to 200-fold reduction in affinity (Fig. 1*A* and Table 1). In contrast, using the stably transfected CHO cell line A9/C7, which expresses large numbers of GMRα only, we found that all mutants exhibited an affinity similar to wild-type GM-CSF (Fig. 1*B* and Table 1).

GM E21R and E21K Antagonize GM-CSF-Mediated Proliferation of Leukemic Cells. Having established that E21R and E21K were able to bind with near wild-type affinity to the

Table 1. Biological activity and receptor binding characteristics of GM-CSF analogues mutated at residue 21

GM-CSF	ED_{50},* ng/ml	K_d ($\alpha\beta_c$),† pM	K_d (α)‡, pM
Wild type	0.01	7	1800
E21A	0.4	730	820
E21Q	0.6	940	1300
E21F	8	1600	1400
E21H	30	1060	800
E21K	—§	1060	1200
E21R	—§	730	1000

*Concentration of GM-CSF analogue stimulating 50% of the maximal, wild-type GM-CSF proliferative response, as measured by [^{3}H]thymidine incorporation by TF-1 cells.

†Mean binding affinities derived from two competition experiments on human neutrophils using 100 pM ^{125}I-labeled rhGM-CSF (^{125}I-rhGM-CSF) and different concentrations of E21 analogues.

‡Mean binding affinities derived from three competition experiments on the CHO cell line A9/C7 using 500 pM ^{125}I-rhGM-CSF and different concentrations of E21 analogues.

§No activity detected up to a concentration of 3000 ng/ml.

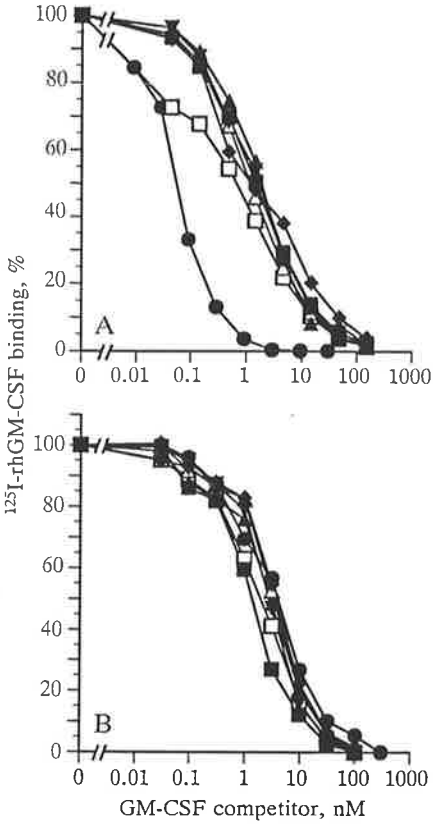

FIG. 1. Comparative binding of wild-type GM-CSF and residue-21 analogues to cells expressing high (GMR$\alpha\beta_c$) (*A*) or low (GMRα) (*B*) affinity GM-CSF receptors. Unlabeled GM-CSF, wild type or mutant protein, was titrated against 100 pM ^{125}I-labeled rhGM-CSF (^{125}I-rhGM-CSF) and 3×10^6 neutrophils per tube in *A* or 500 pM ^{125}I-rhGM-CSF and 5.5×10^5 A9/C7 cells per tube in *B*. Symbols represent competition by wild-type GM-CSF (●), E21A (□), E21Q (△), E21F (◆), E21H (▼), E21K (▲), and E21R (■). The values are expressed as percent ^{125}I-rhGM-CSF bound and are the means of duplicate determinations.

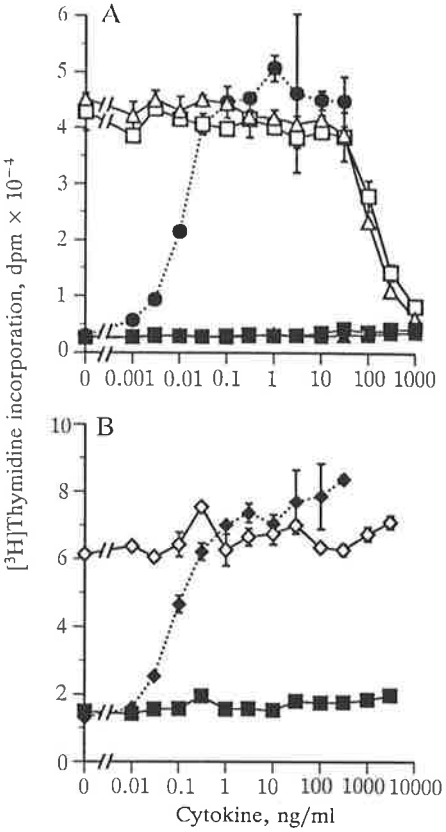

FIG. 2. E21R and E21K antagonize GM-CSF (*A*)- but not IL-3 (*B*)-mediated proliferation of TF-1 cells. Titrations of GM-CSF (●), IL-3 (◆), E21R (■), and E21K (▲) are shown. (*A*) In antagonistic experiments, E21R (□) and E21K (△) were titrated against GM-CSF at 0.03 ng/ml. (*B*) Specificity was determined by titration of E21R (◇) against IL-3 at 0.3 ng/ml. Each value represents the mean of triplicate determinations, and error bars represent the SEM.

GMRα but were unable to deliver a biological signal, we tested these analogues for their ability to antagonize the activity of wild-type GM-CSF. In a TF-1 cell proliferation assay, E21R and E21K were titrated against GM-CSF at 0.03 ng/ml, a dose that stimulates near-maximal TF-1 cell proliferation. We found that E21R and E21K completely antagonize the activity of GM-CSF, requiring a 7000-fold (200 ng/ml) and 5000-fold (150 ng/ml) excess, respectively, to reach 50% inhibition (Fig. 2*A*). When E21R was titrated against IL-3 at 0.3 ng/ml, as a specificity control, we found no detectable antagonism of IL-3 activity by E21R (Fig. 2*B*).

We next examined the ability of GM E21R to antagonize the GM-CSF-mediated proliferation of fresh leukemic cells from patients with AML by titrating GM E21R against GM-CSF at 0.3 ng/ml. The results summarized in Table 2 show that E21R was able to antagonize the GM-CSF-mediated proliferation of all three AMLs tested with a 2000- to 3000-fold (600–900 ng/ml) excess required to reach 50% inhibition.

GM E21R and E21K Antagonize GM-CSF Activation of Neutrophils. Since GM-CSF is a strong stimulator of inflammatory cells, we examined the ability of E21R and E21K to antagonize GM-CSF stimulation of superoxide production by human neutrophils. GM E21R was titrated against GM-CSF at 1 ng/ml or TNF-α at 3 ng/ml as a specificity control. We found that E21R antagonized the activity of GM-CSF, requiring a 300-fold (300 ng/ml) excess to reach 50% inhibition (Fig. 3*A*). However, there was no detectable antagonism of TNF-α even at a 10,000-fold excess.

To determine whether this antagonism could also be observed on glycosylated GM-CSF, which is the form that prevails *in vivo* (44), we tested E21R and E21K for their ability to antagonize glycosylated GM-CSF stimulation of superoxide production by neutrophils. A titration of GM E21R and E21K against glycosylated GM-CSF at 3 ng/ml produced in CHO cells showed that a 30-fold excess (90 ng/ml) of E21R and a 70-fold excess (200 ng/ml) of E21K were required to reach 50% inhibition of GM-CSF stimulation (Fig. 3*B*).

DISCUSSION

We describe here the discovery of two GM-CSF analogues, GM E21R and GM E21K, which are antagonists of GM-CSF function. *E. coli*-derived E21R and E21K completely antagonized the proliferative activity of GM-CSF on leukemic cells and the activation of human neutrophils by GM-CSF. These effects were specific in that IL-3-mediated cell proliferation or TNF-α-mediated neutrophil activation was not inhibited. These antagonists should help our understanding of the basic mechanism of GM-CSF receptor activation and have clinical potential for therapeutic intervention in inflammatory diseases and leukemias where GM-CSF may play a pathogenic role.

The exact mechanism of GM-CSF receptor activation is not known, but it appears to involve the low affinity binding of GM-CSF to GMRα and association of this complex with β_c, leading to high-affinity binding and signaling (23, 24).

Table 2. E21R antagonizes the GM-CSF-dependent proliferation of primary human myeloid leukemias

Cell	[^{3}H]Thymidine incorporation,* dpm	ED_{50} (GM-CSF)†, ng/ml	IC_{50} (E21R)‡, ng/ml
AML 1	6,400	0.02	900
AML 2	29,000	0.15	300
AML 3	2,900	0.07	500
TF-1	44,000	0.01	200

*Maximum GM-CSF-stimulated [^{3}H]thymidine incorporation.
†Concentration of GM-CSF stimulating 50% of the maximal proliferative response.
‡Concentration of E21R producing 50% inhibition of cell proliferation stimulated by GM-CSF at 0.3 ng/ml.

Considerable evidence indicates, however, that the low-affinity binding of GM-CSF to an $\alpha\beta_c$ receptor complex is sufficient to trigger a biological response, perhaps through an allosteric interaction. Thus, human GM-CSF binds with low affinity to murine cell lines expressing the endogenous murine β_c chain and the transfected human GMRα chain and elicits a proliferative response (25, 26). Similarly CHO cell-derived human GM-CSF E21R lacks high-affinity binding yet elicits a biological response (27). In all these cases the reduced binding affinity correlated with a reduced biological potency. In a most extreme example, the murine GM-CSF mutant E21A binds with only low affinity yet triggers a full biological response with wild-type potency (28). We show here that low-affinity binding does not necessarily lead to receptor activation. The *E. coli*-derived analogues E21R and E21K fully bound the GMRα but did not stimulate leukemic cell proliferation or neutrophil activation. This shows that GMRα binding can be dissociated from receptor activation and illustrates the feasibility of constructing cytokine antagonists that can interact with and sequester the major binding chain of a receptor complex.

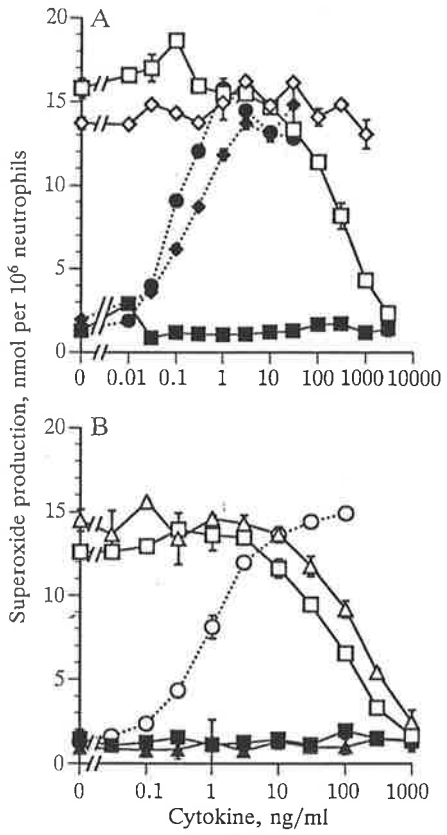

FIG. 3. E21R antagonizes GM-CSF- but not TNF-α-mediated stimulation of human neutrophils (*A*), and both E21R and E21K also antagonize neutrophil stimulation by CHO cell-derived GM-CSF (*B*). (*A*) Titrations of *E. coli*-derived wild-type GM-CSF (●), TNF-α (◆), and E21R (■) are shown. In antagonistic experiments, E21R was titrated against *E. coli*-derived GM-CSF (□) at 1 ng/ml or TNF-α (◇) at 3 ng/ml. (*B*) Titrations of CHO cell-derived wild-type GM-CSF (○), E21R (■), and E21K (▲) are shown. In antagonistic experiments, E21R (□) or E21K (△) was titrated against CHO cell-derived GM-CSF at 3 ng/ml. Each value represents the mean of triplicate determinations, and error bars represent the SEM.

The lack of agonistic activity of E21R and E21K suggests that these mutations have abolished the interaction of GM-CSF with β_c. This is also supported by the inability of E21R to cross-compete for ^{125}I-labeled IL-3 binding (unpublished data) or to antagonize IL-3 activity (Fig. 2*B*). However, E21A, E21Q, E21F, and E21H exhibited agonistic activity and stimulated function with different potencies despite binding to GMRα with essentially the same affinity as E21R and E21K. This suggests a weak residual interaction with β_c, which is unable to enhance the measurable affinity of ligand binding to GMR$\alpha\beta_c$ over binding to GMRα alone. These data suggest that Glu-21 of GM-CSF directly interacts with β_c and that a charge reversal at this position has the most deleterious effect on the interaction with β_c. A direct interaction between GM-CSF Glu-21 and β_c is also supported by the ability of β_c mutants Y365A, H367A, and I368A to partially restore high-affinity binding of E21R (unpublished data).

The lack of agonistic activity exhibited by *E. coli*-derived E21R was surprising in view of the weak agonism exhibited by CHO cell-derived E21R (27, 36). One possibility is that the folding of *E. coli*-derived E21R differs from CHO cell-derived E21R. Although the possibility of gross structural alterations is unlikely given that both E21R and E21K bind fully to GMRα (Fig. 1*B*), we have observed a reduced stability for *E. coli*-derived E21R compared to *E. coli*-derived wild-type GM-CSF (unpublished data). A second possibility is that the carbohydrate of CHO cell-derived GM-CSF provides contact with β_c (45), which is not measurable by binding experiments. However, glycosylated GM-CSF exhibits lower specific activity than nonglycosylated GM-CSF (44, 46). Last, the carbohydrate on CHO cell-derived E21R might prevent an active repulsion of β_c caused by the charge reversal, or it may allow a conformational change in the α chain or the β_c chain to occur by masking the positive charge of the arginine group.

The observed IC_{50} values of the E21R and E21K antagonists are typically around 200 ng/ml, which is 10-fold higher than the concentration required to achieve 50% occupancy of the neutrophil $\alpha\beta_c$ receptor (Fig. 1*A*). This presumably reflects the degree of GMRα that must be occupied by antagonist to block wild-type signaling. This is consistent with previous reports that maximum biological effects of colony-stimulating factors can be exerted with steady-state receptor occupancy levels as low as 10% (47). The IC_{50} values of E21R and E21K exhibit a considerable variation in terms of excess of antagonist over agonist required for 50% inhibition, ranging from 7000-fold (Fig. 2*A*) to 30-fold (Fig. 3*B*). This probably reflects differences in the ED_{50} values of wild-type GM-CSF in these biological assays, which range from 0.01 ng/ml (Fig. 2*A*) to 1 ng/ml (Fig. 3*B*). The difference can partially be accounted for by the effect of carbohydrate on wild-type GM-CSF activity (44) but also appears to reflect differences in the concentration of GM-CSF required to trigger receptor activation in different cell types.

Potential applications of GM-CSF antagonists *in vivo* are in the management of inflammatory conditions exacerbated by abnormal levels of GM-CSF and inhibition of tumor cell growth. The detection of elevated mRNA for GM-CSF but not other cytokines in the bronchoalveolar lavage of atopic patients (11) suggests a role for GM-CSF antagonists in the treatment of allergic reactions. In rheumatoid arthritis, the presence of GM-CSF in synovial fluid (12) and the activated phenotype of the neutrophils in this fluid (13) provide another

scenario for therapeutic intervention with GM-CSF antagonists. The demonstration that E21R and E21K inhibit the GM-CSF-mediated growth of leukemic cells *in vitro* raises the possibility that these analogues may also be effective antagonists of leukemic cell proliferation mediated by GM-CSF produced in either a paracrine or autocrine manner *in vivo* (16, 19).

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A human interleukin 3 analog with increased biological and binding activities

(hemopoiesis/growth factors/function/allergy)

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ABSTRACT **Human interleukin 3 (IL-3) variants generated by site-directed mutagenesis were analyzed in multiple biological and binding assays to identify residues critical for IL-3 activity. Two mutants carrying substitutions in the predicted hydrophilic region within the first α-helix, [Ala^{21},Leu^{22}]IL-3 and [Ala^{21},Leu^{22},Ala^{25}]IL-3 showed loss of biological activity and high-affinity binding. Mutants in a second predicted hydrophilic region, [Ala^{44},Leu^{45},Ala^{46}]IL-3 and [Ala^{44},Ala^{46}]IL-3, however, showed similar biological and binding activities to wild-type IL-3. Mutations in a C-terminal hydrophilic region that overlaps the fourth predicted α-helix led to either loss or gain of function. IL-3 analogs [Glu^{104},Asp^{105}]-, [Leu^{108}]-, [Asn^{108}]-, [Thr^{108}]-, and [Ala^{101},Leu^{108}]IL-3 were less active than wild-type IL-3, whereas [Ala^{101}]IL-3 and [Val^{116}]IL-3 were 2- to 3-fold more potent. Significantly, the double mutant [Ala^{101},Val^{116}]IL-3 exhibited a 15-fold greater potency than native IL-3. Receptor binding studies showed that [Ala^{101},Val^{116}]IL-3 exhibited increased binding to the high- and low-affinity receptors of monocytes. These results show the generation of an IL-3 analog with increased biological and binding activities and support a model where the C terminus of IL-3 interacts with the α chain of the IL-3 receptor, making this region a useful focus for the development of more potent IL-3 agonists or antagonists.**

Interleukin 3 (IL-3) is a T-cell-derived glycoprotein of 133 amino acids that is central in the control of hemopoiesis and inflammation (1). *In vitro* studies showed that IL-3 stimulates the proliferation and differentiation of normal hemopoietic cells of different lineages directly (2–5) and in combination with lineage-restricted growth factors (6), granulocyte–macrophage colony-stimulating factor (GM-CSF), and mast cell growth factor (7). *In vivo*, IL-3 alone elevates the numbers of several hemopoietic cell lineages in the blood (8–10) and can also act synergistically with GM-CSF (11). Because of its pleiotropic effects, IL-3 appears ideally suited for clinical application in situations where extensive bone marrow reconstitution is required. On the other hand, IL-3 is likely to play a regulatory role in inflammatory conditions through its activation of mature cell function. For example, IL-3 can affect the migration of monocytes to sites of inflammation by modulating their adherence to endothelial cells (12), and through its enhancement of histamine release on basophils, IL-3 can significantly exacerbate allergic reactions (13, 14).

Despite its multiple biological activities, it is not clear how IL-3 exerts its effects on various cell types. Cell surface receptors for IL-3 have been identified on several primary cells (14–17). However, information regarding residues of IL-3 required for binding to these receptors and for eliciting a biological response is scarce. This type of information is essential for understanding the structural basis for the multiple biological activities of IL-3 and for the design of more potent agonists and of antagonists that can block the effect of IL-3 in situations such as allergic reactions. Recent experiments have shown that residues 33 and 111 are important for the proliferative activity of IL-3 on the MO-7 cell line (18, 19), suggesting that different regions of the IL-3 molecule can participate in function. We show here that specific substitutions in the N terminus of IL-3 lead to loss of multiple functions and binding, whereas substitutions in the C terminus lead to a decrease or increase in activities. Significantly, we found that the double mutant [Ala^{101},Val^{116}]IL-3 exhibited 15-fold greater biological and binding activities. This region of IL-3 may constitute a useful focus for the design and construction of further IL-3 analogs with more powerful agonistic or with antagonistic activities.

MATERIALS AND METHODS

Site-Directed Mutagenesis of Human IL-3. Human IL-3 mutants were constructed by either site-directed mutagenesis (20) on a synthetic human IL-3 cDNA in an M13 vector (21) or polymerase chain reaction (PCR) mutagenesis (22). A two-part PCR using three primers (22) was used to create mutants in the expression vector pJLA$^+$IL-3 as described (21). IL-3 analogs created by site-directed mutagenesis were subcloned into *Bam*HI–*Eco*RI sites of pJL4 (21). Wild-type IL-3 and the mutant [Ala^{101},Val^{116}]IL-3 were also subcloned, using a PCR to generate *Hin*dIII and *Bam*HI sites, into the *Escherichia coli* expression vector pINIIIOmpH$_3$ (gift of R. Kastelaine and A. Shanafelt, DNAX), a derivative of the vector pINIIIOmpA2 (23). For each IL-3 analog, the complete cDNA was sequenced to confirm the presence of the desired mutation and the absence of other mutations that could have been introduced by PCR.

Expression of IL-3 and Its Analogs. IL-3 analogs in the pJL4 expression vector were transiently transfected in COS cells as described (24). Wild-type IL-3 and the analog [Ala^{101},Val^{116}]IL-3 were expressed in the *E. coli* system after induction by isopropyl β-D-thiogalactoside; protein was recovered from the periplasmic space by osmotic shock (25) and partially purified by gel filtration. IL-3 protein detection was carried out by Western blot analysis using a rabbit anti-human IL-3 (gift from S. Clark, Genetics Institute, Cambridge, MA) and visualized by autoradiography after the addition of ^{125}I-labeled protein A (26).

Quantitation of IL-3 Protein. The amount of IL-3 protein present in COS cell supernatants was quantitated by a competitive radioimmunoassay (RIA) using human IL-3 (a gift from L. Park, Immunex, Seattle) labeled with ^{125}I and an anti-IL-3 serum (gift from S. Clark, Genetics Institute) as

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Abbreviations: IL-3, interleukin 3; CML, chronic myeloid leukemia; GM-CSF, granulocyte–macrophage colony-stimulating factor.

described (27). Wild-type IL-3 and [Ala^{101},Val^{116}]IL-3 produced in *E. coli* were also quantitated directly by scanning densitometry (28). Briefly, wild-type IL-3, [Ala^{101},Val^{116}]IL-3, or RNase standards were electrophoresed over a concentration range of 0.5–5 μg, and the gel then was stained with Coomassie brilliant blue R250 and analyzed using an LKB-Pharmacia Ultrascan XL scanning laser densitometer. Data analysis was performed with GSXL densitometer software. The protein concentrations of the unknown samples were calculated using the area under the peak. In some cases direct protein quantitation was also performed by HPLC peak integration by calculating the area under the IL-3 peak using the extinction coefficient of 0.83 arbitrary units·ml·mg^{-1}. An IL-3 preparation (gift from Genetics Institute) at 0.6 μg/ml (by amino acid analysis) measured 0.59 ± 0.1 μg/ml (mean ± SD) by scanning laser densitometry and 0.6 ± 0.07 μg/ml by RIA. In parallel, an [Ala^{101},Val^{116}]IL-3 concentration of 1.45 ± 0.06 μg/ml by scanning laser densitometry compared with 1.32 ± 0.08 μg/ml by HPLC peak integration and 1.35 ± 0.2 μg/ml by RIA.

Stimulation of Hemopoietic Cell Proliferation. Two types of assay were performed:

(*i*) *Colony assay.* CD34-positive bone marrow cells were purified with the monoclonal antibody MY10 and anti-mouse IgG antibodies coupled to magnetic beads (Dynard, Oslo); 10^3 cells were cultured in the presence of IL-3 analogs.

(*ii*) *Proliferation of chronic myeloid leukemic (CML) cells.* CML cells incorporated [^{3}H]thymidine in response to IL-3 as described (29). Data are expressed in cpm, and each point is the mean of six replicates.

Stimulation of Human Monocyte and Basophil Function. Monocytes were purified from the peripheral blood of normal donors as described (16) and IL-3-mediated adhesion was measured by an isotopic method as described (12). Basophil histamine release was measured as described (14).

Radioreceptor Assay. Human IL-3 (gift from L. Park, Immunex) was radioiodinated by the ICl method (30) and used in competitive binding experiments as described (16, 31). The results are expressed as percent competition, where 100% is the competition observed in the presence of a 100-fold excess of wild-type IL-3.

Statistical Analysis. Each mutant was tested over a range of concentrations using three to six replicates per point, and the concentration of IL-3 analog giving 50% maximal response was determined. Percent potency was calculated by dividing the concentration of wild-type IL-3 giving 50% maximal response by the concentration of IL-3 mutant giving 50% maximal response × 100%. Significance was established by one-way analysis of variance using the 50% values of at least three experiments for each assay.

RESULTS

Predictive studies of human IL-3 structure (32, 33) indicated that this molecule contains four α-helices and several hydrophilic regions (Fig. 1). Since these regions have been implicated in function and receptor binding of related growth factors such as GM-CSF (24, 34–36) and growth hormone (37), they were targeted for mutagenesis. IL-3 cDNA mutants were expressed transiently in COS cells, and Western blot analysis revealed that IL-3 mutants and wild-type IL-3 exhibited similar size and levels of glycosylation (data not shown).

Biological Analysis of N-Terminal Mutations. Mutation of residues Asp^{21}, Glu^{22}, and Thr^{25} located in a hydrophilic region of the predicted first α-helix of IL-3 to Ala, Leu, and Ala, respectively, was predicted to reverse the hydropathy of this region (Fig. 1). The two IL-3 mutants tested, [Ala^{21},Leu^{22}]IL-3 and [Ala^{21},Leu^{22},Ala^{25}]IL-3 were unable to stimulate the proliferation of CML cells, monocyte adherence, and histamine release from basophils (Table 1) or to bind to the high-affinity IL-3 receptor of monocytes (Table 2).

Mutations in a different hydrophilic region comprising residues Asp^{44}, Gln^{45}, and Asp^{46} also led to a predicted inversion of hydropathy. In this case, the IL-3 mutants [Ala^{44},Ala^{46}]- and [Ala^{44},Leu^{45},Ala^{46}]IL-3 exhibited properties similar to wild-type IL-3 in stimulating CML cell proliferation, monocyte adherence, and basophil histamine release (Table 1).

Biological Analysis of C-Terminal Mutations. Initial deletion mutagenesis revealed that the 13 most C-terminal residues of IL-3 were not essential for activity (Table 1). However, deletions into the predicted fourth α-helix illustrated by analogs IL-3-(1–118) and -(1–116) caused a dramatic decrease in activity (data not shown), emphasizing the essential nature of this helix for IL-3 function. Substitution mutagenesis within and adjacent to the fourth α-helix resulted in IL-3 analogs with modified activities. Replacement of Asp^{101} with Ala caused a decrease in the predicted hydrophilic nature of

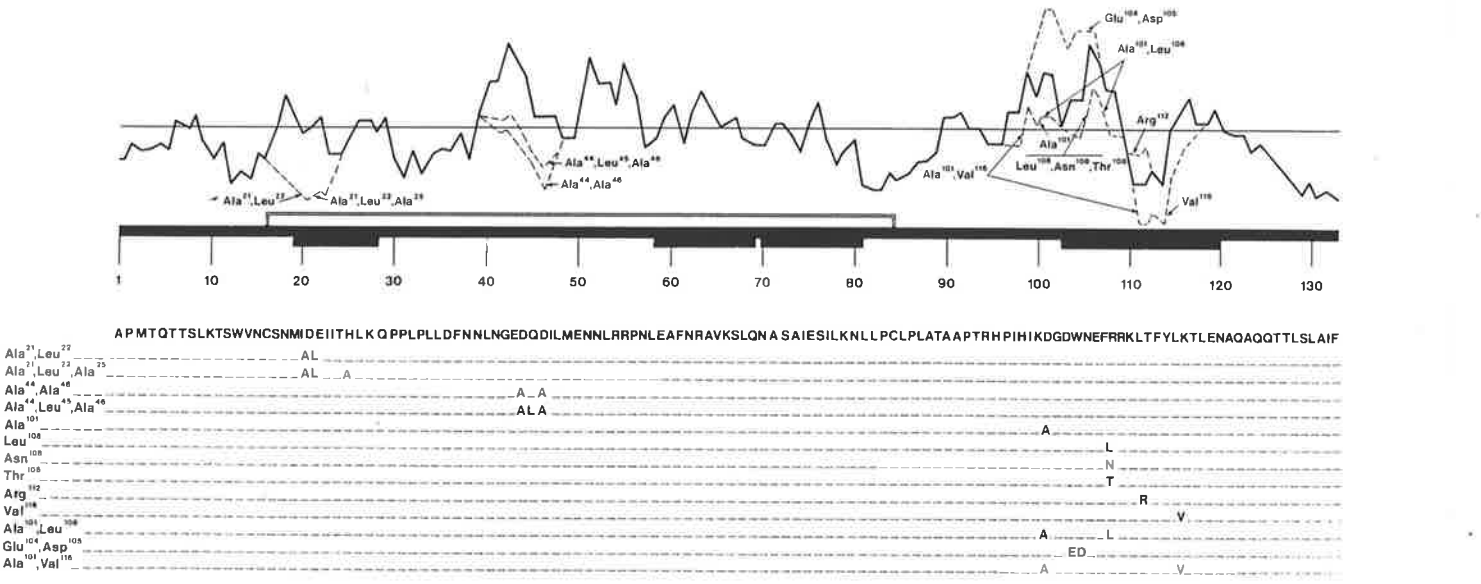

FIG. 1. Hydropathy profile and α-helix prediction of human IL-3. Hydropathy profile was determined using the method of Hopp and Woods (32), and the α-helical boundaries were based on heptad symmetry predictions (33). The four α-helices are represented by the solid rectangular boxes. The open double line joining Cys^{16} to Cys^{84} indicates the disulfide bridge. The IL-3 analogs generated by site-directed mutagenesis and the predicted changes to the hydropathy profile are indicated.

Table 1. Relative potencies of human IL-3 analogs

Analog	Proliferation of CML cells		Monocyte adherence		Basophil histamine release	
	% potency	*P* value	% potency	*P* value	% potency	*P* value
N-terminal mutations						
[Ala^{21},Leu^{22}]IL-3	<10	<0.01	<10	<0.01	<10	<0.01
[Ala^{21},Leu^{22},Ala^{25}]IL-3	<10	<0.01	<10	<0.01	<10	<0.01
[Ala^{44},Ala^{46}]IL-3	103 (1.4)	NS	77 (2.0)	NS	116 (1.6)	NS
[Ala^{44},Leu^{45},Ala^{46}]IL-3	72 (1.6)	NS	81 (1.9)	NS	136 (1.8)	NS
C-terminal mutation(s)						
IL-3-(1–120)	148 (2.1)	NS	ND		181 (1.5)	0.04
[Ala^{101}]IL-3	186 (1.4)	0.05	305 (1.7)	<0.02	259 (2.3)	0.02
[Leu^{108}]IL-3	40 (1.8)	<0.01	49 (1.8)	<0.01	49 (1.2)	<0.05
[Asn^{108}]IL-3	21 (2.2)	0.05	ND		ND	
[Thr^{108}]IL-3	15 (3.2)	<0.01	ND		ND	
[Arg^{112}]IL-3	70 (1.4)	NS	225 (n = 1)		110 (2.2)	NS
[Val^{116}]IL-3	235 (1.4)	0.01	590 (1.9)	<0.01	290 (2.6)	<0.01
[Glu^{104},Asp^{105}]IL-3	24 (1.6)	<0.01	45 (1.2)	<0.01	5 (2.2)	<0.01
[Ala^{101},Leu^{108}]IL-3	<10	<0.01	15 (n = 1)		14 (2.6)	<0.05
[Ala^{101},Val^{116}]IL-3	1153 (1.1)	<0.01	1480 (2.0)	<0.01	1437 (1.5)	<0.01

Percent potency was calculated by dividing the concentration of wild-type IL-3 giving 50% maximal response by the concentration of IL-3 mutant giving 50% maximal response × 100. The numbers in parentheses indicate the SD of the mean after logarithmic transformation. The *P* value represents the level of significance between IL-3 analogs and wild-type IL-3 obtained by performing one-way analysis of variance. ND, not determined; NS, not significant.

this region as did the replacement of Arg^{108} with Leu, Asn, or Thr (Fig. 1). However, whereas the mutant [Ala^{101}]IL-3 showed a 2- to 3-fold enhancement in IL-3 function, the mutants [Leu^{108}]IL-3, [Asn^{108}]IL-3, and [Thr^{108}]IL-3 exhibited a 2- to 5-fold decrease in biological activity (Table 1). The double mutant [Ala^{101},Leu^{108}]IL-3 exhibited low activity, indicating the dominant effect of position 108. This may reflect the fact that position 108 but not position 101 is predicted to be part of the fourth α-helix and thus is less tolerant to substitution mutagenesis. Replacement of residues Trp^{104} and Asn^{105} with Glu and Asp, respectively, led to an IL-3 analog with predicted higher hydrophilicity in this region (Fig. 1) but with significantly decreased biological activity (Table 1). Substitutions in a predicted hydrophobic region caused either no change in function as illustrated by [Arg^{112}]IL-3 or a 3- to 6-fold increase in biological activity as shown by [Val^{116}]IL-3.

Binding Properties of IL-3 Analogs. The results showed a correlation between biological activity and high-affinity binding (Table 2). The inactive analogs [Ala^{21},Leu^{22}]IL-3 and [Ala^{21},Leu^{22},Ala^{25}]IL-3 did not show detectable binding; the mutants with decreased biological activity [Glu^{104},Asp^{105}]IL-3 and [Leu^{108}]IL-3 showed decreased binding; the mutants with unaltered activity IL-3-(1–120) and [Arg^{112}]IL-3 showed similar K_d values; and the two analogs [Ala^{101}]IL-3 and [Val^{116}]IL-3 that were more active than wild-type IL-3 also exhibited increased binding affinity (Table 2). These two mutations were then incorporated into a single molecule and tested in biological and binding assays.

Table 2. Binding properties of human IL-3 analogs tested on the high-affinity IL-3 receptors of human monocytes

Analog	n	K_d	*P* value
Wild type	8	1.15×10^{-11} M	
[Ala^{21},Leu^{22}]IL-3	4	—	
[Ala^{21},Leu^{22},Ala^{25}]IL-3	3	—	
[Ala^{44},Ala^{46}]IL-3	1	5×10^{-11} M	
IL-3-(1–120)	3	1.8×10^{-11} M	NS
[Ala^{101}]IL-3	5	7.5×10^{-12} M	<0.05
[Leu^{108}]IL-3	1	3×10^{-11} M	
[Arg^{112}]IL-3	1	1.3×10^{-11} M	
[Val^{116}]IL-3	5	5.4×10^{-12} M	<0.05
[Glu^{104},Asp^{105}]IL-3	1	1.04×10^{-10} M	
[Ala^{101},Val^{116}]IL-3	5	8.2×10^{-13} M	<0.01

n, Number of experiments; NS, not significant. The K_d values were calculated from a titration curve using increasing concentrations of IL-3 analogs to compete for the binding of 100 pM ^{125}I-labeled IL-3 by using the LIGAND program. Each concentration of competitor was tested in duplicate; 2–4 × 10^6 monocytes per point were used. *P* values were calculated as described in Table 1. —, No competition was observed up to a 100-fold excess concentration of IL-3 mutant.

Biological and Binding Properties of [Ala^{101},Val^{116}]IL-3. The mutant [Ala^{101},Val^{116}]IL-3 was 10- to 20-fold more potent than wild-type IL-3 at stimulating CML cell proliferation (Fig. 2*A* and Table 1) and bone marrow colonies (Fig. 2*B*). Greater potency by [Ala^{101},Val^{116}]IL-3 was also observed in the stimulation of monocyte adherence (Fig. 3*A* and Table 1) and basophil histamine release (Fig. 3*B* and Table 1). This increased potency was reflected in the increased ability of [Ala^{101},Val^{116}]IL-3 to recognize the high-affinity IL-3 receptor of monocytes (Table 2).

Because high-affinity IL-3 receptors contain a low-affinity receptor binding chain and a convertor β chain (38), it was important to establish whether [Ala^{101},Val^{116}]IL-3 was exhibiting increased affinity as a result of increased binding to the low-affinity IL-3 component. To determine this, we used ^{125}I-labeled IL-3 at 2 nM, a concentration at which the majority of occupied receptors in human monocytes are of the low-affinity class (16, 31). For these experiments, [Ala^{101},Val^{116}]IL-3 and wild-type IL-3 were expressed in *E. coli*, partially purified by gel filtration, and tested in conjunction with purified *E. coli*-derived IL-3 (gift from S. Clark, Genetics Institute) for their ability to inhibit the binding of 2 nM ^{125}I-labeled IL-3 to human monocytes. A quantitative inhibition binding experiment showed that [Ala^{101},Val^{116}]IL-3 has a 15-fold greater affinity than wild-type IL-3 in binding to human monocytes under low-affinity conditions (Fig. 4).

DISCUSSION

We show here the construction of a human IL-3 analog with increased biological and binding properties. [Ala^{101},Val^{116}]IL-3 exhibited an ≈15-fold greater potency than native IL-3 at stimulating bone marrow cells to form day-14 colonies, leukemic cell proliferation, and monocyte adhesion and at enhancing the release of histamine from basophils. Paralleling

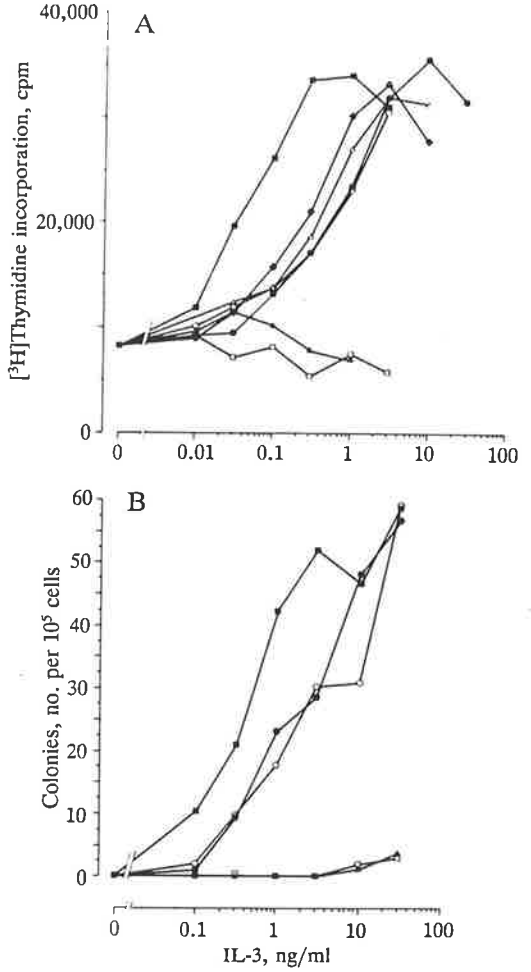

FIG. 2. Titration of [Ala^{101},Val^{116}]IL-3 and other IL-3 analogs for their ability to stimulate cell proliferation. (*A*) [^{3}H]Thymidine incorporation in CML cells. Each value is the mean of six replicates. (*B*) Bone marrow colony formation after 14 days in culture. Each value represents the mean of three replicates. ●, Wild-type IL-3; ◆, [Ala^{101}]IL-3; △, [Val^{116}]IL-3; ■, [Ala^{101},Val^{116}]IL-3; ○, [Arg^{112}]IL-3; □, [Ala^{21},Leu^{22},Ala^{25}]IL-3; ▲, [Ala^{21},Leu^{22}]IL-3.

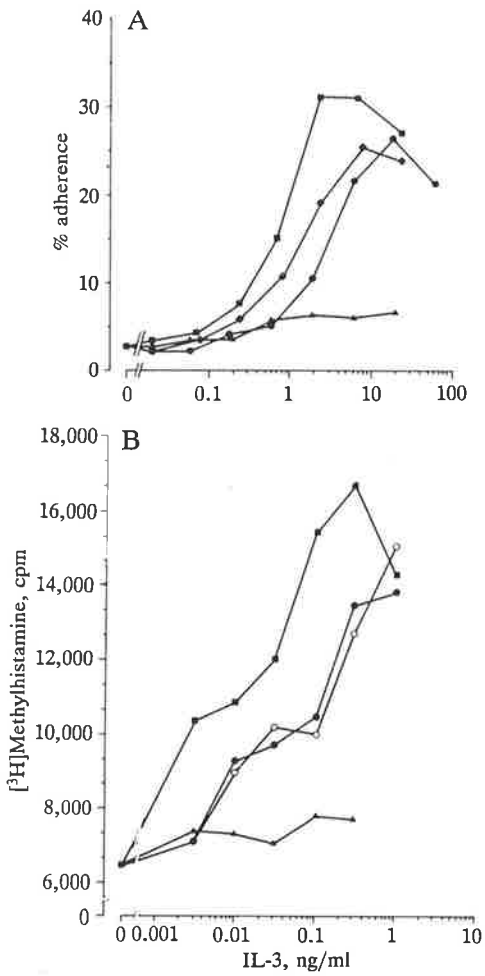

FIG. 3. Titration of [Ala^{101},Val^{116}]IL-3 and other IL-3 analogs for their ability to stimulate monocyte adherence (*A*) and basophil histamine release (*B*). The values represent the mean of five replicates. ●, Wild-type IL-3; ◆, [Ala^{101}]IL-3; ■, [Ala^{101},Val^{116}]IL-3; ▲, [Ala^{21},Leu^{22}]IL-3; ○, [Arg^{112}]IL-3.

this increased function, [Ala^{101},Val^{116}]IL-3 also bound with 15-fold greater affinity to the high-affinity and low-affinity IL-3 receptors of monocytes.

These results show that the potential to generate IL-3 analogs with increased potency appears to be restricted so far to substitution of residues at positions 101 and 116, inasmuch as mutations at positions 104, 105, and 108 (Tables 1 and 2) and replacement of residues Lys^{110} or Leu^{111} (19) and Glu^{106} (39) greatly reduce the potency of IL-3. These results are consistent with other structure–function studies on IL-3 (40) and on GM-CSF and IL-5 where the C terminus has been shown to be required for functional integrity (34–36, 41) and raise the possibility that analogous mutations to [Ala^{101},Val^{116}]IL-3 in GM-CSF and IL-5 may also lead to more potent agonists.

The N terminus of IL-3 is also likely to contribute to function and binding. Replacements and deletions in the N terminus of IL-3 result in decreased activity (18, 39) except for one IL-3 analog, [Gly^{33}]IL-3, that exhibited a 14-fold enhanced biological activity, albeit without increased binding capacity. The results shown here with the analogs [Ala^{21},Leu^{22},Ala^{25}]IL-3 and [Ala^{21},Leu^{22}]IL-3 showing greatly reduced IL-3 binding and activity also illustrate the involvement of the N terminus. On the other hand, the analogs [Ala^{44},Leu^{45},Ala^{46}]IL-3 and [Ala^{44},Ala^{46}]IL-3 showed similar potency to wild-type IL-3 (Table 1). This difference may reflect different structural constraints in that positions 21 and 22 are predicted to be part of an α-helix and positions 44–46 are not (Fig. 1) or that residues 21 and 22 are directly involved in binding and function. In support of the latter, it is worth noting that both IL-3 and GM-CSF interact with their respective specific α chains (38, 42) and with a β chain that is common to both ligands (38, 43). Glu^{22} is in a predicted exposed face of the first α-helix of IL-3 and is in an analogous position to Glu^{21} in GM-CSF (33). Since the mutation of Glu^{21} in GM-CSF impaired binding to the β chain while fully retaining binding to the α chain (24), it is likely that similar substitutions at position 22 in IL-3 will also selectively affect binding to the β chain.

The mechanism by which [Ala^{101},Val^{116}]IL-3 stimulates function with greater potency is not known but is probably related to its increased binding to high-affinity IL-3 receptors (Table 2). Since these are composed of at least two chains, a low-affinity α chain and a convertor β chain (38), it was important to determine to which component of [Ala^{101},Val^{116}]-IL-3 was preferentially binding. The finding that in monocytes, and under low-affinity conditions, [Ala^{101},Val^{116}]IL-3 exhibited a 15-fold higher affinity than wild-type IL-3 suggests that the mutated residues are involved in binding to the IL-3 receptor α chain.

The construction of the more potent analog [Ala^{101},-Val^{116}]IL-3 has important implications. (*i*) It argues that the

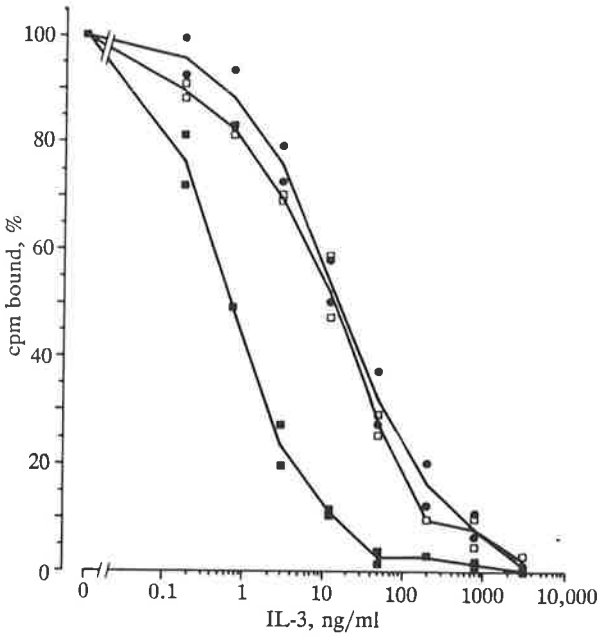

FIG. 4. Competition for binding to low-affinity IL-3 receptors of monocytes by partially purified *E. coli*-derived wild-type IL-3 (● and □) and [Ala^{101},Val^{116}]IL-3 (■). The purified wild-type IL-3 (□) was a gift from S. Clark. ^{125}I-labeled IL-3 was used at 2 nM. Duplicate determinations were performed with 4×10^6 monocytes per point.

C-terminal region of IL-3 and probably of related cytokines such as GM-CSF and IL-5 are involved in binding to low-affinity (α chain) receptors. (*ii*) This type of mutant with increased potency may be useful in situations such as hemopoietic reconstitution where clinical trials are already showing a beneficial effect of IL-3 (44). (*iii*) The identification of residues involved in IL-3 binding to the receptor α chain may facilitate the construction of IL-3 antagonists for use in allergic reactions where IL-3 can exacerbate these conditions (45) and in follicular cell tumors where IL-3 binds and promotes their growth (46).

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Biochemistry

Nuclear proteins interacting with the promoter region of the human granulocyte/macrophage colony-stimulating factor gene

(DNA-binding proteins/gene expression/tissue specificity/growth factors/hemopoiesis)

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ABSTRACT **The gene for human granulocyte/macrophage colony-stimulating factor (GM-CSF) is expressed in a tissue-specific as well as an activation-dependent manner. The interaction of nuclear proteins with the promoter region of the GM-CSF gene that is likely to be responsible for this pattern of GM-CSF expression was investigated. We show that nuclear proteins interact with DNA fragments from the GM-CSF promoter in a cell-specific manner. A region spanning two cytokine-specific sequences, cytokine 1 (CK-1, 5′ GAGATTC-CAC 3′) and cytokine 2 (CK-2, 5′ TCAGGTA 3′) bound two nuclear proteins [nuclear factor (NF)-GMa and NF-GMb] from GM-CSF-expressing cells in gel retardation assays. NF-GMb was inducible with phorbol 12-myristate 13-acetate and accompanied induction of GM-CSF message. NF-GMb was absent in cell lines not producing GM-CSF, some of which had other distinct binding proteins. NF-GMa and NF-GMb eluted from a heparin-Sepharose column at 0.3 and 0.6 M KCl, respectively. We hypothesize that the sequences CK-1 and CK-2 bind specific proteins and regulate GM-CSF transcription.**

Human granulocyte/macrophage colony-stimulating factor (GM-CSF) is a 22-kDa glycoprotein that stimulates the formation of granulocyte, macrophage, granulocyte/macrophage, and eosinophil colonies from normal bone marrow progenitor cells *in vitro* (1). GM-CSF has direct action also on the function of mature peripheral blood granulocytes (2, 3). The gene encoding human GM-CSF is 2.5–3 kilobases long (4, 5) and maps to the long arm of chromosome 5 (6).

Antigen- or mitogen-activated T cells and T-cell lines produce relatively high levels of GM-CSF (7–9). Other cytokines such as interleukin 1 and tumor necrosis factor activate the expression of the GM-CSF gene in endothelial cells (10). Primary human stromal cells can also be induced to produce GM-CSF (11).

It appears that there may be multiple forms of control of GM-CSF production, both at a transcriptional and posttranscriptional level. mRNA stability has been shown to be involved in controlling the induction of mouse GM-CSF in macrophages (12), and a sequence in the 3′-untranslated region is responsible for the instability of GM-CSF mRNA (13). It has been reported (14) that a 650-base-pair (bp) fragment from the promoter region of the human GM-CSF gene was involved in T-cell-specific expression of the gene and was only active in phytohemagglutinin/phorbol 12-myristate 13-acetate (PMA)-stimulated T cells.

Computer analysis of the mouse and human GM-CSF genes has shown that the most highly conserved sequences are in the promoter region and in the 3′-noncoding sequence of the mRNA (5), indicating the potential importance of these regions in the regulation of expression of these genes. Sequences shared with other cytokines can also be found especially in the promoter region of the genes (5, 15, 16). Sequences within this promoter region, especially those sequences shared with other cytokine genes, may be the binding sites for nuclear proteins that confer cell specificity or inducibility on the GM-CSF gene.

In this study, we have examined the interaction of the GM-CSF promoter with nuclear proteins from cells that express GM-CSF and from cells where GM-CSF is not produced. We have also used synthetic oligonucleotides to examine the interaction of nuclear proteins with conserved sequences in the GM-CSF promoter. We report cell-specific interactions with promoter regions of the GM-CSF gene.

MATERIALS AND METHODS

DNA Probes. A 650-bp *Pst* I fragment from the promoter region of the human GM-CSF gene (Fig. 1*a*) was cloned into pUC18 and was the source of all fragments used in gel retardation assays (the GM-CSF genomal clone was a gift from J. Gasson, School of Medicine, University of California, Los Angeles). The fragments routinely used were a 445-bp *Sac* I fragment that was end-labeled with T4 DNA polymerase (Pharmacia, Uppsala, Sweden) (17) and a 199-bp *Dde* I–*Sac* I fragment derived from the 3′ end of the larger *Sac* I fragment (Fig. 1*a*) that was radiolabeled by end-filling with the Klenow fragment of DNA polymerase 1 (Bresa, Adelaide, Australia) (17).

Two complementary 41-bp oligonucleotides were synthesized with *Eco*RI ends (Fig. 1*b*). Each oligonucleotide was end-labeled with [γ-^{32}P]ATP (Bresa) and polynucleotide kinase (Pharmacia). The radiolabeled oligonucleotides were annealed by heating to 100°C for 3 min in 25 mM Tris·HCl, pH 7.6/150 mM NaCl and then cooling at room temperature for 15 min. Unlabeled oligonucleotides were also annealed as described above to give a final concentration of 10 ng/μl and used as specific competitors in the binding reactions.

Cell Lines. U5637 is a human bladder carcinoma cell line that constitutively produces GM-CSF and granulocyte colony-stimulating factor (G-CSF) (18). HUT78 is a T-lymphoblastoid cell line derived from a patient with Sezary syndrome (19). SP2/10-Ag14 is a mouse myeloma cell line (20) and LiBr is a human melanoma cell line (21). GM-CSF mRNA can be detected in U5637 and HUT78 cells but not in LiBr and SP2 cells (M.F.S., unpublished results).

All cell lines were routinely grown in RPMI medium supplemented with 10% (vol/vol) fetal calf serum. Cells were harvested at 10^6 cells per ml or at 80% confluence for nonadherent and adherent cell lines, respectively, following treatment for 6 hr with PMA at 10 ng/ml. Untreated cells were grown for the same period of time but without PMA.

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Abbreviations: GM-CSF, granulocyte/macrophage colony-stimulating factor; PMA, phorbol 12-myristate 13-acetate; G-CSF, granulocyte colony-stimulating factor; IL-3, interleukin 3; NF, nuclear factor.

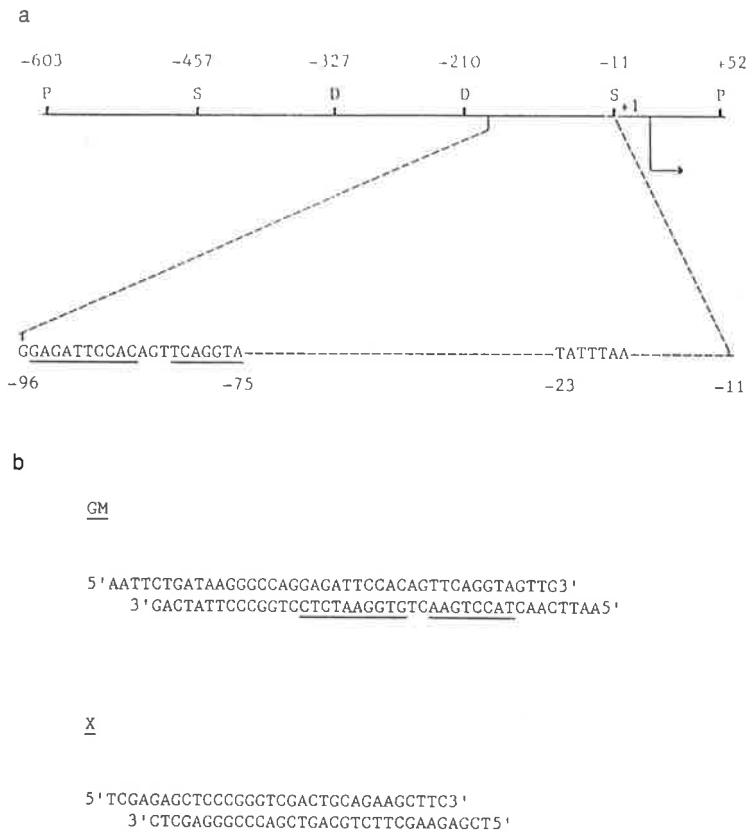

FIG. 1. (*a*) Restriction enzyme map of the promoter region of the human GM-CSF gene. The sequence is numbered from the start of transcription (+1). The sequence between the *Sac* I site at position −11 and position −96 is expanded to show both the sequence conserved between the cytokine genes and the TATA box sequence. P, *Pst* I; S, *Sac* I; D, *Dde* I. (*b*) Sequences of the 41-bp synthetic oligonucleotides (GM) that span the conserved cytokine sequence (GAGATTCCAC). Conserved regions are underlined. Sequence of the unrelated 32-bp oligonucleotide X is also shown.

Preparation of Nuclear Extracts. Nuclei were prepared as described by Dignam *et al.* (22). Nuclear proteins were extracted by constant agitation for 30 min with 0.5 M KCl at 4°C. Following centrifugation at 20,000 rpm for 30 min (Beckman rotor TL100.3), the supernatant was dialyzed against three changes of TM buffer containing 100 mM KCl for 12–16 hr. [TM buffer is 50 mM Tris·HCl (pH 7.6), 12.5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 20% (vol/vol) glycerol; ref. 23]. The protein extracts were stored at −70°C. Protein concentration was determined using the Bio-Rad protein assay (Bio-Rad).

Gel Retardation Assay. For binding reactions, 0.1–1.0 ng of radiolabeled fragment (5–10,000 cpm) was mixed with 1–3 μg of nuclear extract in a final volume of 20 μl containing 25 mM Tris·HCl (pH 7.6), 6.25 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol, 10% (vol/vol) glycerol, and 80–200 mM KCl. Poly(dI·dC) (0.5–3 μg) was used in all reactions as nonspecific competitor. Specific competitors were added to each reaction mixture as described in individual experiments. The reaction mixtures were analyzed on 5% polyacrylamide gels in low-ionic-strength buffer (24). The gels were preelectrophoresed at 20 V/cm for 2 hr and electrophoresed at the same voltage for 1–2 hr. Following electrophoresis the gels were dried and autoradiographed either overnight or for 1–2 days.

Binding reactions with synthetic oligonucleotides were as described above. The retardation patterns were analyzed on 11% polyacrylamide gels in 0.5× TBE (1.0× TBE = 50 mM Tris borate, pH 8.3/1 mM EDTA).

Heparin-Sepharose Column Chromatography. A 1-ml heparin-Sepharose (Pharmacia) column was equilibrated with TM buffer containing 100 mM KCl (TM.1). Six milligrams of crude nuclear extract from PMA-treated U5637 cells was loaded onto the column in the same buffer. Following extensive washing with TM.1, the bound protein was eluted in a stepwise fashion with 3 ml of 0.2 M, 3 ml of 0.3 M, and 3 ml of 0.6 M KCl in TM buffer. The eluates from each salt concentration were collected and dialyzed into TM.1, and the protein concentration was estimated with the Bio-Rad assay. The fractions were tested for binding activity to the synthetic oligonucleotides.

RESULTS

Specific Interaction of Nuclear Proteins with the Promoter Region of the GM-CSF Gene. A 445-bp *Sac* I fragment from the GM-CSF promoter region (Fig. 1*a*) was radiolabeled and mixed with nuclear extracts prepared from the U5637 bladder carcinoma cell line that had been treated for 6 hr with PMA at 10 ng/ml. Gel retardation assays resulted in a single retarded band (Fig. 2*A*), which was specifically blocked by competition with 100 ng of unlabeled *Sac* I fragment but not with 500 ng of poly (dI·dC) (Fig. 2*A*, compare lanes 2 and 4).

To define more precisely the promoter region responsible for the protein–DNA interaction, competition experiments were carried out with a number of smaller fragments spanning the 445-bp region. The binding could be successfully blocked by competition with a 199-bp *Dde* I–*Sac* I fragment (Fig. 2*A*, lane 6) comprising the region closest to the transcription initiation point (Fig. 1*a*). Fragments further upstream did not compete successfully in the retardation reactions. Fig. 2*A* (lanes 8 and 9) shows the inability of a 130-bp *Pst* I–*Sac* I fragment (positions −603 to −457) to block the binding by competition.

When binding reactions were carried out with radiolabeled 199-bp *Dde* I–*Sac* I fragment and nuclear extract from PMA-stimulated U5637 cells, one main retarded complex was observed (Fig. 2*B*, lane 1). A fainter complex, migrating closer to the free DNA was seen on longer exposure of the gels to x-ray film (Fig. 2*B*). The formation of these complexes could be specifically blocked by competition with 100 ng of unlabeled fragment (Fig. 2*B*, lane 2).

Comparison of Promoter DNA–Protein Interactions from Various Cell Lines. To determine if any proteins interacting

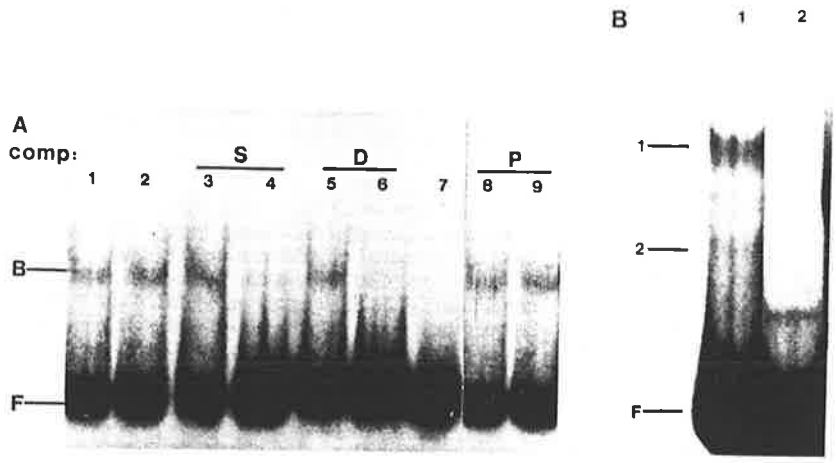

FIG. 2. Binding of nuclear proteins from PMA-treated U5637 cells to DNA fragments from the human GM-CSF gene. (*A*) Gel retardation assays using a 445-bp *Sac* I fragment. Radiolabeled fragment (1 ng) and 2 μg of nuclear protein were used in each reaction mixture: Lane 1 had 100 ng of poly(dI·dC), and lanes 2–7 had 500 ng of poly(dI·dC). The following competitor DNAs (comp.) were used. Lanes: 3, 10 ng of *Sac* I fragment (S); 4, 100 ng of S; 5, 10 ng of *Dde* I–*Sac* I fragment (D); 6, 100 ng of D; 7, no protein extract; 8, 10 ng of a 130-bp *Pst* I–*Sac* I fragment (P); and 9, 100 ng of P. B, DNA–protein complex; F, free DNA. (*B*) Gel retardation assay using a radiolabeled 199-bp *Dde* I–*Sac* I fragment and 2 μg of nuclear protein. Lanes: 1, no competitor; 2, 100 ng of *Dde* I–*Sac* I fragment. Each lane had 500 ng of poly(dI·dC). Autoradiographic exposure was 2 days.

with the 445-bp or 199-bp GM-CSF promoter fragments are cell-type-specific, we made nuclear protein extracts from U5637, HUT78, and SP2 cell lines treated for 6 hr with PMA at 10 ng/ml. Fig. 3*A* shows the retarded bands obtained with extracts from each cell line following binding to the 445-bp *Sac* I fragment. U5637 and HUT78 extracts show the same retarded bands, with the major band corresponding to the U5637 band described above (band 3). A weaker band migrating higher on the gel is also visible in both extracts (band 2). The SP2 extract, however, shows a retarded band (band 1) that migrates higher on the gel than either retarded complex from the other cell lines.

We also examined the interaction between these extracts and the smaller 199-bp fragment. The three cell lines examined showed different retardation patterns (Fig. 3*B*). The main retarded band observed with U5637 cell extracts (band 1) was also present in extracts from the other two cell lines but at reduced levels. HUT78 cell extracts generated a different band (band 2) as did SP2 cell extracts (band 3). Band 2 was seen in some U5637 cell extracts. These results show that each of these cell lines contains a distinct set of proteins capable of interacting with the GM-CSF promoter.

A Conserved Cytokine-Specific Sequence Binds Nuclear Proteins. Two complementary oligonucleotides (each 41 bp long) spanning the sequence 5′ GAGATTCCAC 3′ (Fig. 1*b*), were synthesized to investigate the interaction of nuclear

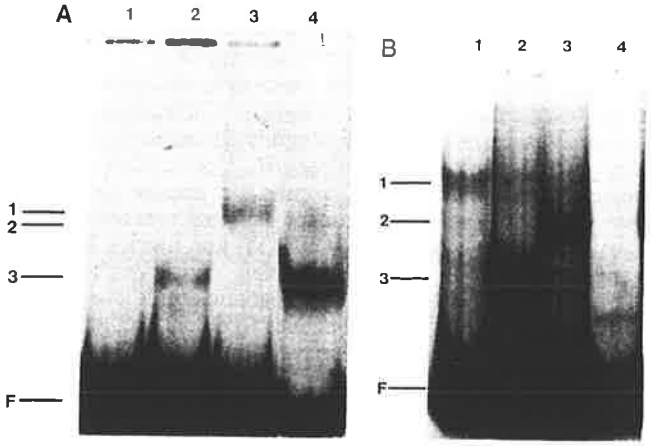

FIG. 3. Gel retardation patterns generated with various cell line extracts. (*A*) Each reaction mixture contained 1 ng of radiolabeled 445-bp *Sac* I fragment, 2.5 μg of nuclear protein, and 1.5 μg of poly(dI·dC). Retarded complexes are numbered, and free DNA fragment is labeled F. Lanes: 1, no protein; 2, U5637 extract; 3, SP2 extract; 4, HUT78 extract. (*B*) Binding reactions were as described in *A* but with the radiolabeled 199-bp *Dde* I–*Sac* I fragment. Lanes: 1, U5637 extract; 2, SP2 extract; 3, HUT78 extract; 4, no protein.

proteins with this sequence. Two specific retarded complexes, labeled a and b in Fig. 4*A*, were generated with extracts from PMA-stimulated U5637 cells. These complexes will be referred to as nuclear factor (NF)-GMa and NF-GMb. The formation of these two complexes could be completely blocked by competition with 50 ng of unlabeled annealed oligonucleotides (Fig. 4*A*, lane 5) but not with the same concentration of an unrelated oligonucleotide (Fig. 4*A*, lane 10) or not with 3 μg of poly(dI·dC) (data not shown). The apparent enhancement of NF-GMa and NF-GMb seen here with increasing concentrations of the nonspecific competitor is not reproducible between experiments. Other retarded complexes migrating higher on the gel or closer to the free DNA are not consistently observed, and their formation cannot be blocked by competition with increasing concentrations of specific competitor (Fig. 4*A*). Identical results were obtained with PMA-stimulated HUT78 extracts (data not shown). Increasing the salt concentration in the binding reactions from 80 mM to 200 mM greatly reduces this nonspecific interaction and enhances the specific interactions by ≈3-fold (data not shown). Subsequent binding reactions were, therefore, carried out at 200 mM KCl.

The two retarded complexes could result either from interaction with multimers of the same protein or with two distinct proteins. The nuclear extract from U5637 cells was fractionated on a heparin-Sepharose column. The proteins eluted from the column with 0.1 M, 0.2 M, 0.3 M, and 0.6 M KCl were tested in retardation assays (Fig. 4*B*). The protein(s) responsible for the NF-GMa complex eluted from the column with 0.3 M KCl and that responsible for NF-GMb eluted with 0.6 M KCl (Fig. 4*B*, lanes 5 and 6 and lanes 7 and 8, respectively), suggesting the involvement of two distinct proteins in these complexes.

Cell-Specific Interactions with the Conserved Cytokine Sequence. We have compared the retardation band patterns obtained with extracts prepared from U5637, HUT78, LiBr, and SP2 cell lines. The extracts were prepared from cells treated for 6 hr with PMA at 10 ng/ml. Both of the specific retarded bands NF-GMa and NF-GMb were obtained using the radiolabeled oligonucleotides and extracts from PMA-treated U5637 and HUT78 cells (Fig. 5*A*, lanes 1 and 2), although HUT78 cell extracts always yielded a 3- to 4-fold higher concentration of the proteins involved in both these complexes. Extracts from the SP2 cell line did not result in either of the specific complexes but gave a diffuse retarded band migrating above NF-GMa (Fig. 5*A*, lane 3). This interaction is blocked by competition with increasing concentrations of poly(dI·dC) (data not shown). Extracts from PMA-treated LiBr cells bound to the GM-CSF-specific oligonucleotide giving rise to NF-GMa but not NF-GMb. The amount of NF-GMa formed with extracts from LiBr

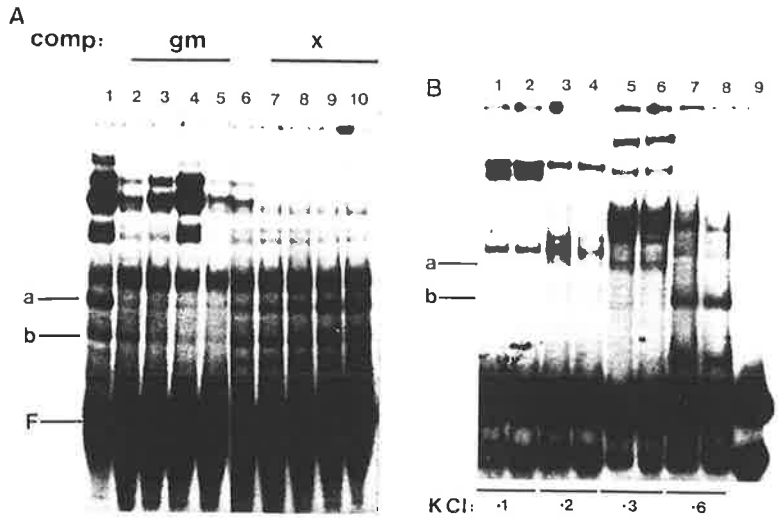

FIG. 4. Interaction of nuclear proteins from PMA-treated U5637 cells with synthetic oligonucleotides. (*A*) Competition assays to determine the specificity of binding to the synthetic 41-bp annealed oligonucleotides. Radiolabeled oligonucleotides (0.2 ng) were mixed with 2 μg of nuclear protein and 2 μg of poly(dI·dC). Increasing concentrations of the specific oligonucleotide (gm) (lanes 2–5) or of an unrelated oligonucleotide (x) (lanes 7–10) were added. Lanes: 1 and 6, no competitor; 2 and 7, 5 ng of competitor; 3 and 8, 10 ng of competitor; 4 and 9, 25 ng of competitor; 5 and 10, 50 ng of competitor. a and b, Specific complexes NF-GMa and NF-GMb, respectively; F, unbound oligonucleotide; comp., competitor. (*B*) Interaction of heparin-Sepharose-fractionated U5637 nuclear extracts with oligonucleotides. The KCl concentration (in M) used for elution is indicated under each lane. Lanes: 1, 3, 5, and 7, 80 mM KCl in the binding reactions; 2, 4, 6, and 8, 200 mM KCl in the binding reactions.

cells was always lower than that seen in U5637 cells by a factor of ≈3 (Fig. 5*A*, lane 4).

Effect of PMA Stimulation on the Formation of NF-GMa and NF-GMb DNA–Protein Complexes. To determine if PMA treatment induced the production of the proteins involved in the two cytokine-specific complexes, extracts were prepared from the U5637 and LiBr cell lines before and after PMA treatment. The retarded patterns obtained with these extracts indicate that in U5637 cells the protein involved in the NF-GMb complex was induced 5- to 10-fold (Fig. 5*B*, compare lanes 1 and 2). The induction seen here in the level of NF-GMa is not consistent between experiments. In LiBr cells this induction was not observed, and no change in the level of NF-GMa was seen (Fig. 5*B*, lanes 3 and 4).

DISCUSSION

The experiments described show that there are cell-specific as well as inducible nuclear proteins that interact with DNA fragments from the GM-CSF promoter.

Nuclear proteins from U5637 cells, a cell line that expresses GM-CSF mRNA, specifically bound to both a 445-bp fragment, spanning a large area of the promoter, and a 199-bp fragment in a region close to the transcription start site. The SP2 cell line, where GM-CSF mRNA is not detectable, has proteins that bound to the promoter region of the GM-CSF gene but formed a complex distinct from that found in U5637 or HUT78 cells, where mRNA for GM-CSF is detectable. Since the human and mouse GM-CSF promoters share >80% of the sequences in the first 350 bp, it seems valid to compare human and mouse cell lines. The presence of cell-specific DNA binding proteins has now been shown for a number of gene promoters where it has been hypothesized that these interactions are involved in cell-specific expression (24–28). It is possible then that the binding proteins from SP2 cells may be interacting with common promoter regions such as the TATA box, not involved in tissue-specific transcription, or with negative regulator regions of the promoter, such as those DNA-binding proteins shown to be involved in the negative regulation of the interferon β gene (29, 30). Some of the factors identified here that bind to the GM-CSF promoter may be involved in the inducible or tissue-specific nature of GM-CSF expression.

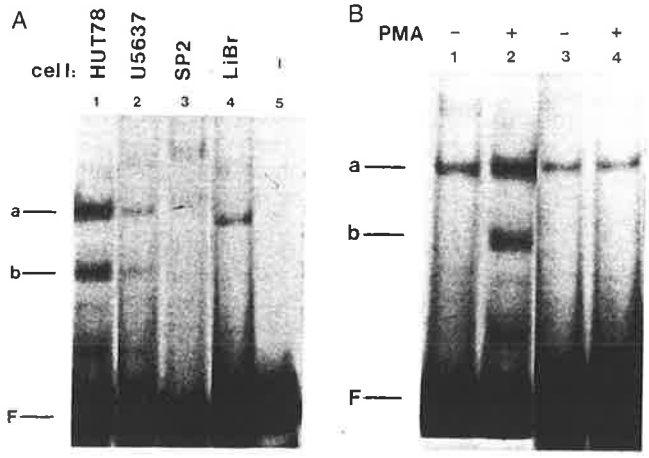

FIG. 5. (*A*) Interaction of nuclear proteins from various PMA-treated cell lines with the synthetic oligonucleotides. Extracts from PMA-treated HUT78 cells (2 μg, lane 1), U5637 cells (2 μg, lane 2), SP2 cells (6 μg, lane 3), and LiBr cells (6 μg, lane 4) were used with 0.2 ng radiolabeled oligonucleotides and no protein (lane 5) are shown. a and b, Specific complexes; F, unbound oligonucleotide. (*B*) Effect of PMA treatment on the formation of the NF-GMa and NF-GMb complexes. Lanes: 1, untreated U5637 cells; 2, PMA-treated U5637 cells; 3, untreated LiBr cells; 4, PMA-treated LiBr cells.

Comparison of the promoter regions from a number of cytokine genes has revealed some sequences that are conserved between these genes and across species (5, 14, 15). One decanucleotide sequence [cytokine 1 (CK-1)], 5′ GRGRTTYCAY 3′ (where R = A or G and Y = C or T) is found in both human and murine interleukin 2, interleukin 3 (IL-3), GM-CSF, and G-CSF genes (Fig. 6). In addition a second sequence [cytokine 2 (CK-2)], 5′ TCAGRTA 3′, lying on the 3′ side of the decanucleotide, is conserved in both human and murine GM-CSF and IL-3 genes (Fig. 6). This sequence is not found in human or murine G-CSF or interleukin 2. The CK-1 sequence is also repeated further upstream in the human GM-CSF (14) and murine IL-3 (32) genes but without the extra flanking conserved sequence (CK-2). We designed our 41-bp oligonucleotide probe spanning the CK-1 and CK-2 sequences to elucidate their role in nuclear protein binding. These two sequences are flanked by GM-CSF sequences that are not conserved in the other genes.

Two DNA–protein complexes that are specific for the 41-bp oligonucleotide spanning these conserved sequences were identified. It would appear that these two complexes are generated by two or more different proteins since the proteins involved in the NF-GMa and NF-GMb complexes are eluted from a heparin-Sepharose column at 0.3 M and 0.6 M KCl, respectively. It has yet to be determined which specific nucleotides are involved in the binding of these proteins.

GENE	SEQUENCE
	-96 -75
hGM-CSF (5)	GGAGATTCCACAGTTCAGGTA
	-108 -88
mGM-CSF (15)	GGAGATTCCACAacTCAGGTA
hIL-3 (31)	GGAGgTTCCAt-G-TCAGaTA
	-115 -97
mIL-3 (32)	GGAGgTTCCAt-G-TCAGaTA
	-198 -178
hIL-2 (33)	aGgGATTtCACctacatccat
	-208 -188
mIL-2 (34)	aGgGATTtCACctaaatccat
	-188 -168
hG-CSF (35)	aGAGATTCCACAaTTtcacaA
	-192 -172
mG-CSF (16)	aGAGATTCCcCgaTTtcacaA

FIG. 6. Conserved sequences found in the promoter region of cytokine genes. CK-1 and CK-2 sequences are underlined. All the sequences have been compared to the human GM-CSF sequence. Nonconserved bases are shown in lowercase letters. Numbering is relative to the transcription start site of each gene (+1). h, Human; m, murine; IL-2, interleukin 2. Numbers in parentheses after gene name refer to reference number in list of references.

The CK-1 sequence has been postulated to account for the coordinate expression of some of these genes in activated T cells (14, 15). However, G-CSF is not expressed in activated T cells (13) despite the fact that its promoter contains a copy of CK-1. It seems unlikely, therefore, that this sequence alone is responsible for T-cell expression of GM-CSF or IL-3. However, the extended conserved sequence of CK-1 and CK-2 noted above between GM-CSF and IL-3 genes may be involved in T-cell expression of these genes. It is noteworthy that IL-3 and GM-CSF are often coordinately expressed following Con A stimulation of murine T-cell clones (36).

Treatment of U5637 cells with PMA results in an increase in GM-CSF mRNA and an increase in the level of the NF-GMb complex but not NF-GMa. The ability to induce the NF-GMb complex in PMA-treated U5637 cells suggests that the protein(s) involved in the NF-GMb complex may be responsible for the inducibility of the GM-CSF gene. However, unstimulated cells also transcribe the GM-CSF gene but have undetectable levels of NF-GMb. Therefore, NF-GMb may not be essential for basal level transcription of the gene in this tumor cell line but could be involved in increasing transcriptional efficiency following stimulation. The situation observed here parallels that found with proteins binding to the octamer motif of the immunoglobulin genes (25, 26). Two proteins, one of which is lymphoid specific, bind to this motif. The lymphoid-specific protein is inducible with lipopolysaccharide (26), as is NF-GMb with PMA in U5637 cells. Extracts from the human melanoma cell line LiBr contain protein(s) that may be equivalent to NF-GMa, but NF-GMb could not be induced by PMA treatment of these cells. Nuclear proteins prepared from the mouse SP2 cell line did not bind specifically to the GM-CSF conserved sequence. It would appear, therefore, that the protein(s) that bind to this GM-CSF sequence are limited in their cellular distribution. The result is consistent with results (14) that show that a GM-CSF-chloramphenicol acetyltransferase fusion gene is expressed in activated T cells but not in a human B-lymphoblastoid cell line. The elucidation of the role of these protein–DNA complexes in GM-CSF transcription awaits analysis of the DNA fragments *in vivo* by using transfection assays.

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A Novel Tumor Necrosis Factor-Responsive Transcription Factor Which Recognizes a Regulatory Element in Hemopoietic Growth Factor Genes

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A conserved DNA sequence element, termed cytokine 1 (CK-1), is found in the promoter regions of many hemopoietic growth factor (HGF) genes. Mutational analyses and modification interference experiments show that this sequence specifically binds a nuclear transcription factor, NF-GMa, which is a protein with a molecular mass of 43 kilodaltons. It interacts with different affinities with the CK-1-like sequence from a number of HGF genes, including granulocyte macrophage colony-stimulating factor (GM-CSF), granulocyte (G)-CSF, interleukin 3 (IL-3), and IL-5. We show here that the level of NF-GMa binding is induced in embryonic fibroblasts by tumor necrosis factor-α (TNF-α) treatment and that the CK-1 sequence from the G-CSF gene is a TNF-α-responsive enhancer in these cells. The NF-GMa protein is distinct from another TNF-α-responsive transcription factor, NF-κB, by several criteria. Firstly, several NF-κB-binding sites, although having sequence similarity with the CK-1 sequence, cannot compete efficiently for NF-GMa binding to CK-1. Secondly, the CK-1 sequence from both G-CSF and GM-CSF does not respond to phorbol ester treatment as would an NF-κB-binding element. These results demonstrate that NF-GMa is a novel transcription factor inducible by TNF-α and binds to a common element in HGF gene promoters.

Hemopoietic growth factors (HGFs) are a family of glycoproteins involved in the stimulation of proliferation and differentiation of progenitor cells and in the activation of function of mature hemopoietic cells (reviewed in reference 28). Expression of the genes encoding these glycoproteins is tightly regulated, with little constitutive production observed. Expression of one or a subset of these genes can, however, be induced by a range of stimuli in different cell types (reviewed in reference 6). The relative level of expression from the genes can also be altered by different stimuli in the same cell type. Antigen or mitogen activation of T cells leads to the increased synthesis of several HGFs, including granulocyte-macrophage colony-stimulating factor (GM-CSF) (50), interleukin 3 (IL-3) (35, 52), and IL-5 (46). Monocytes and macrophages can produce GM-CSF, granulocyte (G)-CSF, and monocyte-CSF following treatment with agents such as lipopolysaccharide (9, 39, 47). Fibroblasts and endothelial cells can be stimulated by tumor necrosis factor-α (TNF-α) or IL-1 to produce GM-CSF, G-CSF, monocyte-CSF, and IL-6 (3, 4, 19, 21, 31, 53). In monocytes, increased mRNA stability may account for induced expression (9), whereas in fibroblasts and endothelial cells, both increased mRNA stability (22) and increased transcriptional activity of the genes (22, 40) are responsible for the increase in HGF expression.

The capacity to produce one or several of these proteins suggests that their transcription could be regulated either individually or as a group. There exist a number of conserved sequences in the promoter regions of many genes, including HGF genes, that are potential transcriptional regulatory sequences. One highly observed sequence which we have termed cytokine 1 (CK-1; 5'GRGR/TTTY/ACY/AN3', in which R = purine, Y = pyrimidine, and N = any nucleotide) has been identified only in the promoter region of several hemopoietic growth-factor genes, including GM-CSF, G-CSF, IL-3, IL-5, IL-2, IL-4, and IL-6 (Table 1) (30, 43, 45). A second sequence, called CK-2 (5'TCAGGTA3'), immediately downstream of CK-1 is restricted to the GM-CSF and IL-3 genes (43). Deletional analyses have agreed that the CK-1 sequence of both the human and mouse GM-CSF promoters appears to have no role in either basal-level expression or in phorbol ester induction in some T-cell lines (31, 32, 37). On the other hand, the CK-2 sequence is required for phorbol ester inducibility of the mouse but not the human gene promoter (32, 37). Both the CK-1 and CK-2 sequences appear to play a role in mouse GM-CSF promoter activation by the transactivator of the human T-cell leukemia virus type 1 ($p40^x$) (15, 32). In contrast, sequences closer to the transcription start site are thought to be responsible for human GM-CSF promoter activation by $p40^x$ and phorbol ester (36, 37). The role of these sequences in gene induction in other cell types has not been extensively investigated, but a recent report suggests that IL-1 induction of GM-CSF in endothelial cells is mediated by a sequence at −63 relative to the CAP site (18).

DNA fragments spanning the conserved CK-1 sequence from the human GM-CSF and G-CSF genes specifically bind a nuclear protein called NF-GMa, which has been found in a number of cell types (43). The NF-GMb protein, which is phorbol ester inducible in the 5637 bladder carcinoma cell line (43), requires the intact CK-2 region of the GM-CSF promoter for binding (44). The CK-1-like sequence in the IL-2 gene (Table 1) has recently been shown to bind the transcription factor NF-κB (23). NF-κB has been shown to mediate phorbol ester inducibility of many genes, including IL-2 (16). Also, NF-κB mediates the TNF-α induction of the

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TABLE 1. Conserved sequences in hemopoietic growth factor gene promoters

HGF gene[a]	Sequence[b]
hGM-CSF (1)	−96 G A G A T T C C A C A G T T C A G G T A −77
hGM-CSF (2)	−357 G g G A T T a C A g −346
mGM-CSF	−108 G A G A T T C C A C A a c T C A G G T A −89
hIL-3 (1)	−126 G A G g T T C C A t − g − T C A G a T A −109
hIL-3 (2)	−331 G A G A T c C C A C −322
mIL-3 (1)	−115 G A G g T T C C A t − g − T g A G a T A −98
mIL-3 (2)	−198 G A G A T T C C A C −189
hIL-2	−208 G g G A T T t C A C −199
mIL-2	−208 G g G A T T t C A C −199
hG-CSF	−188 G A G A T T C C A C −179
mG-CSF	−192 G A G A T T C C c C −181
hIL-5	−223 a A G A T T C t t C −214
hIL-6	−136 a g G t T T C C A a −127
hIL-4 (R)	−178 G A a A T T a C A C −169

[a] The HGF gene names are abbreviated as in the text, with the addition of hIL-4 (human IL-4). h, Human; m, mouse; R, sequence in the reverse orientation relative to the direction of transcription. The numbers in parentheses, (1) and (2), refer to two copies of the sequence in the same promoter.

[b] The altered bases, when compared with the GM-CSF sequence, are shown in lowercase letters. The numbers above the sequence refer to the distance from the transcription start site.

human immunodeficiency virus (HIV) enhancer and the IL-2 receptor α-chain gene (25, 38). Since genes containing the CK-1 sequence are also phorbol-12-myristate-13-acetate (PMA) and TNF inducible, the relationship between NF-GMa and NF-κB is of interest.

In the present study, we have characterized the transcription factor NF-GMa and suggest that it is involved in the control of HGF gene transcription. The NF-GMa protein has a molecular mass of 43 kilodaltons and binds with a range of affinities to the CK-1 sequence from several HGF genes. It is distinct from a second GM-CSF promoter-binding protein, NF-GMb, which we show binds to the adjacent CK-2 sequence. In primary embryonic fibroblasts, the level of NF-GMa transiently increases with TNF-α treatment and the G-CSF CK-1 sequence acts as a TNF-responsive enhancer in transfection experiments. Although there is sequence similarity between the binding sites for NF-GMa and NF-κB, these two proteins show high-affinity binding to distinct sets of genes. Transfection experiments have also shown that although the CK-1 sequence is a TNF-α-inducible enhancer, unlike NF-κB it does not respond to phorbol ester.

MATERIALS AND METHODS

Oligonucleotides and plasmids. All oligonucleotides were synthesized on an Applied Biosystems model 381A DNA synthesizer, and full-length material was purified by polyacrylamide gel electrophoresis. The sequences of the oligonucleotides are described for individual experiments. Following treatment with kinase and annealing (27), double-stranded oligonucleotides were cloned into the Bluescript SK(−) vector (Stratagene Inc.) by using *Eco*RI or *Sma*I cloning sites. Clones containing single or multiple copies of the oligonucleotides were selected, and the sequences and orientations were checked by double-stranded sequencing (5). pSKCK-1/2 and pSKCK-1 contained a single copy of the GM-CSF and G-CSF oligonucleotides, respectively (Fig. 1A), and were used to generate probes for gel retardation, modification interference, and UV cross-linking. Single or multiple inserts of the G-CSF or multiple inserts of the GM-CSF oligonucleotides (Fig. 1A) were excised from Bluescript clones by using *Bam*HI-*Hin*dIII and were directionally cloned into pBLCAT2 (26) upstream of the thymidine kinase (tk) promoter and the chloramphenicol acetyltransferase (CAT) reporter gene to generate the following constructs for transfection. pCK-1(4+) and pCK-1(4−) contained four copies of the G-CSF CK-1 sequence, and pCK-1/2(5+) and pCK-1/2(5−) contained five copies of the GM-CSF CK-1-CK-2 sequence (see Fig. 6A), with plus and minus indicating opposite orientations. To generate pM1(3) and pM4(3), three copies of the M1 and M4 mutant oligonucleotides, respectively, were cloned upstream of the tk promoter as described above. The IL-2 probe was as previously described (23).

Cell lines. 5637 is a human bladder carcinoma cell line that constitutively produces low levels of GM-CSF and G-CSF. Treatment with PMA increases the levels of GM-CSF and G-CSF mRNA and protein (49). HUT78 is a T-lymphoblastoid cell line (12) which synthesizes low levels of GM-CSF and responds to PMA with increased synthesis of GM-CSF mRNA (unpublished observations). Primary embryonic fibroblasts were obtained from Flow Laboratories, Inc., and used at passages 16 to 25 in all experiments. All cell lines were routinely grown in RPMI medium supplemented with

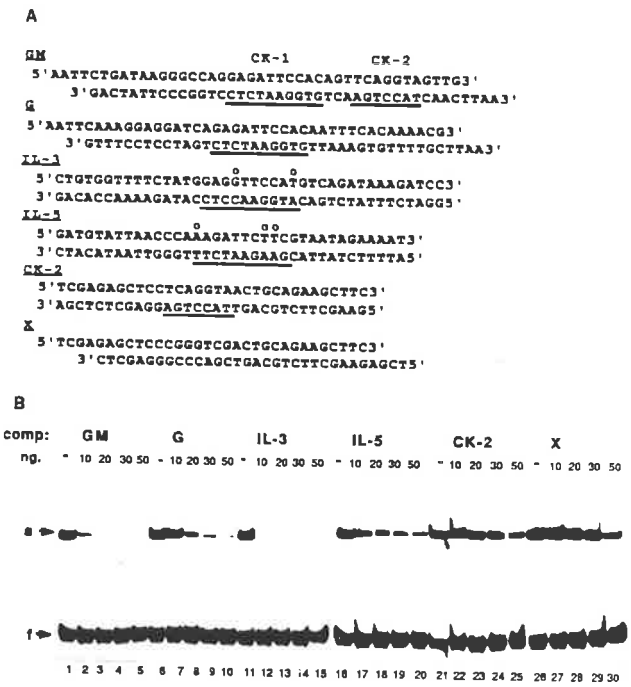

FIG. 1. NF-GMa binding to the CK-1 sequence from several HGF genes. (A) Sequences of the double-stranded synthetic oligonucleotides spanning the CK-1-CK-2 sequences of the GM-CSF, G-CSF, IL-3, and IL-5 genes which were used in competition experiments. The CK-2 sequence from GM-CSF was placed in a random DNA sequence to generate the CK-2 oligonucleotide. X is a random polylinker DNA fragment used as a nonspecific competitor. The CK-1 and CK-2 regions are underlined, and the base changes within these regions compared with the GM-CSF sequence are indicated (O). (B) Gel retardation binding assay using 1 μg of heparin-Sepharose-enriched NF-GMa from HUT78 T cells and radiolabeled GM sequence as a probe (0.2 ng). Binding reactions contained either no competitor (lanes 1, 6, 11, 16, 21, and 26) or GM (lanes 2 through 5), G (lanes 7 through 11), IL-3 (lanes 12 through 15), IL-5 (lanes 17 through 20), CK-2 (lanes 22 through 25), or X (lanes 27 through 30) in the increasing nanogram amounts shown for each lane. The NF-GMa complex is indicated by the letter a and the unbound DNA by the letter f.

10% fetal calf serum. Cells were harvested for extract preparation at 10^6 cells per ml or at 80% confluence for nonadherent and adherent cell lines, respectively. TNF-α (100 U/ml) was added to the fibroblast cells in fresh medium, and cells were harvested for nuclear extract preparation at different times following treatment.

Preparation of nuclear extracts and gel retardation assays. Nuclei were prepared from all cell types as described by Dignam et al. (8). Nuclear proteins were prepared as previously described (43), except that extraction of the nuclei with 0.5 M KCl was for 60 min at 4°C. The protein extracts were stored at −70°C in TM.1 (50 mM Tris hydrochloride [pH 7.6], 12 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 20% glycerol, 100 mM KCl) (17). Protein concentration was determined by the method of Bradford (2). Purified NF-κB was prepared as described by Lenardo et al. (23).

Probes for gel retardation were prepared as previously described (43) or by end labeling of restriction fragments from pSKCK-1 or pSKCK-1/2 plasmids. Competitor DNAs were prepared by annealing the appropriate synthetic oligonucleotide pairs in 25 mM Tris hydrochloride (pH 7.6)–150 mM KCl at a final concentration of 10 ng/μl. For NF-GMa-binding reactions, 0.1 to 0.5 ng of radiolabeled double-stranded oligonucleotides or restriction fragment (5,000 to 10,000 cpm) was mixed with 1 to 3 μg of crude nuclear extract or the indicated amount of enriched or purified material in a final volume of 20 μl containing 25 mM Tris hydrochloride, pH 7.6, 6.25 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol, 10% glycerol, 200 mM KCl. Poly(dI:dC) (0.5 to 2.0 μg) was used as nonspecific competitor in the reactions. For binding competition experiments, specific competitor DNAs were always mixed with the reaction before addition of the radiolabeled probe. The reactions were analyzed on 12% polyacrylamide gels in 0.5× TBE (43). NF-κB binding was detected as previously described (23).

UV cross-linking. For UV cross-linking experiments, probes were generated by primed synthesis on collapsed double-stranded DNA (5) by using 50 μM 5-bromodeoxyuridine and [α-^{32}P]dATP and by using pSKCK-1/2. The DNA was then cleaved with *Eco*RI, and the 40-base-pair (bp) insert was purified on polyacrylamide gels. Binding reactions for UV cross-linking were carried out as described above, but in a final volume of 50 μl, by using 6 to 10 μg of nuclear extract and 1 ng of radiolabeled probe containing 5-bromodeoxyuridine. Following UV irradiation (340 nm) for 15 min, complexes were separated on the standard gel retardation system. The wet gel was exposed to X-ray film for 6 to 12 h, and the area of the gel containing the retarded band was excised and incubated in sodium dodecyl sulfate load buffer for 30 min at 37°C. The gel slice was placed directly in the well of a 10% Laemmli protein gel, overlaid with 1% agarose, and electrophoresed at 30 mA for 6 h. The gel was stained with Coomassie blue and destained to visualize the markers, dried, and exposed to X-ray film for 1 to 3 days.

Modification interference. Modification interference reactions were carried out by the method of Gilman et al. (13), except that both A and G residues were modified with formic acid for 5 to 10 min prior to the binding reactions. Concentrated protein fractions, from heparin-Sepharose chromatography of crude nuclear extract (43), containing either NF-GMa or NF-GMb were used. Probes were generated by end labeling either the *Hin*dIII or *Bam*HI sites in pSKCK-1 or pSKCK-1/2 and excising the labeled fragment with the opposite enzyme. The modified end-labeled fragments were used in scaled-up gel retardation assays with either 10 or 5 μg of protein enriched for NF-GMa or NF-GMb, respectively.

Transfection of cells. Both fibroblasts and 5637 cells were plated at a density of 1×10^6 to 1.5×10^6 cells per 100-mm dish and grown for 2 to 3 days. Plasmid DNA (10 μg per dish) was transfected by using DEAE dextran (400 μg/ml for 2 h) and a 10% dimethyl sulfoxide shock (41). Cells were treated 24 h later with TNF-α (100 U/ml) or PMA (20 ng/ml) for 12 to 16 h. Cytoplasmic extracts were prepared, and CAT assays were carried out by using 25 to 50 μg of cell extract per assay (14). Percent CAT conversion was determined by scintillation counting of thin-layer chromatography plate areas containing [^{14}C]chloramphenicol and its acetylated derivatives. Relative CAT activity was determined within each experiment from the value obtained for pBLCAT2.

RNA analysis. Total cytoplasmic RNA was prepared from 5637 cells that had either been treated with PMA for 6 h or left untreated. Northern (RNA) blots (15 μg per track of RNA) were probed with a 600-bp insert from pSP6GM-CSF cDNA clone (20) labeled by random priming (10).

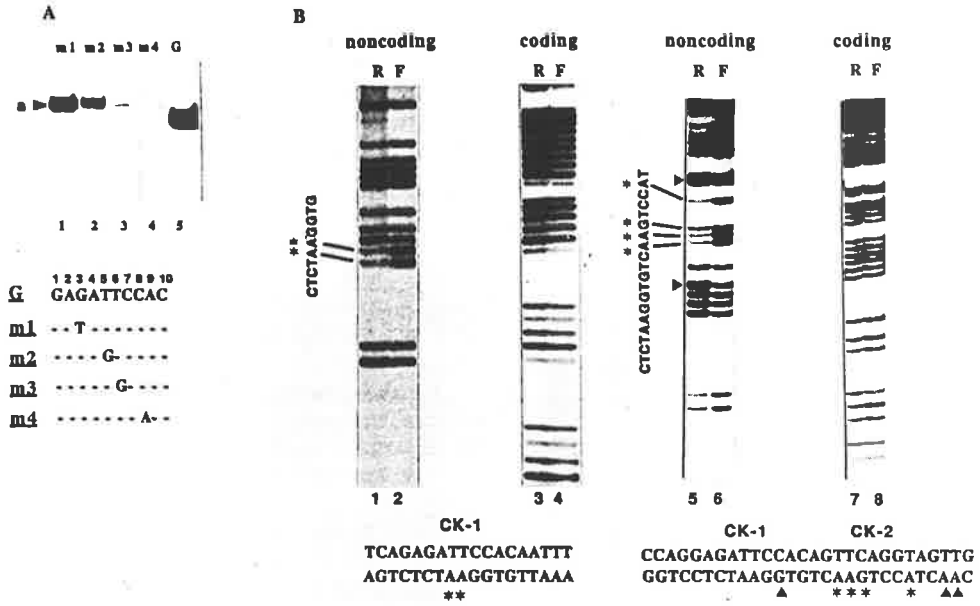

FIG. 2. Binding requirements for NF-GMa and NF-GMb. (A) Single-point mutations were generated within the CK-1 sequence of the G-CSF gene, and each mutant was tested for its ability to generate the NF-GMa complex from heparin-Sepharose-enriched HUT78 cell extract. Only the retarded complex NF-GMa is shown. Lane 1, Mutant 1 (m1); lane 2, mutant 2 (m2); lane 3, mutant 3 (m3); lane 4, mutant 4 (m4); lane 5, G-CSF CK-1 sequence. The complexes formed with mutant sequences migrate more slowly on the gel because they are all 30 bp long compared with 40 bp for the G-CSF sequence. The single-point mutations are shown under the G-CSF CK-1 sequence. Flanking sequences were maintained as in the G-CSF flanking sequence. (B) Modification interference experiments with NF-GMa-enriched (lanes 1 through 4) and NF-GMb-enriched HUT78 extract (lanes 5 through 8). Free DNA (F) and retarded DNA protein complex (R) were eluted from a scaled-up retardation gel, and the piperidine cleavage pattern was analyzed on a 6% denaturing polyacrylamide gel. A 70-bp DNA fragment containing the G-CSF CK-1 sequence from pSKCK-1 (see Materials and Methods) (lanes 1 through 4) or the GM-CSF CK-1/2 sequence excised from pSKCK-1/2 (lanes 5 through 8) was end labeled on either the noncoding (lanes 1, 2, 5, and 6) or coding strands (lanes 3, 4, 7, and 8) and used as probes in the binding assays. The modified bases which interfered with (*) or enhanced (▲) NF-GMa or NF-GMb binding are indicated. The positions of the CK-1 and CK-2 sequences are shown.

RESULTS

NF-GMa binds to several CK-1-containing DNA fragments. We have previously shown that two DNA-protein complexes, NF-GMa and NF-GMb, are formed with crude nuclear extract from PMA-stimulated 5637 cells binding to a short DNA fragment spanning the highly conserved CK-1 (5′GAGATTCCAC3′) and CK-2 (5′TCAGTCA3′) sequences of the human GM-CSF promoter (43). Only the NF-GMa complex is formed when a fragment of DNA from the G-CSF promoter containing CK-1 but not CK-2 is used as a probe in gel retardation assays (44). Double-stranded oligonucleotides spanning the CK-1 sequence in GM-CSF, G-CSF, IL-3, and IL-5 (Fig. 1A) surrounded by their naturally occurring flanking sequences were tested on retardation gels for their ability to compete for NF-GMa binding to a radiolabeled GM-CSF CK-1 sequence. NF-GMa enriched by heparin-Sepharose chromatography was used in all binding studies (43). Each CK-1 sequence was found to compete for NF-GMa binding to GM-CSF but with varying affinities (Fig. 1B). The relative affinity of each sequence, as determined by densitometer scanning of the gels, was IL-3 > GM-CSF > G-CSF > IL-5. The CK-2 sequence from GM-CSF placed in a random altered context and an unrelated piece of polylinker DNA did not significantly compete for NF-GMa binding (Fig. 1B). These results imply that CK-1 is the binding site for NF-GMa.

CK-1 is the binding site for NF-GMa. To define more precisely the binding requirements for NF-GMa, we introduced single-base mutations into the most highly conserved bases in the CK-1 sequence from G-CSF (Fig. 2A) and examined the ability of these mutant CK-1 sequences to bind NF-GMa. Each mutation reduced, to some degree, the ability of the sequence to bind NF-GMa. Mutation of either the T residue at position 6 to a G or the C residue at position 8 to an A reduced the binding affinity by >90% (Fig. 2A). Altering residue 5 (T to G) or residue 3 (G to T) also inhibited binding by 60 and 50%, respectively (Fig. 2A). The ability of these mutant CK-1 sequences to compete for binding of NF-GMa to the G-CSF CK-1 sequence parallels their ability to bind NF-GMa (data not shown). Single-base alterations have, therefore, defined several important nucleotides for NF-GMa binding to CK-1.

In order to further define the contact points of NF-GMa with the DNA, modification interference experiments were carried out. Modification of the central two A residues on the noncoding strand of the CK-1 sequence yielded a reproducible, albeit incomplete, interference pattern (Fig. 2B). Both of these bases were previously shown to affect binding when mutated (Fig. 2A).

CK-2 is the binding site for NF-GMb. Mutations within the CK-2 sequence have been shown to abolish NF-GMb but not NF-GMa binding to the GM-CSF sequence (44). Modification interference experiments were carried out with heparin-Sepharose-enriched NF-GMb (43). The interference pattern generated with NF-GMb was centered over the CK-2 sequence on the coding strand of the DNA fragment (Fig. 2B). Depurination at four bases within the sequence interfered with binding, whereas modification of some flanking bases

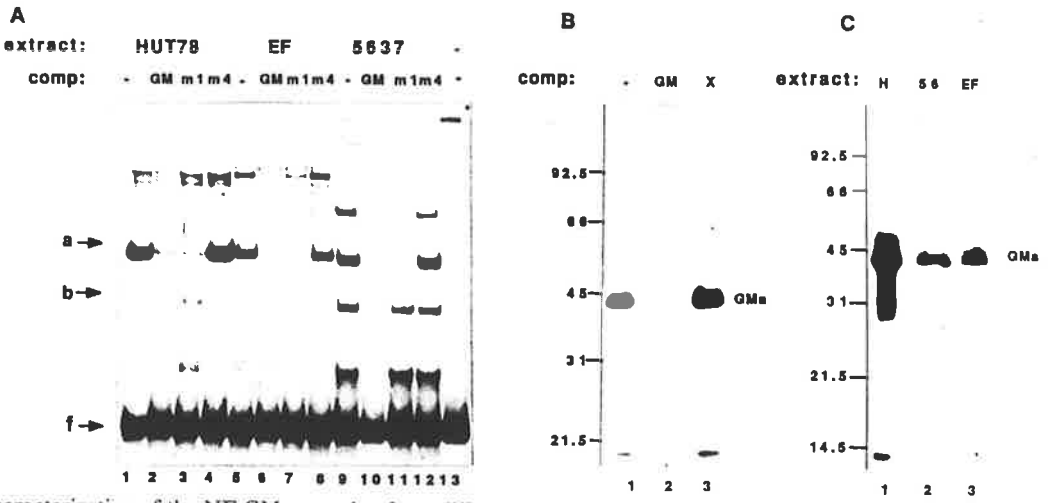

FIG. 3. Characterization of the NF-GMa complex from different cell types. (A) Binding reactions used the GM-CSF radiolabeled probe (0.2 ng) (Fig. 1A) and 2 μg of protein extract from HUT78 cells (lanes 1 through 4), embryonic fibroblasts (EF) (lanes 5 through 8), and 5637 bladder carcinoma cells (lanes 9 through 12). Lane 13 had no protein added to the binding reaction. For competition, 30 ng of GM (lanes 2, 6, and 10), mutant 1 (m1) (lanes 3, 7, and 11), and mutant 4 (m4) (lanes 4, 8, and 12) (Fig. 2A) was used. NF-GMa and NF-GMb are labeled a and b respectively, and f is unretarded DNA. (B) UV cross-linking experiments to determine the protein component(s) of the NF-GMa complex. Binding reactions contained 1 ng of 5-bromodeoxyuridine-labeled GM-CSF probe from pSKCK-1/2 and 5 μg of HUT78 extract. The cross-linked complexes were excised from a standard retardation gel and analyzed on a 12% sodium dodecyl sulfate-polyacrylamide gel. Binding reactions contained no competitor (lane 1), 50 ng of GM oligonucleotide (lane 2), or 50 ng of X oligonucleotide (lane 3). The positions and sizes of molecular weight markers are shown. (C) Sodium dodecyl sulfate-polyacrylamide gel of UV cross-linked NF-GMa complex from HUT78 cells (H, lane 1), 5637 cells (56, lane 2) and embryonic fibroblasts (EF, lane 3). Binding conditions were as described above.

appeared to enhance binding (Fig. 2B). These results show that the two proteins, NF-GMa and NF-GMb, form distinct complexes with adjacent DNA sequences.

Characterization of the protein(s) involved in the NF-GMa complex. We have previously detected NF-GMa binding in several cell types, including the bladder carcinoma cell line 5637, the lymphoblastoid cell line HUT78, and the melanoma cell line LiBr (43). The NF-GMa complex from the previously characterized 5637 and HUT78 cells and also from primary embryonic fibroblasts, in which low levels of NF-GMa can be detected, behaved in an identical manner in binding competition experiments with both wild-type and mutant CK-1 sequences (Fig. 3A). The identical behavior of the NF-GMa complex from different cell lines suggested that the same binding site and the same protein(s) are involved in the NF-GMa complex in each cell line.

To identify the molecular weight of the protein(s) responsible for forming the NF-GMa complex, a modification of the UV cross-linking method of Wu et al. (51) was used. A 40-bp fragment of DNA containing the G-CSF CK-1 sequence, labeled with [^{32}P]dATP and 5-bromodeoxyuridine, was used as a probe in a scaled-up binding reaction. Following exposure to UV light, the NF-GMa complex was separated on a standard polyacrylamide retardation gel. The NF-GMa complex was excised and reelectrophoresed on a 12% sodium dodecyl sulfate-polyacrylamide gel. A major protein band with a molecular mass of 43 kDa was identified on the protein gel (Fig. 3B). When excess unlabeled CK-1 competitor was added to the binding reaction, the 43-kDa protein band was eliminated, but a similar excess of an unrelated DNA fragment had no effect (Fig. 3B). In addition, complexes formed from extracts of HUT78, 5637, and fibroblast cells contained a single protein band of the same molecular weight, as determined by UV cross-linking (Fig. 3C). These results indicate that the NF-GMa complex involves at least one binding protein of 43 kDa.

NF-GMa and NF-κB have distinct binding requirements. The CK-1-like sequence from the IL-2 gene (11) has previously been regarded as a potential NF-GMa-binding site (43). Recently, however, this IL-2 sequence has been shown to bind with high affinity the transcription factor NF-κB, (16, 23). The IL-2 NF-κB-binding site differs from the GM-CSF CK-1 sequence by only two bases (Table 1). In order to determine whether NF-κB-binding sites can compete for binding of NF-GMa to the CK-1 sequence, competition experiments were carried out with increasing concentrations of unlabeled GM-CSF CK-1 sites or NF-κB-binding sites from the HIV enhancer (34), the IL-2 gene promoter (23), or the H2k^b gene H2TF1-binding site (1) (Fig. 4A). Although the homologous CK-1 sequence abolished the binding at 50-fold molar excess of cold DNA, none of the NF-κB-binding sites competed efficiently for NF-GMa binding (Fig. 4A). At high concentrations (>30 ng), the H2TF1-binding site showed a low level of competition (Fig. 4A).

We also tested the ability of purified NF-κB protein (23) to bind to the CK-1 sequence. Specific binding of NF-κB was detected to the IL-2 NF-κB site, but no retarded complexes were formed with the CK-1 sequence from either GM-CSF or G-CSF (Fig. 4B). Purified NF-GMa, on the other hand, bound with very low affinity to the NF-κB site from both the HIV enhancer and the IL-2 gene (data not shown). These data show that NF-GMa is distinct from NF-κB and that the IL-2 site has different binding properties to other CK-1-like sequences in hemopoietic growth factor genes.

The G-CSF CK-1 sequence acts as a TNF-α-responsive element in fibroblasts. Since several HGF genes which contain a CK-1-like sequence are TNF-α inducible in fibroblasts, we examined the possibility that NF-GMa and its binding site might play a role in this induction. Nuclear extracts prepared from primary human embryonic lung fibroblasts contained low levels of NF-GMa-binding activity compared with that previously seen in a number of perma-

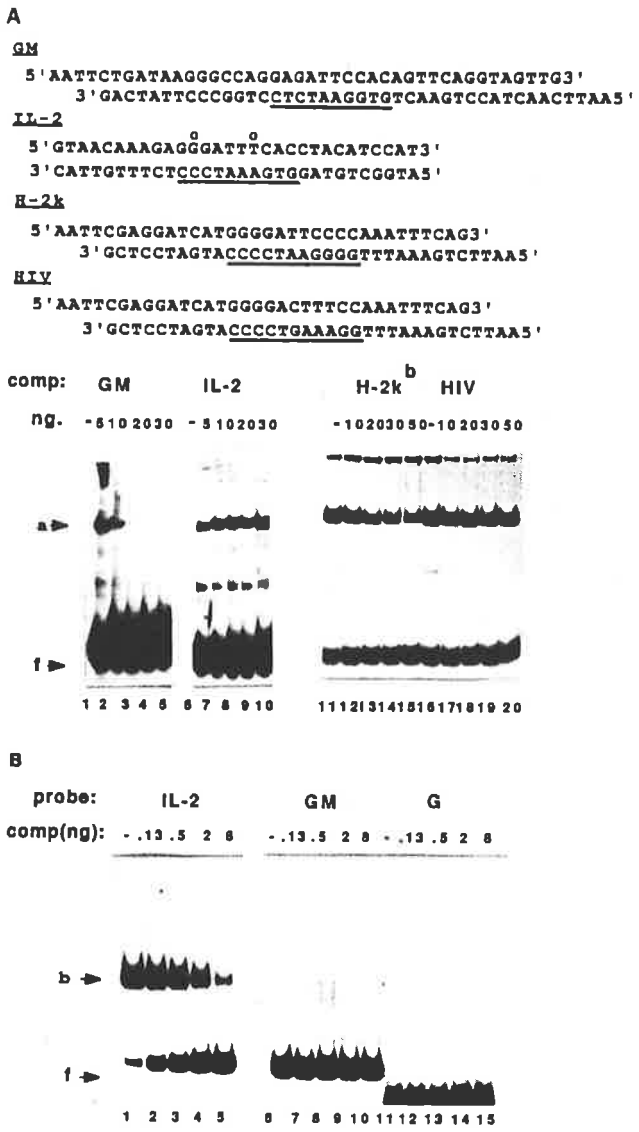

FIG. 4. NF-GMa and NF-κB have distinct binding sites. (A) Sequences of oligonucleotides from the GM-CSF, IL-2, H-2k^b genes, and the HIV enhancer containing either CK-1 (GM) or NF-κB-binding sites (IL-2, H-2k^b, and HIV), with the relevant sequences underlined. The base differences between the GM-CSF CK-1 and the IL-2 NF-κB sites are shown (○). Protein extract from HUT78 cells (2 μg) was used with the GM-CSF CK-1 sequence (0.2 ng) to generate the NF-GMa complex (a). Unlabeled competitor for GM (lanes 2 through 5), IL-2 (lanes 7 through 10), H-2k^b (lanes 12 through 15), and HIV (lanes 17 through 20) was added to binding reactions in the nanogram amounts shown on the lanes. Lanes 1, 6, 11, and 16 had no competitor added. (B) Binding of purified NF-κB from bovine spleen to a DNA fragment containing the IL-2 NF-κB sequence (lanes 1 through 5), the GM-CSF CK-1/CK-2 sequence (lanes 6 through 10), or the G-CSF CK-1 sequence (lanes 11 through 15). Competitor IL-2 NF-κB sequence was added to binding reactions in the nanogram quantities indicated. Lanes 1, 6, and 11 had no competitor added. NF-κB complex (b) and the unbound DNA (f) are indicated.

nent cell lines in which GM-CSF and G-CSF could be induced (Fig. 5A). A time-dependent transient increase in the amount of NF-GMa was seen following treatment with TNF-α (100 U/ml) (Fig. 5A). Densitometric scanning of tracks from four independent experiments showed that the maximum increase of fivefold was seen at 6 to 8 h of TNF-α treatment. By 24 h the amount of NF-GMa had returned to pretreatment levels (Fig. 5A). A similar time course of TNF induction of NF-GMa was observed in human umbilical vein endothelial cells (data not shown).

To determine whether the increased levels of NF-GMa described above are associated with increased transcriptional activity of the CK-1 sequence, single and multiple copies of the G-CSF CK-1 sequence were cloned upstream of the tk promoter and the CAT gene in the pBLCAT2 vector (26) (Fig. 5B). CAT activity was measured following transient transfection into fibroblasts. An increase of 12-fold (average of nine experiments) in CAT activity was observed following TNF-α treatment of cells transfected with a plasmid containing four copies of the CK-1 sequence [pCK-1(4+)] (Fig. 5C). A single copy of the CK-1 sequence [pCK-1(+)] gave low levels of CAT activity, but an average threefold increase in CAT levels was observed following TNF-α treatment. pM1(3) and pM4(3) (Fig. 5B), plasmids containing three copies of mutant 1 and mutant 4, respectively (Fig. 1A), were also transfected into fibroblasts and tested for their TNF responsiveness. Transcription from pM1(3) but not pM4(3) was induced approximately fourfold by TNF-α treatment (Fig. 5D), a result which corresponds with the ability of these mutants to bind NF-GMa (Fig. 1A). Cells transfected with the vector alone had undetectable CAT levels and did not respond to TNF-α treatment (Fig. 5C). The TNF-α response of the CK-1 sequence was both time- and dose-dependent, with maximum induction levels reached at 16 to 24 h and 100 U of TNF-α per ml (data not shown). These results show that the CK-1 sequence from G-CSF is a TNF-α-responsive element and that the response is probably mediated by increased levels of NF-GMa.

CK-1 sequences respond to TNF-α but not PMA in fibroblasts. We have shown here that the CK-1 sequence from the G-CSF gene is a TNF-α-responsive enhancer in embryonic fibroblasts. The NF-κB site from both the IL-2R α chain gene, the HIV enhancer, or the immunoglobulin κ gene are not only TNF-α responsive (25, 38) but also respond to phorbol ester in T cells and fibroblasts (38, 42). It appears, however, that the CK-1 sequence is not required for phorbol ester induction of the GM-CSF promoter in T cells (32). We have tested multiple copies of the CK-1 sequence from G-CSF cloned in either orientation upstream of the tk promoter (Fig. 6A) for their ability to respond to either TNF-α or PMA following transfection into primary embryonic fibroblasts. As shown above, multiple copies of the CK-1 sequence can respond to TNF-α and they are also functional in either orientation upstream of the tk promoter in pBLCAT2 (Fig. 6B). However, PMA treatment of cells containing these constructs showed no CAT activity after either 6 or 16 h of exposure to PMA (Fig. 6B). A plasmid containing four copies of the IL-2 NF-κB site (Fig. 4A) showed a weak response to PMA in these cells (data not shown). The CK-1 sequence was also unresponsive to PMA in the 5637 bladder carcinoma cell line, in which we have previously shown that NF-GMa-binding activity does not change with PMA treatment (43) (Fig. 6B). The same concentration (20 ng/ml) of PMA can, however, induce both NF-GMb-binding activity (43) and GM-CSF mRNA in 5637 cells (Fig. 6C) and also both GM-CSF and G-CSF in embryonic fibroblasts (22).

DISCUSSION

The transcriptional regulation of HGF gene expression is likely to be complex, differing from one cell type to another

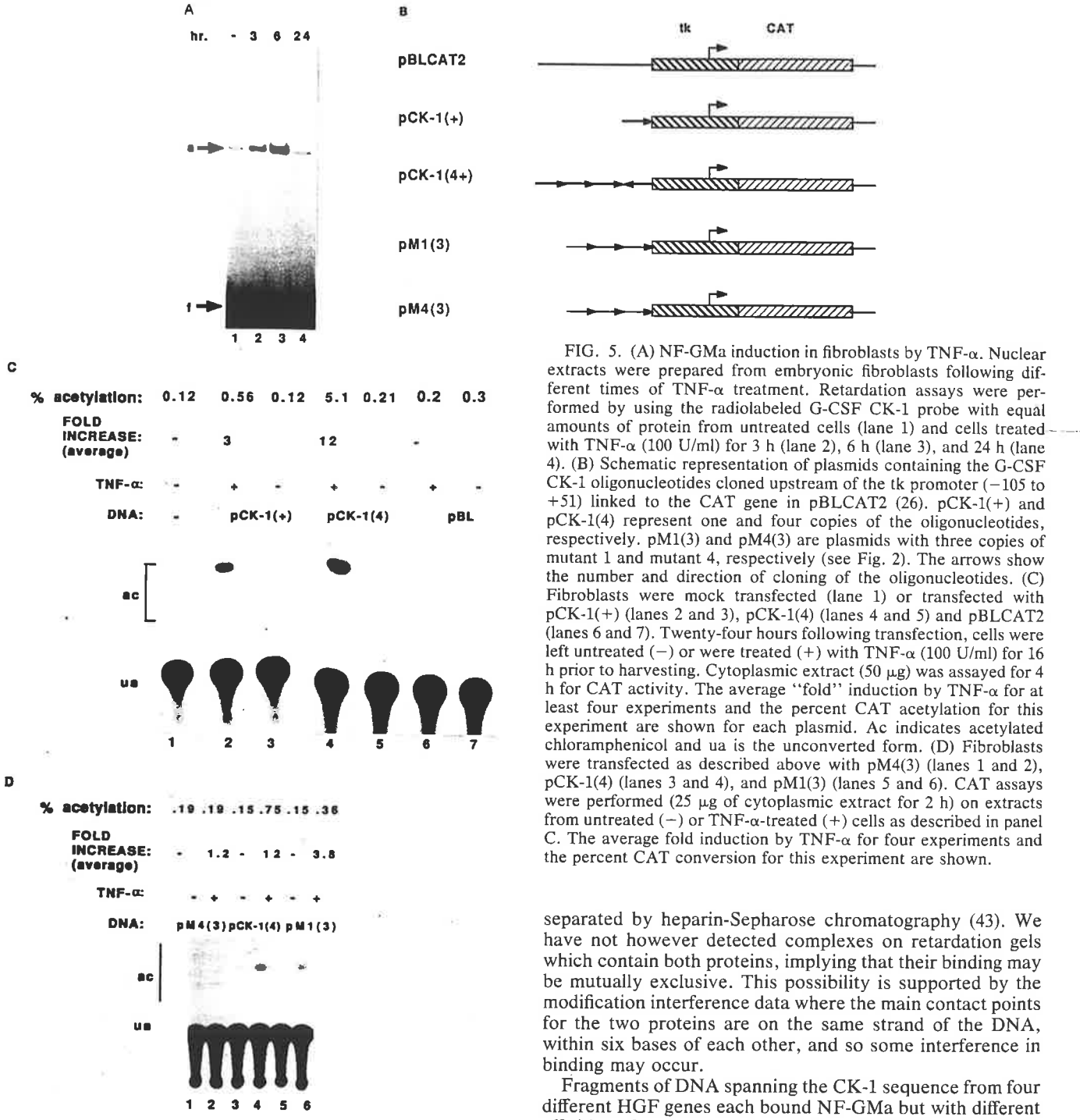

FIG. 5. (A) NF-GMa induction in fibroblasts by TNF-α. Nuclear extracts were prepared from embryonic fibroblasts following different times of TNF-α treatment. Retardation assays were performed by using the radiolabeled G-CSF CK-1 probe with equal amounts of protein from untreated cells (lane 1) and cells treated with TNF-α (100 U/ml) for 3 h (lane 2), 6 h (lane 3), and 24 h (lane 4). (B) Schematic representation of plasmids containing the G-CSF CK-1 oligonucleotides cloned upstream of the tk promoter (−105 to +51) linked to the CAT gene in pBLCAT2 (26). pCK-1(+) and pCK-1(4) represent one and four copies of the oligonucleotides, respectively. pM1(3) and pM4(3) are plasmids with three copies of mutant 1 and mutant 4, respectively (see Fig. 2). The arrows show the number and direction of cloning of the oligonucleotides. (C) Fibroblasts were mock transfected (lane 1) or transfected with pCK-1(+) (lanes 2 and 3), pCK-1(4) (lanes 4 and 5) and pBLCAT2 (lanes 6 and 7). Twenty-four hours following transfection, cells were left untreated (−) or were treated (+) with TNF-α (100 U/ml) for 16 h prior to harvesting. Cytoplasmic extract (50 μg) was assayed for 4 h for CAT activity. The average "fold" induction by TNF-α for at least four experiments and the percent CAT acetylation for this experiment are shown for each plasmid. Ac indicates acetylated chloramphenicol and ua is the unconverted form. (D) Fibroblasts were transfected as described above with pM4(3) (lanes 1 and 2), pCK-1(4) (lanes 3 and 4), and pM1(3) (lanes 5 and 6). CAT assays were performed (25 μg of cytoplasmic extract for 2 h) on extracts from untreated (−) or TNF-α-treated (+) cells as described in panel C. The average fold induction by TNF-α for four experiments and the percent CAT conversion for this experiment are shown.

and involving the interaction of a number of regulatory nuclear proteins. We have characterized one of these nuclear factors with a binding specificity apparently restricted to HGF genes and which appears to mediate TNF-α inducibility in fibroblasts. This protein, NF-GMa, appears as a 43-kDa protein from several cell types. Mutation analyses and modification interference data both show that the CK-1 sequence is the binding site for NF-GMa. NF-GMb clearly represents a distinct protein which binds to the adjacent CK-2 sequence.

These two proteins can bind independently to DNA when separated by heparin-Sepharose chromatography (43). We have not however detected complexes on retardation gels which contain both proteins, implying that their binding may be mutually exclusive. This possibility is supported by the modification interference data where the main contact points for the two proteins are on the same strand of the DNA, within six bases of each other, and so some interference in binding may occur.

Fragments of DNA spanning the CK-1 sequence from four different HGF genes each bound NF-GMa but with different affinities. Sequences both within and flanking the CK-1 sequence may play some role in this altered affinity. The G-CSF and GM-CSF sequences have identical CK-1 sequences but quite divergent flanking sequences and an approximately twofold difference in affinity for NF-GMa. The IL-3 CK-1 sequence appears to have the highest affinity, and IL-5 has the lowest affinity for NF-GMa. The IL-5 sequence is one of the least conserved CK-1 regions, with three base changes, when compared with GM-CSF CK-1 (Table 1). A series of 3-bp substitutions within the 3′ flanking sequence of the GM-CSF CK-1 region (i.e., across the CK-2 region) did not significantly alter NF-GMa binding (44).

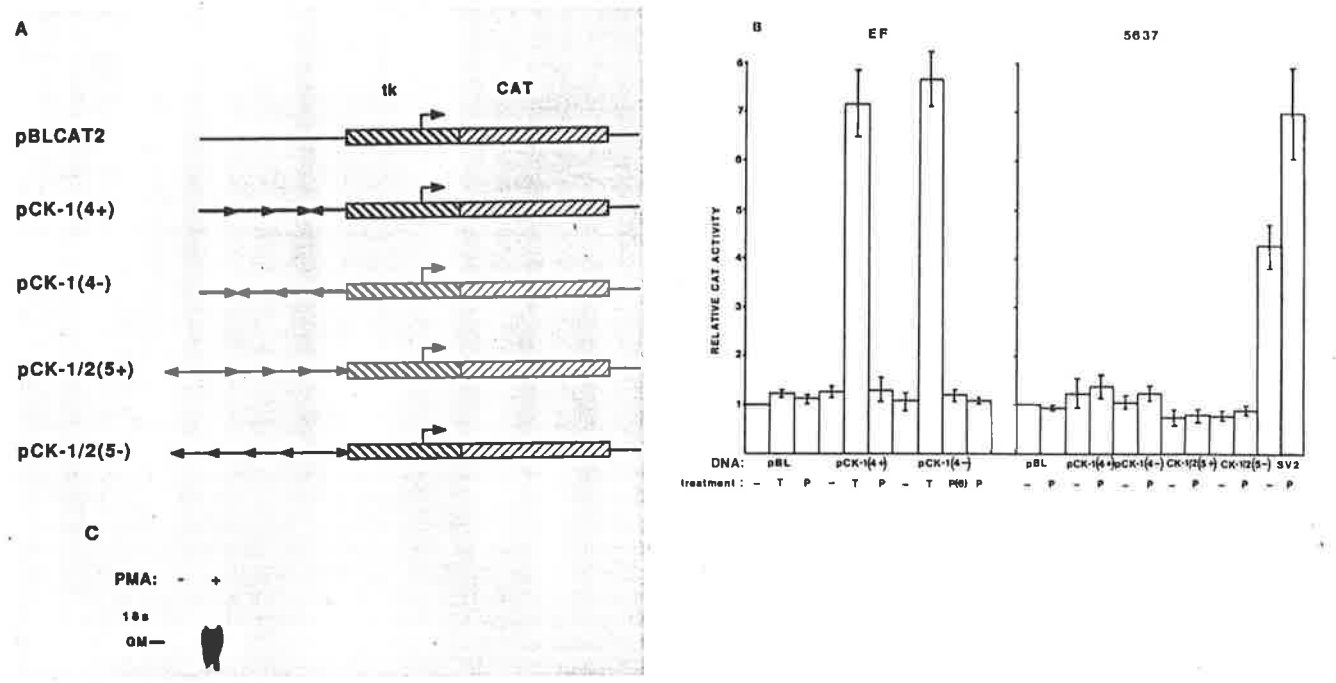

FIG. 6. The CK-1 sequence is TNF-α but not PMA responsive. (A) Schematic representation of plasmid constructs containing multiple copies of either CK-1 from G-CSF [pCK-1(4)] or CK-1/2 from GM-CSF [pCK-1/2(5)] cloned in either orientation (+ and −) upstream of the tk promoter (−105 to +51) linked to the CAT gene in pBLCAT2. The arrows show the number of copies and the direction of the sequences. (B) CAT activity measured in either embryonic fibroblasts of 5637 cells following transfection with the plasmids in panel A. Cells were treated 24 h after transfection with either TNF-α (T) (100 μ/ml) or PMA (P) (20 ng/ml) for 12 to 16 h prior to harvesting. CAT activity was measured and is expressed relative to the level of CAT activity in unstimulated cells transfected with the pBLCAT2 plasmid. The figures shown are the mean (± standard error) of at least three separate transfection experiments. Absolute CAT conversion was on average 4.5% for pCK-1(4+) following TNF-α treatment. (C) Northern blot analysis of mRNA prepared from 5637 cells either untreated (−) or treated with PMA (20 ng/ml) for 6 h (+). Total cytoplasmic RNA (15 μg) was loaded per track. Equivalent tracks were stained with ethidium bromide to ensure equal amounts of RNA in each sample. The ≃1-kilobase GM-CSF mRNA and the position of the 18s ribosomal RNA band are indicated. The blot was probed with a 650-bp GM-CSF cDNA fragment labeled to approximately 1×10^9 cpm/μg by random priming.

Single-base changes within the NF-GMa transcription factor-binding site or perhaps altered flanking sequences can, therefore, significantly alter nuclear factor-binding affinity and ultimately transcriptional efficiency of the DNA fragment.

The physiological significance of the conservation of this sequence across the family of HGF genes and the changes in affinity between different genes for NF-GMa binding is not clear but suggests a mechanism for coregulation. There is no evidence for the coordinate regulation of all the CK-1-containing HGF genes. There is, however, some evidence for the coordinate induction of subsets of these genes. For example, GM-CSF, G-CSF, and IL-6 mRNAs are induced following TNF-α treatment of fibroblasts and endothelial cells (40, 53) and some mouse T-cell clones coordinately express GM-CSF and IL-3 (20). We have shown that the CK-1 sequence from the G-CSF gene is a TNF-α-responsive element in fibroblasts. Levels of NF-GMa-binding activity are also induced by TNF-α and probably function in mediating the response of the CK-1 sequence to TNF-α. This sequence may be involved, therefore, in regulating the expression of several CK-1-containing genes in cells exposed to monocyte products such as TNF-α. However, all CK-1-containing genes are not TNF-α responsive and IL-3 mRNA cannot be detected in endothelial cells following TNF-α treatment (35). In addition, IL-1 induction of GM-CSF in endothelial cells is not affected by deletion of the CK-1 sequence (18), although basal-level expression is altered. The promoter context in which the CK-1 sequence occurs may be important in controlling its function, and adjacent binding proteins may block CK-1/NF-GMa activity. Such a situation has been described for the NF-κB binding sites from the immunoglobulin κ gene and the IL-2R α-chain gene (7). Although these sequences appear to bind NF-κB with equal affinity, the IL-2R sequence does not respond to phorbol esters in Jurkat T cells and so in vitro binding cannot be equated with in vivo activity (7).

We compared the binding specificity of NF-GMa with that of another TNF-α-responsive transcription factor, NF-κB (17, 25, 38). In spite of the sequence similarity between the NF-GMa- and NF-κB-binding sites, especially those present in the GM-CSF (NF-GMa) and IL-2 (NF-κB) genes (Table 1), our experiments showed that each transcription factor has a distinct set of binding sites and that NF-GMa binding appears to be confined to HGF-type genes. DNA binding and transcriptional activity of NF-κB is induced by PMA in both fibroblasts (41) and T cells (34, 36). Costimulation of Jurkat cells with TNF-α and PMA leads to superinduction of NF-κB (36), implying that these two agents act through independent mechanisms.

Unlike NF-κB-binding sites, the CK-1 sequence from G-CSF did not respond to phorbol ester treatment in embryonic fibroblasts. Similar results were obtained in 5637 cells, in which the CK-1 sequence from both G- and GM-CSF was not PMA inducible. In both of these cell types, G- and GM-CSF mRNA levels are increased following PMA treatment (Fig. 5C [22]). Part of this response is at the level of transcription, since it has been reported that PMA induces a

three- to fourfold increase in transcriptional activity from the GM-CSF gene in embryonic fibroblasts (22). It seems clear that the transcriptional response to PMA is not mediated via the CK-1 sequence or NF-GMa, since the binding ability of this protein does not change with PMA treatment in a number of cell types (43; Shannon and Ryan, unpublished data). A similar situation appears to apply in T cells, in which deletion of the CK-1 sequence from the promoters of both the mouse (called CLE-1) and human GM-CSF genes does not effect basal-level expression or phorbol ester inducibility in Jurkat cells (15, 32, 37).

It is more difficult to explain the lack of response of the GM-CSF CK-1-CK-2 sequence to PMA in 5637 cells, since the CK-2-binding protein NF-GMb is induced by PMA in these cells (43). However, it has been shown that the mouse CK-2 sequence (called CLE-2) alone does not confer PMA inducibility on the basal-level promoter but that the 3′ flanking G+C-rich sequence is also required (32). NF-GMb may not function alone to respond to PMA but in concert with a second protein(s) which binds to the G+C-rich sequence.

Although NF-GMa and NF-κB respond to some stimuli in common, they can be distinguished both by their binding specificities and their transcriptional response to phorbol esters. It appears that NF-κB plays a central function during T-cell activation in the induction of IL-2 and the IL-2R α-chain genes (16, 25), although the role played by the κB site alone in the IL-2R sequence is unclear (7). On the other hand, the NF-GMa-binding site does not have a clear role in T cells (32, 37) but could play an important role in induction of G-CSF, GM-CSF, and other HGF genes in fibroblasts. The TNF-α response appears to be cell specific also, since the CK-1 sequence does not respond to TNF in Jurkat T cells (Shannon et al., unpublished data). This function may be relevant both in the bone marrow stroma and at sites of inflammation in local inflammatory responses. In the bone marrow stroma, fibroblasts or endothelial cells may respond to extracellular stimuli via NF-GMa to produce HGF proteins required for steady-state hemopoiesis. TNF produced by infiltrating monocytes could augment the inflammatory response by inducing localized cells to produce HGF proteins, which in turn could activate the cytotoxic or phagocytic functions of peripheral granulocytes or macrophages (24, 29, 48). Cloning of the gene for NF-GMa, an apparently HGF-specific transcription factor, will enable us to determine in more detail its relationship with NF-κB and to elucidate signal transduction mechanisms involved in its activation by TNF-α or other agents.

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Immunology

The granulocyte–macrophage colony-stimulating factor/interleukin 3 locus is regulated by an inducible cyclosporin A-sensitive enhancer

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ABSTRACT **Granulocyte–macrophage colony-stimulating factor (GM-CSF) and interleukin 3 (IL-3) are pleiotropic hemopoietic growth factors whose genes are closely linked and induced in T lymphocytes in a cyclosporin A (CsA)-sensitive fashion. Since we found that the human GM-CSF and IL-3 proximal promoters were not sufficient to account for the observed regulation of these genes, we mapped DNase I hypersensitive sites across the GM-CSF/IL-3 locus in the Jurkat human T-cell line to identify additional regulatory elements. We located an inducible DNase I hypersensitive site, 3 kb upstream of the GM-CSF gene, that functioned as a strong CsA-sensitive enhancer of both the GM-CSF and IL-3 promoters. Binding studies employing Jurkat cell nuclear extracts indicated that four sites within the enhancer associate with the inducible transcription factor AP1. Three of these AP1 elements lie within sequences that also associate with factors resembling the CsA-sensitive, T cell-specific transcription factor NFAT. We provide additional evidence suggesting that an AP1-like factor represents one of the components of NFAT. We propose that the intergenic enhancer described here is required for the correctly regulated activation of both GM-CSF and IL-3 gene expression in T cells and that it mediates the CsA sensitivity of the GM-CSF/IL-3 locus.**

Granulocyte–macrophage colony-stimulating factor (GM-CSF) and interleukin 3 (IL-3) are tightly regulated cytokines that direct the proliferation, differentiation, and function of a variety of hemopoietic cells and their precursors (1). Their genes are closely linked; they are just 10 kb apart on human chromosome 5 (2). Both genes are expressed in most T lymphocytes and T-cell lines following activation of the T-cell antigen receptor and other surface molecules (1).

The T-cell-receptor-dependent induction of GM-CSF, IL-3, and several other cytokine genes can be blocked by the powerful immunosuppressant cyclosporin A (CsA) (3, 4). The mechanism of action of CsA is well documented only for the interleukin 2 (IL-2) gene, which has a promoter that is strongly induced during T-cell activation (5–9). CsA blocks signals transmitted by the T-cell receptor, which mediate the induction of specific transcription factors such as NFAT that associate with the IL-2 promoter. NFAT is a T cell-specific CsA-inhibitable transcription factor induced early in the course of T-cell activation; it is essential for the activation of the IL-2 gene (6–9). The GM-CSF and IL-3 genes, however, appear to be subject to a more complex mode of regulation than IL-2. We observed that the proximal promoters of these genes responded poorly to signals normally delivered via the T-cell receptor and were relatively insensitive to CsA (see below). This suggests that a distal regulatory element(s) may participate in the activation of the GM-CSF/IL-3 locus and be a target for CsA inhibition.

Transcriptional regulatory elements frequently adopt altered chromatin structures, termed DNase I hypersensitive (DH) sites, which can be detected within nuclei on the basis of their enhanced accessibility to DNase I (10). DH sites most likely represent nucleosome-free regions where specific DNA binding proteins associate with DNA and exclude nucleosomes. In this study, we mapped DH sites across the human GM-CSF/IL-3 locus and located an inducible DH site† in the intergenic region, which functioned as a powerful CsA-inhibitable enhancer.

MATERIALS AND METHODS

Plasmid Construction. pHGM0.6 was created by inserting the multiple cloning site AGCTGATATCTCGAGATC-TGGGCCCGGATCCTGCAGGCCTAAGCTTCGCGAG-GTCACC into the *Hin*dIII site at the 5′ end of the 0.6-kb fragment of the human GM-CSF promoter in the chloramphenicol acetyltransferase (CAT) reporter gene plasmid pCSFp1+ (11). Subsequent derivatives of pHGM0.6 incorporating additional fragments of the human GM-CSF/IL-3 locus contained 2.7-kb *Bgl* II/*Hin*dIII (pHGM3.3), 2.5-kb *Bam*HI/*Hin*dIII (pHGM3.1), 2.0-kb *Bgl* II/*Hin*dIII (pHGM2.6), or 0.7-kb *Bgl* II (pHGMB716) fragments of λJ1-16 DNA (2) inserted into the corresponding sites upstream of the promoter in pHGM0.6. pHGMN3 contained three head-to-tail copies of the GM550 element GATCTCTTATTATGACTCTTGCTT-TCCTCCTTTCA in the *Bgl* II site of pHGM0.6. pHIL3 contained a 5.2-kb *Hin*dIII/*Ban* II fragment of λ-66 DNA (a gift from S. Clark and Y.-C. Yang) from upstream of the human IL-3 gene inserted into the *Hin*dIII/*Sal* I sites of pBLCAT3 (12). pHIL3B716 had the 716-bp *Bgl* II fragment inserted into the *Hin*dIII site of pHIL3. All DNA fragments were inserted in the same orientation relative to the promoters as they exist in the GM-CSF/IL-3 locus.

DH Site Analyses. Nuclei isolations and DNA digestions were performed essentially as described (5). Briefly, nuclei were isolated from Jurkat cells by lysis in 0.1% Nonidet P-40 and resuspended to ≈0.4 mg/ml (optical density at 260 nm of an aliquot in 1 M NaOH = 10 absorbance units/ml). Aliquots of nuclei were digested for 3 min at 22°C with DNase I (Worthington) at 2–20 units/ml in nuclei isolation buffer containing 1 mM $CaCl_2$. DNA was purified, digested with *Eco*RI, electrophoresed on a 0.8% agarose gel, blotted onto a Hybond N membrane (Amersham), and hybridized to the ^{32}P-labeled 1.4-kb *Bam*HI fragment of λJ1-16 DNA indicated

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Abbreviations: GM-CSF, granulocyte–macrophage colony-stimulating factor; IL-2 and IL-3, interleukins 2 and 3; CsA, cyclosporin A; DH, DNase I hypersensitive; CAT, chloramphenicol acetyltransferase; PMA, phorbol 12-myristate 13-acetate.

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†The sequence reported in this paper has been submitted in the GenBank data base (accession no. L07488).

in Fig. 1. One representative sample was selected from each DNase I titration. The extent of DNase I cutting at the DH site was determined by quantitation of ^{32}P emissions with a Molecular Dynamics PhosphorImager.

Analyses of mRNA Expression. GM-CSF and IL-3 mRNA accumulation, relative to β-actin, was determined by RNase protection assay (13). SP6 RNA polymerase transcription templates for the preparation of ^{32}P-labeled RNA probes used in RNase protection assays contained the terminal 207 bp of the human GM-CSF gene (gift from G. Goodall), the 301-bp *Sma* I/*Pst* I fragment of the human IL-3 gene, and the 130-bp *Pst* I/*Sma* I fragment of the human β-actin gene. mRNA levels were quantitated with a Molecular Dynamics PhosphorImager. The levels of IL-3 and GM-CSF mRNA were normalized as the ratio over the level of β-actin detected in each assay and displayed as a percentage of the maximum level induced in each series of experiments.

Cell Transfections. Jurkat cells (5×10^6) were transfected by electroporation with 10 μg of DNA and cultured for 16–24 h before stimulating with phorbol 12-myristate 13-acetate (PMA) at 20 ng/ml plus 2 μM A23187, in the presence or absence of 0.1 μM CsA. After 6 h the cells were washed to remove the stimulus and cultured for 16–24 h in fresh medium before harvesting cytoplasmic protein for CAT assay (14). Relative CAT activity and standard errors were calculated from at least four experiments by using unstimulated pHGM0.6 set at a value of 1 as a reference. At least two independently derived clones of each construct were tested. The fold induction in response to PMA and A23187 was calculated relative to each unstimulated construct.

Gel Electrophoretic Mobility Shift Assays. Mobility shift assays were performed as described (15) using 4% polyacrylamide gels in 25 mM Tris borate/0.5 mM EDTA. Nuclear extracts were prepared as described (16) from Jurkat cells with and without stimulation for 3 h with PMA at 20 ng/ml and 2 μM A23187. In each assay, 4 μg of nuclear protein, 5 μg of poly(dI-dC), and 0.2 ng of DNA probe were incubated for 25 min at 22°C in 18 μl of 30 mM NaCl, 30 mM KCl, 10% glycerol, 1 mM EDTA, 1 mM dithiothreitol, 20 mM Hepes (pH 7.9), 0.1 mM phenylmethylsulfonyl fluoride, aprotinin at 5 μg/ml, and leupeptin at 5 μg/ml. Assays in Fig. 5*b* were performed in the presence and absence of 25 ng of specific competitor DNA. GM170, GM330, GM400, and GM550 probes and competitors corresponded to the DNA segments indicated in Fig. 2*b*. The human IL-2 gene NFAT probe and competitor (gift of G. Crabtree) encompassed the sequence GGAGGAAAAACTGTTTCATACAGAAGGCGT. The human stromelysin gene AP1 probe and competitor encompassed the sequence GCAAGGATGAGTCAAGCTGCGGGTGATCC. The nonspecific DNA competitor had the sequence TCGCCAATGAGCTCCCGGGTCGACTGCAGAAGCTTC.

RESULTS

An Inducible DH Site Lies Upstream of the GM-CSF Gene. Elements required for the correct regulation of the GM-CSF/IL-3 locus were sought by mapping DH sites (10) in the Jurkat human T-cell line before and after stimulation with PMA and the Ca^{2+} ionophore A23187. These stimuli mobilize protein kinase C and increase free cytoplasmic Ca^{2+}, respectively, and thus mimic at least some aspects of T-cell receptor activation (1). We mapped DH sites across ≈40 kb of DNA extending from an *Eco*RI site 12 kb upstream of the IL-3 gene to a *Bgl* II site 12 kb downstream of the GM-CSF gene. Just one inducible DH site, located 2.8–3.0 kb upstream of the GM-CSF gene, was detected in Jurkat cells (Figs. 1*a* and 2*a*); this site was also inducible in peripheral blood T cells (not shown). In addition, at least seven constitutive DH sites were detected in the vicinity of the IL-3 gene (Fig. 2*a*; data not shown).

Maximal induction of the DH site upstream of the GM-CSF gene required two signals, provided here by PMA and the Ca^{2+} ionophore, as did the induction of GM-CSF and IL-3 gene mRNA accumulation (Fig. 1*a*). The DH site appeared 1–2 h before the onset of GM-CSF and IL-3 mRNA accumulation (Fig. 1*b*), and its induction was blocked by both the transcription inhibitor dichlorobenzimidazole riboside and the translation inhibitor cycloheximide (Fig. 1*a*). Significantly, the immunosuppressant CsA, which inhibited both GM-CSF and IL-3 transcription, also blocked induction of the DH site (Fig. 1*a*). The DH site required ongoing induction of nuclear factors for its continued presence, since a significant decrease in intensity occurred 4 h after either addition of CsA or removal of the stimulus (Fig. 1*c*).

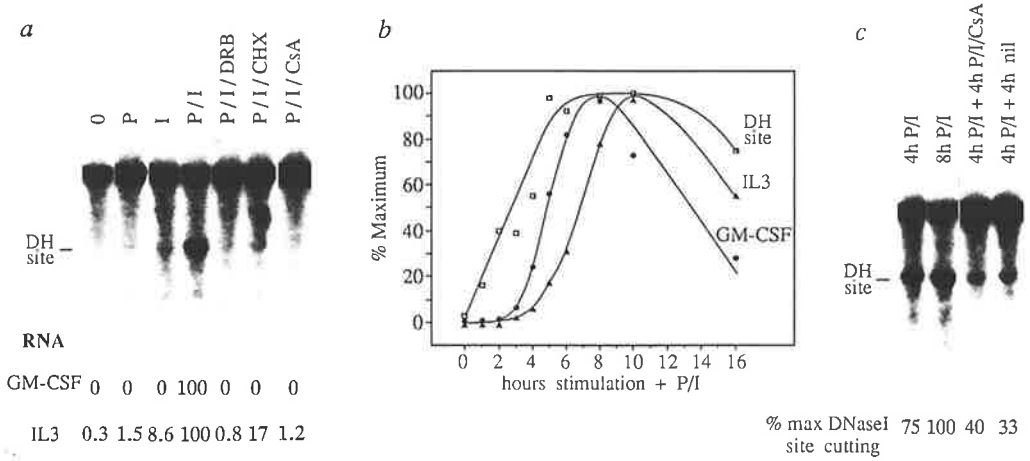
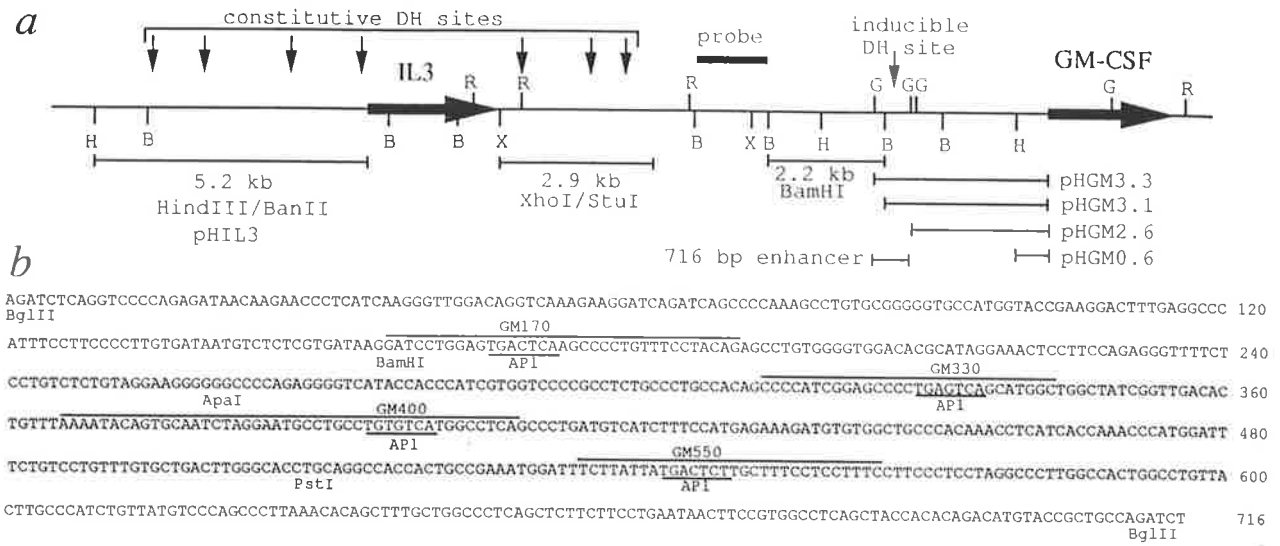


FIG. 1. An inducible DH site exists upstream of the GM-CSF gene. (*a*) Induction of the DH site and mRNA expression in Jurkat cells following a 4-h incubation with the indicated stimuli. The upper band is the intact 9.4-kb *Eco*RI fragment encompassing the GM-CSF gene, while the lower band is a 3.9-kb subfragment generated by DNase I digestion (see Fig. 2*a*). The numbers below indicate relative accumulation of GM-CSF and IL-3 mRNA in the same cultures. (*b*) Time course of induction of the DH site (□) and GM-CSF (●) and IL-3 (▲) mRNA accumulation after stimulation with PMA and Ca^{2+} ionophore. (*c*) The stability of the DH site following induction was tested by either adding CsA after 4 h of an 8-h PMA/Ca^{2+} ionophore stimulation period or by removing the stimulus at 4 h and incubating for an additional 4 h in medium alone (nil). The first lane provides a measure of the extent of DH site formation after just 4 h of stimulation. 0, unstimulated; P, PMA at 20 ng/ml; I, 2 μM Ca^{2+} ionophore A23187; DRB, 100 μM dichlorobenzimidazole riboside; CHX, cycloheximide at 20 μg/ml; CsA, 0.1 μM. Inhibitors were added 10 min before PMA and A23187.



AGATCTCAGGTCCCCAGAGATAACAAGAACCCTCATCAAGGGTTGGACAGGTCAAAGAAGGATCAGATCAGCCCCAAAGCCTGTGCGGGGGTGCCATGGTACCGAAGGACTTTGAGGCCC 120

ATTTCCTTCCCCTTGTGATAATGTCTCTCGTGATAAGGATCCTGGAGTGACTCAAGCCCCTGTTTCCTACAGAGCCTGTGGGGTGGACACGCATAGGAAACTCCTTCCAGAGGGTTTTCT 240

CCTGTCTCTGTAGGAAGGGGGGCCCCAGAGGGGTCATACCACCCATCGTGGTCCCCGCCTCTGCCCTGCCACAGCCCCATCGGAGCCCCTGAGTCAGCATGGCTGGCTATCGGTTGACAC 360

TGTTTAAAATACAGTGCAATCTAGGAATGCCTGCCTGTGTCATGGCCTCAGCCCTGATGTCATCTTTCCATGAGAAAGATGTGTGGCTGCCCACAAACCTCATCACCAAACCCATGGATT 480

TCTGTCCTGTTTGTGCTGACTTGGGCACCTGCAGGCCACCACTGCCGAAATGGATTTCTTATTATGACTCTTGCTTTCCTCCTTTCCTTCCCTCCTAGGCCCTTGGCCACTGGCCTGTTA 600

CTTGCCCATCTGTTATGTCCCAGCCCTTAAACACAGCTTTGCTGGCCCTCAGCTCTTCTTCCTGAATAACTTCCGTGGCCTCAGCTACCACACAGACATGTACCGCTGCCAGATCT 716

FIG. 2. Location and sequence of the inducible DH site. (*a*) Restriction enzyme map of the human GM-CSF/IL-3 locus displaying the location of DH sites (vertical arrows). The map was derived from the phage clones λJ1-16 (2) and λ-66 (obtained from Y.-C. Yang and S. Clark) and the plasmid pCH5.2 (11). The solid bar above the map represents the DNA probe used to map the inducible DH site. The bars below indicate DNA fragments tested in subsequent transcriptional analyses. H, *Hin*dIII; B, *Bam*HI; R, *Eco*RI; X, *Xho* I; G, *Bgl* II. (*b*) DNA sequence of the 716-bp *Bgl* II fragment spanning the DH site. AP1-like elements are underlined, whereas DNA probes and competitors used in subsequent electrophoretic mobility shift assays are indicated above the sequence. The DH site lies between the *Apa* I and *Pst* I sites. The DNA sequence was determined using an Applied Biosystems automated sequencer, by sequencing each strand of overlapping DNA clones derived from λJ1-16.

GM-CSF and IL-3 Are Regulated by an Inducible CsA-Sensitive Enhancer. The inducible DH site was tested for transcriptional regulatory activity in transient transfection assays in Jurkat cells. Segments of up to 3.3 kb of DNA, extending upstream from the GM-CSF gene transcription start site, were linked to the CAT reporter gene (pHGM0.6–pHGM3.3; Fig. 2*a*). DNA segments that include the DH site (pHGM3.3 and pHGM3.1) had little constitutive activity, but mediated an 80- to 95-fold induction of CAT in response to PMA plus Ca^{2+} ionophore (Fig. 3*a*). In contrast, very weak induction of CAT was supported by GM-CSF promoter segments lacking the DH site (pHGM2.6 and pHGM0.6; Fig. 3*a*). Furthermore, a 716-bp *Bgl* II fragment of DNA encompassing the DH site functioned as a powerful inducible enhancer when linked directly to the 0.6-kb GM-CSF proximal promoter fragment in both its normal (pHGMB716; Fig. 3*b*) and reverse orientation (not shown). No additional enhancers were detected upon testing a 2.2-kb *Bam*HI fragment immediately upstream of the inducible DH site or a 2.9-kb *Xho* I/*Stu* I fragment encompassing three constitutive DH sites just downstream of the IL-3 gene (Fig. 2*a*; data not shown).

The enhancer was also required for efficient induction of the IL-3 promoter in transient transfection assays. A CAT gene plasmid containing 5.2 kb of the IL-3 promoter and upstream DNA, including four upstream DH sites, had a moderate basal activity, but was only weakly induced by PMA and the Ca^{2+} ionophore (pHIL3; Figs. 2*a* and 3*b*). However, a reduced basal activity and a 10-fold induction of CAT was obtained when the 716-bp enhancer was coupled to the IL-3 promoter, 5.2 kb upstream of the CAT gene (pHIL3B716; Fig. 3*b*). Similar results were obtained when the enhancer was placed either upstream of the promoter or downstream of the CAT gene in an IL-3 promoter/CAT gene construct containing just the 315-bp *Stu* I/*Ban* II fragment from immediately upstream of the IL-3 transcription start site (data not shown).

The enhancer appeared to be the major target for CsA in the GM-CSF/IL-3 locus, since CsA suppressed the enhancer-driven activation of both promoters (pHGMB716 and pHIL3B716; Fig. 3*b*). In contrast, CsA had little inhibitory effect on the GM-CSF promoter alone (pHGM0.6; Fig. 3*b*) and unexpectedly led to a slightly increased induction of both the 5.2-kb (pHIL3; Fig. 3*b*) and 315-bp (data not shown) IL-3 promoter constructs used in this study.

The Enhancer Binds AP1 and NFAT. The 716-bp enhancer fragment was sequenced and found to encompass four consensus binding sequences for the inducible CsA-resistant transcription factor AP1 (17, 18) (Fig. 2*b*). Two of these sites (located at positions 170 and 330) match perfectly the TGAGTCA AP1 consensus, while the other two (located at positions 400 and 550) have a single base mismatch. The AP1 element at position 550 is, however, identical to a functional AP1 element in the IL-2 promoter (19, 20) (Fig. 4). Furthermore, this AP1 element is contained within a broader region (segment GM550; Fig. 2*b*) homologous to an NFAT site located in a more distal region of the IL-2 promoter (Fig. 4). NFAT could, therefore, also participate in the CsA-sensitive activation of the GM-CSF and IL-3 genes in Jurkat cells. Indeed, three linked copies of the GM550 segment functioned as a strong inducible enhancer inhibited by CsA, when placed upstream of the GM-CSF promoter (pHGMN3; Fig. 3*b*).

To identify the transcription factors that mediate enhancer function, we assayed fragments of the enhancer in electrophoretic mobility shift assays with Jurkat cell nuclear extracts prepared from unstimulated cells and cells stimulated in the presence and absence of CsA (Fig. 5*a*). Complexes induced by PMA and Ca^{2+} ionophore were formed with four DNA segments encompassing AP1 sites (GM170, GM330, GM400, and GM550 as in Fig. 2*b*). Each probe formed a CsA-resistant complex that comigrated with a stromelysin gene (21) AP1 complex. Furthermore, all four AP1 sites were protected by recombinant c-jun protein (18), a component of AP1, in DNase I footprinting assays (data not shown). Three of the probes (GM170, GM330, and GM550) also formed inducible CsA-sensitive complexes that comigrated with an inducible complex formed with the distal NFAT site from the human IL-2 gene (6, 8, 22). The IL-2 NFAT probe formed two inducible CsA-inhibitable complexes similar to those detected with a mouse IL-2 gene NFAT site (22). The lower of these complexes comigrated with AP1 and may be a previously described component of NFAT that lacks AP1 (22). All

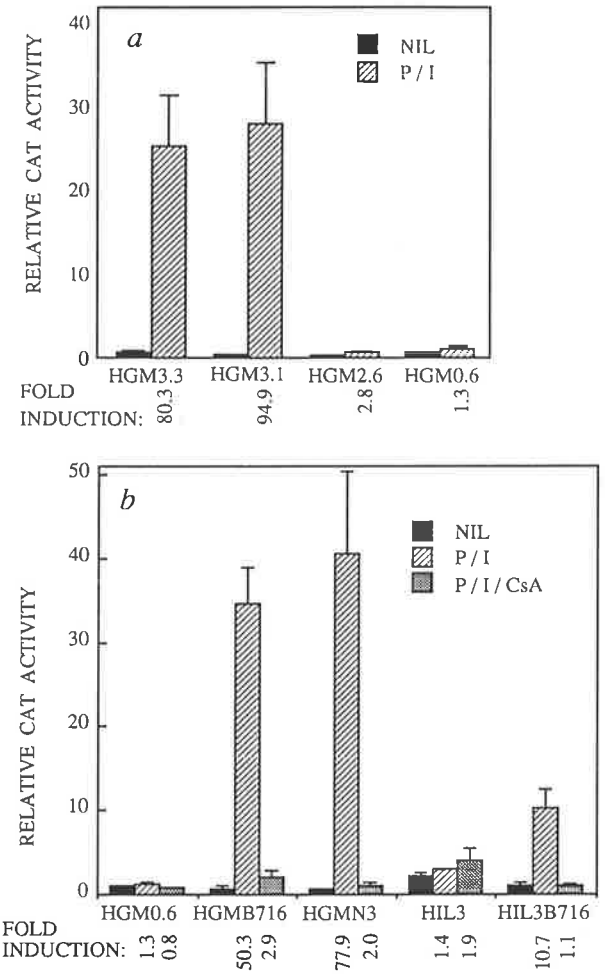


FIG. 3. Identification of a transcriptional enhancer encompassing the inducible DH site. (*a*) Stepwise deletions of a 3.3-kb segment of DNA upstream of the GM-CSF gene reveal an inducible enhancer located between 3.1 and 2.6 kb upstream. CAT gene constructs contained 3.3, 3.1, 2.6, or 0.6 kb of DNA from upstream of the GM-CSF transcription initiation site. (*b*) The 716-bp *Bgl* II fragment located 2.6–3.3 kb upstream of the GM-CSF gene mediates inducible CsA sensitive activation of the GM-CSF (pHGMB716) and IL-3 (pHIL3B716) promoters. pHIL3 contains 5.2 kb of DNA from immediately upstream of the IL-3 gene transcription start site. pHGMN3 contains three copies of GM550 (Fig. 2*a*) inserted upstream of the GM-CSF promoter in pHGM0.6. P, PMA; I, A23187; NIL, no additions.

six probes also bound constitutively expressed factors, which were not investigated further as they may be nonspecific.

The nature of the inducible complexes was further examined by using the stromelysin gene AP1 site and the IL-2 gene NFAT site as specific competitors (Fig. 5*b*). The AP1 competitor largely or completely inhibited binding of AP1-like factors to all four AP1 sites in the enhancer. The formation of the upper NFAT-like complexes was, in each case, inhibited not only by the NFAT competitor but also by the AP1 competitor. Conversely, the GM170, GM330, and GM550 NFAT-like elements efficiently inhibited the binding of both

```
IL2 AP1 site                    -144 AAGAGTCAT -136
                                     *********
GM550 NFAT/AP1  566 GAAAGGAGGAAAGCAAGAGTCATAATA  540
                    ***********     *  *****
IL2 NFAT site  -282 GAAAGGAGGAAAAACTGTTTCATACAG -256
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FIG. 4. Alignment of the GM550 NFAT/AP1-like element located at positions 566–540 in the enhancer with AP1 and NFAT binding-sites in the human IL-2 promoter (5). Stars indicate regions of identity.

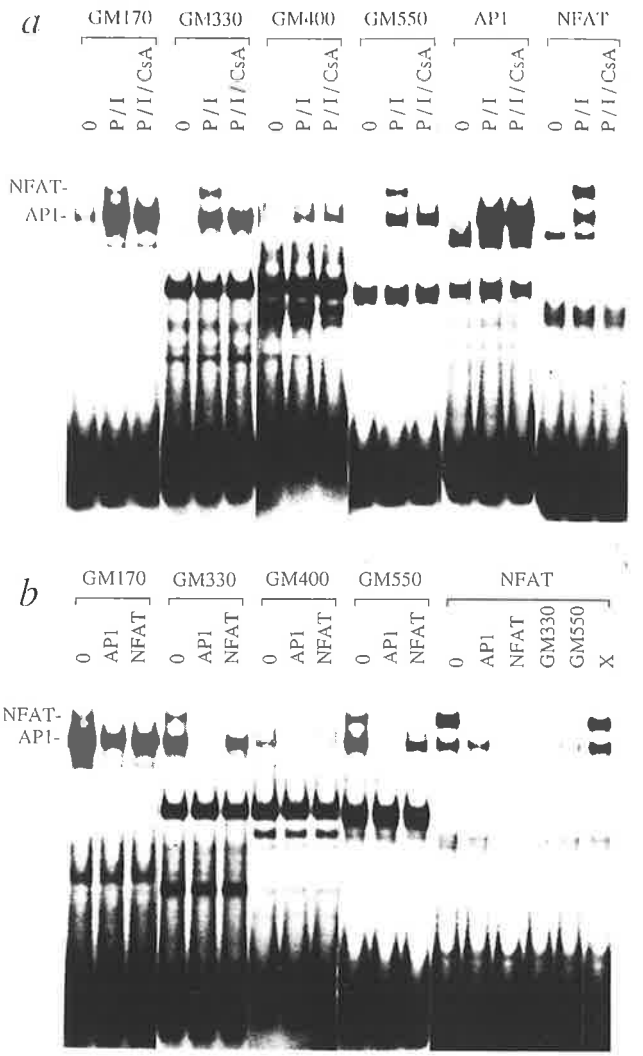

FIG. 5. Electrophoretic mobility shift assays of AP1 and NFAT-like elements in the enhancer. (*a*) Demonstration of inducible CsA-resistant AP1-like binding to the GM170, GM330, GM400, and GM550 elements and inducible CsA-sensitive NFAT-like binding to the GM170, GM330, and GM550 elements of the enhancer. The two right-hand panels served as controls and used the human stromelysin gene AP1 element (21) and the human IL-2 gene distal NFAT element (6–9). Each probe was assayed with nuclear extracts prepared from Jurkat cells that were either unstimulated (0), stimulated with PMA and Ca^{2+} ionophore (P/I), or stimulated with PMA and Ca^{2+} ionophore in the presence of 0.1 μM CsA (P/I/CsA). (*b*) Specific inhibition of AP1 and NFAT complexes in stimulated cell nuclear extracts by unlabeled competitor DNA. The probes are listed above, and the competitors are identified below each probe. X represents a nonspecific competitor.

of the NFAT-like complexes to the IL-2 gene NFAT site, whereas an unrelated DNA competitor (X) did not inhibit formation of any of the AP1 or NFAT-like complexes (Fig. 5*b*; data not shown).

DISCUSSION

Functional analyses of potential regulatory elements in the GM-CSF/IL-3 locus revealed a single enhancer that may normally be required for the coinduction of these genes in T cells. In Jurkat cells, the enhancer appeared to be the only element within this locus that responded significantly to stimulation by PMA and Ca^{2+} ionophore. Induction of the DH site within the enhancer preceded the onset of IL-3 and GM-CSF mRNA accumulation, suggesting that activation of

the IL-3 and GM-CSF proximal promoters requires prior activation of the enhancer.

Activation of the enhancer appeared to involve remodeling of its chromatin structure and binding of specific transcription factors. Since DH site induction and enhancer activation occurred in response to PMA and A23187, the enhancer is a likely target for protein kinase C and Ca^{2+}-transduced signals resulting from activation of the T-cell receptor. Consistent with this view is the observation that AP1 and NFAT, which interact with the enhancer, are both induced in T cells after T-cell receptor stimulation. Interestingly, the enhancer may also function in the repression of basal transcription.

DNA binding and competition studies strongly suggested that four sites within the enhancer associate, to differing degrees of affinity, with the AP1 family of transcription factors. Two of these sites (GM170 and GM330) perfectly match the AP1 consensus and formed AP1-like complexes almost as efficiently as the stromelysin gene AP1 element used as a reference in this study. The GM550 AP1 element, which is identical to the IL-2 gene AP1 site, bound AP1 less efficiently, whereas the GM400 AP1 element bound AP1 only very weakly. Further studies are required to determine whether all of these AP1 sites are functional components of the enhancer.

Good evidence was obtained suggesting that the T cell-specific transcription factor NFAT associates with three sites in the enhancer. Each site satisfied the operational definition of NFAT sites, in that they formed inducible CsA-sensitive complexes resembling NFAT and competed with an IL-2 gene NFAT site for NFAT binding. At least one of these sites (GM550) functioned as a powerful inducible, CsA-inhibitable enhancer element (pHGMN3; Fig. 3*b*).

Induction of NFAT occurs via the assembly of two distinct components, in the following fashion. A preexisting cytosolic component (NFATc) migrates in a CsA-inhibitable fashion to the nucleus, where it associates with a newly synthesized component (NFATn) that is not inhibited by CsA (9). While the identity of NFATc is unknown, Jain *et al.* (22) have proposed that NFATn is an AP1-like factor. Accordingly, we and others (22) have observed that AP1 DNA competitors inhibit both NFAT and AP1 binding. Interestingly, all three NFAT-like elements defined here encompass AP1 sites. From this we not only conclude that an AP1-like factor is an essential component of the NFAT complex but further predict that the AP1-like component of NFAT interacts directly with AP1-like consensus elements in at least some NFAT sites. Note that the distal IL-2 NFAT site also includes an AP1-like element (TGttTCA; Fig. 4), which appears unable to bind AP1 independently, but may represent the site of interaction with an AP1-like component of the NFAT complex.

NFAT binding sites also typically encompass homopurine segments, and an ets-like GGA core sequence (23) within this region may be essential (22). Significantly, the GM550 and IL-2 NFAT sites share identity across 12 bp of the homopurine segment. The GM330 NFAT site also retains the GGA motif in the same position relative to the AP1-like motif as it exists in the IL-2 and GM550 sites, whereas the GM170 NFAT site includes a conserved AGGAAA motif at a slightly greater distance. NFATc is a good candidate for the protein that interacts with the purine region, although it is yet to be established whether NFATc is itself a DNA binding protein. We and others have, however, observed formation of a second faster migrating complex with IL-2 NFAT probes (Fig. 5; ref. 22), which is CsA-sensitive and does not appear to contain AP1 and could therefore represent the NFATc component.

Unlike the IL-2 NFAT site, the GM170, GM330, and GM550 NFAT sites each had the capacity to bind AP1 independently of NFAT. These sites may represent an additional class of NFAT sites that retain some function in cells other than T cells, which lack the T cell-specific protein NFATc. It will be of interest to determine whether the enhancer mediates activation of the GM-CSF gene in the wide range of cell types that express GM-CSF but lack NFAT.

The enhancer can also account for the CsA sensitivity of the locus, via mechanisms similar to those previously observed for the IL-2 gene (5–9), which involve inhibition of induction of NFAT and suppression of a DH site. The enhancer remains the only element identified in the locus that is both highly inducible and inhibitable by CsA. By inhibiting the enhancer, and consequently GM-CSF and IL-3 expression, CsA may be suppressing elements of the immune response that contribute to graft rejection, autoimmunities, and allergic diseases such as asthma (24).

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Human Granulocyte-Macrophage Colony-Stimulating Factor Enhancer Function Is Associated with Cooperative Interactions between AP-1 and NFATp/c

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The promoter of the human granulocyte-macrophage colony-stimulating factor gene is regulated by an inducible upstream enhancer. The enhancer encompasses three previously defined binding sites for the transcription factor NFAT (GM170, GM330, and GM550) and a novel NFAT site defined here as the GM420 element. While there was considerable redundancy within the enhancer, the GM330, GM420, and GM550 motifs each functioned efficiently in isolation as enhancer elements and bound NFATp and AP-1 in a highly cooperative fashion. These three NFAT sites closely resembled the distal interleukin-2 NFAT site, and methylation interference assays further defined GGA(N)$_5$TCA as a minimum consensus sequence for this family of NFAT sites. By contrast, the GM170 site, which also had conserved GGA and TCA motifs but in which these motifs were separated by 15 bases, supported strong independent but no cooperative binding of AP-1 and NFATp, and this site functioned poorly as an enhancer element. While both the GM330 and GM420 elements were closely associated with the inducible DNase I-hypersensitive site within the enhancer, the GM420 element was the only NFAT site located within a 160-bp *Hin*cII-*Bal*I fragment defined by deletion analysis as the essential core of the enhancer. The GM420 element was unusual, however, in containing a high-affinity NFATp/c-binding sequence (TGGAAAGA) immediately upstream of the sequence TGACATCA which more closely resembled a cyclic AMP response-like element than an AP-1 site. We suggest that the cooperative binding of NFATp/c and AP-1 requires a particular spacing of sites and that their cooperativity and induction via independent pathways ensure very tight regulation of the granulocyte-macrophage colony-stimulating factor enhancer.

The expression of the closely linked GM-CSF and IL-3 genes is induced in T cells at the transcriptional level in response to activation of the T-cell receptor and other surface molecules (19, 23). GM-CSF and IL-3 form part of the cytokine network directing the proliferation, differentiation, and function of hemopoietic cells (23). In humans, the expression of IL-3 is largely restricted to T cells, while the expression of GM-CSF is much more widespread, with GM-CSF typically being expressed in tissues at sites of inflammation.

In human T cells, the IL-3/GM-CSF locus is regulated by a CsA-sensitive enhancer that increases the inducible activities of both the GM-CSF and IL-3 promoters (8). The enhancer coincides with an inducible DH site located 3 kb upstream of the GM-CSF gene, within the 10.5-kb region that separates the IL-3 and GM-CSF genes (Fig. 1A). The enhancer increases the activity of the GM-CSF promoter by an order of magnitude and responds to signals that mimic activation of the T-cell receptor. Transfection studies with Jurkat T cells indicate that activation of the enhancer depends upon both induction of Ca^{2+} flux and mobilization of protein kinase C and that this activation is blocked by CsA (8). This same pattern of CsA-sensitive induction exists for the transcription factor NFAT (nuclear factor of activated T cells) (28, 30, 31), which was previously shown to mediate the CsA-sensitive activation of the IL-2 promoter (21). It is therefore significant that the only transcription factor-binding sites previously identified within the enhancer are three NFAT-binding sites and a weak AP-1-binding site (8) (Fig. 1B).

NFAT is a heterogeneous complex that typically contains members of both the NFAT and AP-1 families of transcription factors (4, 8, 17, 18, 24, 26), and the induction of NFAT consequently requires at least two distinct activation pathways (17, 33). The NFAT family currently consists of two closely related proteins, termed NFATp and NFATc (22, 25), that share a conserved DNA-binding domain which itself resembles the conserved DNA-binding domain of the Rel/NF-κB family of transcription factors (24, 28). NFATp and NFATc are expressed in the cytoplasm in T cells and translocate to the nucleus in response to increases in cytosolic Ca^{2+} through a process requiring the CsA-inhibitable phosphatase calcineurin (7, 27). Although NFATp is expressed constitutively in the cytoplasm, NFATc is reported to be induced at the transcriptional level upon T-cell activation (25). NFATp and NFATc have similar sizes and can bind independently to the IL-2 NFAT site at a low affinity to generate indistinguishable complexes in gel mobility shift assays. In the nucleus, NFATp and NFATc typically associate with NFAT sites in conjunction with members of the AP-1 family of proteins (4, 18, 26), even though NFAT sites sometimes have very poor AP-1-binding sites (28). AP-1 ordinarily binds to the consensus sequence TGAGTCA as a heterodimer of a member of the Fos family

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and a member of the Jun family of proteins (1, 9). The AP-1 proteins are induced principally via the Ras and protein kinase C pathways, thus accounting for the dependence of NFAT upon both the Ca^{2+} and protein kinase C pathways, which are normally induced upon T-cell receptor activation (21, 24, 33).

The three previously defined NFAT sites found in the GM-CSF enhancer each resemble the distal IL-2 promoter NFAT site in that they have loosely conserved purine-rich segments positioned upstream of AP-1-like motifs (8, 28). The IL-2 promoter and GM-CSF enhancer NFAT sites are different, however, in their relative affinities for NFATp/c and AP-1 (8). Our previous binding studies (8) demonstrated that while the human distal IL-2 NFAT element is a moderate-affinity binding site for NFATp or NFATc, it lacks the ability to bind AP-1 independently. In contrast, the GM170, GM330, and GM550 NFAT sites in the GM-CSF enhancer each function as moderate- to high-affinity AP-1 sites, retaining the ability to bind AP-1 in the absence of NFATp or NFATc (8). The GM330 and GM550 sites do not, however, appear to be efficient NFATp/c-binding sites (8).

In this study we examined DNA elements that contribute to enhancer function. We defined the essential core of the enhancer and showed that it contained potential binding sites for the T-lymphotropic transcription factor CBF (16) and a novel NFAT site that encompassed a CRE-like sequence in place of the more usual AP-1 motif. Three of the four NFAT sites in the enhancer bound AP-1 and NFATp in a highly cooperative fashion. This class of NFAT sites appeared to require a particular spacing between AP-1- and NFATp/c-binding sites for cooperative binding and enhancer function.

MATERIALS AND METHODS

Abbreviations. The following abbreviations are used in this paper: DH, DNase I hypersensitive; CsA, cyclosporin A; CRE, cyclic AMP response element; PMA, phorbol 12-myristate 13-acetate; CAT, chloramphenicol acetyltransferase; IL-2, interleukin-2; GM-CSF, granulocyte-macrophage colony-stimulating factor; CBF, core-binding factor; and HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

Oligonucleotides. Oligonucleotide duplexes used as probes and competitors and in the construction of reporter plasmids had the following sequences, with complementary single-stranded regions used for cloning shown in lowercase for the upper strand only: GM170, gatcCTGTAGGAAACAGGGGCTTGAGTCACTCCAG; GM330, gatcCCCCCATCGGAGCCCCTGAGTCAGCATGGCG; GM400, gatcTCTAGGAATGCCTGCCTGTGTCATGGCCTCA; GM420, gatcCCATCTTTCTCATGGAAAGATGACATCAGGG; GM420 dCRE, gatcCCATCTTTCTCATGGAAAGATAGCACTAGGG; GM430, gatcTCACACATCTTTCTCATGGAAAGATGA; GM550, gatcTGAAAGGAGGAAAGCAAGAGTCATAATAAGA; IL-2 NFAT, gatcCGAAAGGAGGAAAAACTGTTTCATACAGAAG; and AP-1, gatcTGGATCACCCGCAGCTTGACTCATCCTTGCA.

Plasmid construction. All of the CAT reporter plasmids were based on the plasmid pHGM (8), which has a 600-bp fragment of the human GM-CSF promoter upstream of the CAT gene. Enhancer fragments and oligonucleotides were inserted into appropriate sites of the multiple cloning site located upstream of the promoter in the same orientation in relation to the promoter as that in which they exist in the GM-CSF enhancer.

Recombinant proteins. wbFos and wbJun were prepared as described previously (1). The plasmid pQE-31#1, used to make a 293-amino-acid truncated fragment of NFATp, was a gift from A. Rao and was engineered by deleting into the *Xho*I-*Sma*I fragment of the mouse NFATp gene in pNFATpXS (22) from the 3′ end. All three recombinant proteins were expressed in *Escherichia coli* with a His_6 tag, solubilized in guanidine HCl, and purified by chromatography on Qiagen Ni^{2+}-nitrilotriacetic acid-agarose according to the manufacturer's instructions.

Antibodies. The antibody R59 was raised against recombinant truncated NFATp prepared from pNFATpXS (22). The antibody 67.1 was raised against peptide 72 of purified mouse NFATp (22). These antibodies were a gift from A. Rao and cross-react between human and mouse NFATp. The 7A6 monoclonal antibody raised against human NFATc residues 1 to 654 (25) was provided by G. Crabtree.

Transfection assays. Enhancer activities were determined by transfecting 10 μg of each CAT reporter construct into 5×10^6 Jurkat T cells and measuring CAT expression after stimulation with 3 μM A23187 (calcium ionophore) and 20 ng of PMA per ml for 6 h in the presence and absence of 0.1 μM CsA, as described previously (8).

Gel electrophoretic mobility shift assays. Gel shift assays and preparation of nuclear extracts were as previously described (8), except that assays employed 5 μg of nuclear extract and 2 μg of poly(dI-dC) in a 15-μl volume. In some instances nuclear extracts were enriched by binding to heparin-Sepharose, washing with 0.15 M KCl, and eluting with 0.45 M KCl before the final dialysis. Assays of nuclear proteins used 5 μg of nuclear extracts prepared from unstimulated cells and cells stimulated for 2 to 3 h with 20 ng of PMA per ml and 2 μM A23187 in the presence and absence of 0.1 μM CsA. Assays of recombinant proteins used 8 ng of wbJun and 5 ng of wbFos as the source of AP-1 (1) and 0.5 ng of truncated NFATp with 100 ng of poly(dI-dC) and 0.2 ng of probe. When indicated, 0.05 μl of 67.1 antiserum, 0.2 μl of R59 antiserum, 0.2 μl of preimmune antiserum, or 0.1 μl of 7A6 ascites was included.

DNase I footprinting. Probes were prepared by 5′ end labelling the *Hin*cII-*Bal*I fragment at either end with [^{32}P]ATP. Nuclear extracts enriched by heparin-Sepharose chromatography were prepared as described above from Jurkat cells stimulated for 2 h with 20 ng of PMA per ml and 2 μM A23187. Approximately 5 ng of probe was incubated for 30 min at 22°C in a 100-μl volume of 20 mM HEPES (pH 7.9)–50 mM KCl–10 mM NaCl–1 mM dithiothreitol–10% glycerol with 25 μg of nuclear extract, 1 μg of poly(dI-dC), and 5 μg of tRNA and then digested with 1 U of DNase I per ml for 30 s at 22°C after addition of $MgCl_2$ to 3 mM and $CaCl_2$ to 0.1 mM. A protein-free DNase I control employed 0.1 U of DNase I per ml. Purified DNA recovered from each digest was examined on a DNA sequencing gel alongside a G+A DNA sequencing reaction.

Methylation interference assays. Probes were prepared from *Xho*I-*Nru*I or *Eco*RV-*Hin*dIII fragments of plasmids in which single copies of the GM170, GM330, GM420, GM430, and GM550 oligonucleotides were inserted at the *Bgl*II site of pHGM. Probes were ^{32}P end labelled by using either the *Hin*dIII or *Xho*I 5′ end and partially methylated at guanine residues with dimethyl sulfate. Methylation interference assays were performed essentially as described previously (3) with modifications (32), with stimulated Jurkat cell nuclear extracts enriched by heparin-Sepharose chromatography. Briefly, gel mobility shift assays were performed as described above (with a fivefold scale-up), the gels were electrotransferred to NA-45 membranes, NFAT complexes and free probe bands were excised, and DNA was eluted and cleaved with 10% piperidine–1 M NaCl–5 mM EDTA before purification and electrophoresis on 10% acrylamide gels containing 8 M urea, 50 mM Tris acetate, 20 mM Na acetate, and 2 mM EDTA, pH 8.

RESULTS

Definition of the GM-CSF enhancer core. A 716-bp *Bgl*II fragment encompasses all of the GM-CSF enhancer elements required for efficient activation of the GM-CSF promoter in transfection assays in the Jurkat human T-cell line (8) (Fig. 1A). We previously defined three NFAT-binding sites in the enhancer, which were named according to their positions in the 716-bp sequence (GM170, GM330, and GM550; Fig. 1B), and a weak AP-1-binding site (termed GM400) that is unlikely to make a significant contribution to enhancer activity (8). To ascertain the relative contributions that different elements of the enhancer make to its inducible activity, stepwise deletions were made into the enhancer from either direction (Fig. 1B). These deletions were designed to remove each of the NFAT sites in turn. Enhancer fragments subcloned into the GM-CSF promoter/CAT reporter gene plasmid pHGM (8) were tested for inducible enhancer activity in transient transfection assays with Jurkat T cells (Fig. 1B). Following transfection, cells were stimulated with a combination of phorbol ester and calcium ionophore to mimic T-cell receptor activation.

Initial deletion analyses indicated that the 425-bp *Bam*HI-*Bal*I subfragment that included the three NFAT sites and the weak AP-1 site (pBB425; Fig. 1B) retained full enhancer activity. Since the 425-bp *Bam*HI-*Bal*I fragment of the enhancer had, if anything, a slightly greater activity than the previously defined 716-bp *Bgl*II fragment, all subsequent enhancer activities have been normalized as a percentage of the activity obtained with the plasmid pBB425 and compared with the activity obtained with pHGM, which contains the promoter alone. The fold induction of each construct relative to an unstimulated control is also shown in Fig. 1B.

Only marginal decreases in activity occurred upon deletion of either the GM170 region or the GM550 region from

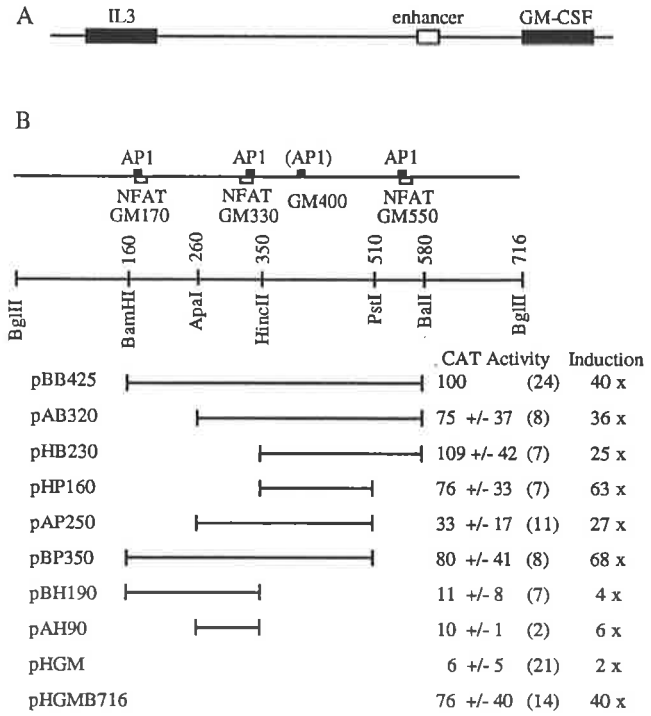

FIG. 1. Expression of GM-CSF enhancer/CAT reporter plasmids in Jurkat cells. (A) Location of the GM-CSF enhancer in the 10.5-kb intergenic region between the IL-3 and GM-CSF genes. (B) Deletion analysis of the 716-bp *Bgl*II fragment of the GM-CSF enhancer. Enhancer fragments coupled to the GM-CSF promoter in the CAT reporter plasmid pHGM were transfected into Jurkat cells. CAT activities of cell lysates were determined after stimulation of the cells for 6 h with 20 ng of PMA per ml and 3 μM A23187. Enhancer subclones were named by using the initial letters of the enzyme sites used for cloning and the approximate lengths of the fragments. The activity of each plasmid was expressed relative to that of pBB425, which was included in each of a total of 15 independent transfection experiments. Activities are given together with the standard deviation, and the number of times each plasmid was assayed is shown in parentheses. The fold induction represents the response of each plasmid relative to that of an unstimulated control for each plasmid.

pBB425, since the plasmids lacking either one of these sites (pAB320 and pBP350) had activities in the same range as those of pBB425 and pHGMB716. Deletion of both the GM170 and GM330 regions from pBB425 also had little effect, indicating that most of the activity of the enhancer was confined to the 230-bp *Hinc*II-*Bal*I fragment (pHB230; Fig. 1B), which retains only one of the three previously defined NFAT sites (GM550). The plasmid pHB230 was 18 times as active as pHGM and supported a 25-fold induction of CAT activity in response to stimulation with PMA and A23187. In contrast, the plasmid pBH190, which encompassed the upstream half of the enhancer, was only twice as active as pHGM even though it encompassed two of the three NFAT sites. Surprisingly, only a one-third decrease in activity was observed when the GM550 site was also deleted from pHB230 to create pHP160. Thus, the 160-bp *Hinc*II-*Pst*I fragment defined here as the essential core of the enhancer retained 76% of the activity obtained with pBB425 but included none of the previously defined NFAT sites. This core fragment was also tightly regulated, since it supported a 63-fold induction of CAT activity upon stimulation, and its activity was reduced by 80% when stimulated in the presence of CsA. Longer enhancer fragments were, however, slightly more sensitive to CsA, since the activities of the plasmids pHGMB716, pBB425, pAB320, pAP250, and pBP350 were reduced by 90 to 98% in the presence of CsA (data not shown).

It is likely that a high degree of redundancy operates in the utilization of activation elements within the enhancer, since the GM170 and GM550 regions did make small contributions to the activity of the enhancer core in plasmids that were already missing one or two NFAT sites. Thus, approximately twofold decreases in activities were observed when the 100-bp *Bam*HI-ApaI fragment was deleted from pBP350 to create pAP250 and when the 70-bp *Bal*I-*Bgl*II fragment was deleted from pAB320 to create pAP250 and from pHB230 to create pHB160. These differences were consistently observed in independent assays.

There may also exist an inhibitory element in the 90-bp *Apa*I-*Hinc*II fragment. The plasmids pBH190 and pAH90 had little activity on their own even though they encompass NFAT sites, and the plasmid pAP250 was only 43% as active as the enhancer core plasmid (pHP160) even though it included the GM330 NFAT site in addition to the core. This observation was reproducible, since pAP250 was consistently much less active than pHP160 in the three assays in which both plasmids were assayed on the same occasion and could thus be compared directly.

The essential core of the enhancer lies within a DH site. The earliest detectable event during the activation of the IL-3/GM-CSF locus is the appearance of the CsA-suppressible DH site within the enhancer (8). To determine whether the DH site was closely associated with either the essential core of the enhancer or any of the NFAT sites, the position of the DH site was finely mapped in nuclei isolated from activated Jurkat cells. Sites of DNase I cleavage were mapped from both directions by Southern blot hybridization analysis by utilizing *Bgl*I sites located 1.9 and 3.6 kb upstream of the GM-CSF gene (Fig. 2).

When mapped from downstream with probe A, the DH site appeared as a broad region approximately 150 to 250 bp across located principally within the essential core of the enhancer. The most intense DNase I cutting occurred between positions 350 and 400 in the sequence of the 716-bp *Bgl*II fragment, and a second zone of DNase I cutting was greatest between positions 450 and 500 (Fig. 2A, arrows). A somewhat protected region existed between positions 400 and 450, which became more visible when mapped in even finer detail from the downstream *Bam*HI site (data not shown).

As might be expected in a region where DNase I cuts frequently, the mapping of DNase I cleavage sites was biased towards the direction from which they were mapped. Thus, probe B revealed the strong site between positions 350 and 400 and an additional frequently cut region between positions 250 and 300. The DH site again appeared as a broad region about 150 bp across with two high-intensity bands (Fig. 2B, arrows) flanking a protected section that in this instance spanned the GM330 element. Consequently, the DH site probably existed as a 250-bp DH region defined by the *Apa*I and *Pst*I sites, with two protected regions coinciding with the GM330 element and a site near position 425.

Two nuclear factors associate with the essential core of the enhancer. Since previously identified transcription factor binding sites could only partially account for the induction of the DH site and the properties of the enhancer, DNase I footprint analysis (10) was used to seek additional regulatory elements within the essential core of the enhancer. Probes were made by labelling the *Hinc*II-*Bal*I fragment of the enhancer at either end and assayed with nuclear extracts prepared from activated Jurkat cells. Two regions within the essential core were partially protected in similar positions on both strands of the sequence (labelled FP on the upper strand shown in Fig. 3). One of these regions encompassed the sequence TGGAAA

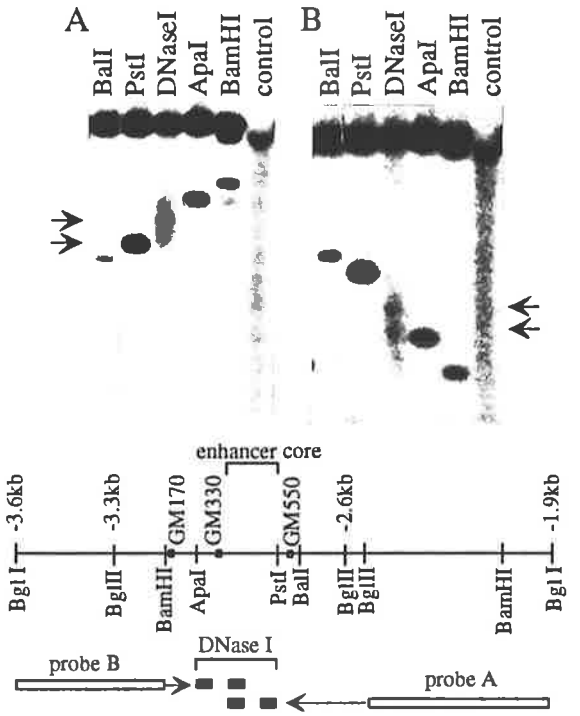

FIG. 2. Fine mapping of the DH site in nuclei isolated from Jurkat cells activated for 7 h with 2 μM A23187 and 20 ng of PMA per ml and digested with 5 μg of DNase I per ml. The DH site was mapped as described previously (8) from downstream (A) and upstream (B) by Southern blot hybridization analysis with probes A and B, respectively, and utilizing *Bgl*I sites located 1.9 (A) and 3.6 (B) kb upstream of the GM-CSF gene. All DNA samples were digested to completion with *Bgl*I. The marker lanes contain intact purified Jurkat cell DNA partially digested with *Bal*I, *Pst*I, *Apa*I, or *Bam*HI. The control lane contains a DNase I digest of intact purified DNA. The same filter was used for both panels. Probes A and B (open boxes) were *Bgl*II-*Bam*HI and *Bgl*II-*Bgl*I fragments originating from the lambda clone J1-16 (8). Areas of enhanced DNase I cleavage within the DH site are indicated by arrows and displayed as solid boxes. The location of the 160-bp *Hinc*II-*Bal*I enhancer core fragment is bracketed.

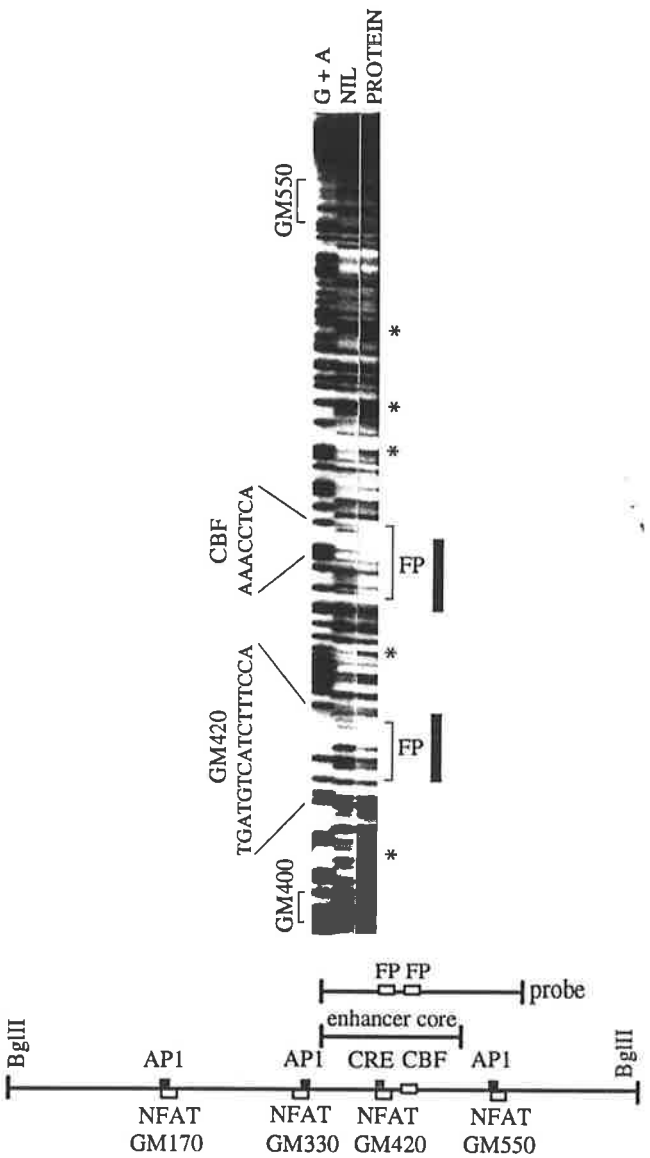

FIG. 3. DNase I footprint analysis of nuclear protein-binding sites within the enhancer core. The data were obtained with a probe labelled on the upper strand, and brackets indicate footprinted (FP) regions protected from DNase I digestion. Solid boxes indicate the relative positions of complementary regions protected on the lower strand (data not shown). Asterisks highlight regions of enhanced DNase I cleavage. The three lanes represent a G+A sequencing reaction, the DNase I digestion products of protein-free DNA (NIL), and a DNase I digestion of probe complexed with activated Jurkat cell nuclear protein.

GATGACATCA, which was subsequently found to be homologous to NFAT sites present elsewhere in the enhancer and in the IL-2 promoter. This element was centered at approximately position 420 in the sequence of the 716-bp *Bgl*II fragment of the enhancer and therefore has been defined here as the GM420 element, in keeping with previously identified motifs in the enhancer. Significantly, this element lies within the DH site between two of the regions of highest-frequency DNase I cleavage (Fig. 2A). Like other NFAT sites, the GM420 element contains a conserved GGA core within a purine-rich region (28). Interestingly, a highly homologous second purine-rich region (GAGAAAGATG) exists as an inverted repeat just downstream of the GM420 element, but this repeated sequence does not contain a GGA core. The GM420 element is unusual, however, in that the conserved purine motif lies upstream of a CRE-like sequence (TGACATCA) (5) rather than upstream of an AP-1 element as found in the other previously identified sites.

The second protected region was centered at position 450 and encompassed two overlapping sequences (TGCCCA CAAACCTCA) that resembled binding sites for CBF, a transcription factor implicated in the regulation of several T-cell-specific genes (16), including that for IL-3 (6). These newly identified CBF- and NFAT-like binding sites may account for the activity of the essential core of the enhancer and cooperate with the other three NFAT sites in the enhancer (summarized in Fig. 3). The GM550 and GM400 regions were not protected in the above-described assay, most likely because they encompass low-affinity factor-binding sites.

The GM420 element encompasses a strong NFATp/c-binding site. To examine the binding of NFATp/c to the GM420 region, we designed a truncated probe (GATCTCACACATC TTTCTCATGGAAAGATGAGATC) (termed here GM430) that included both the conserved purine motif TGGAAAGA and the inverted repeat sequence GAAAGATG (underlined) but lacked an intact CRE. We tested binding of the GM430 probe in gel mobility shift assays with nuclear extracts prepared from the Jurkat and HSB-2 human T-cell lines. In each case the GM430 probe associated with an inducible CsA-inhibitable

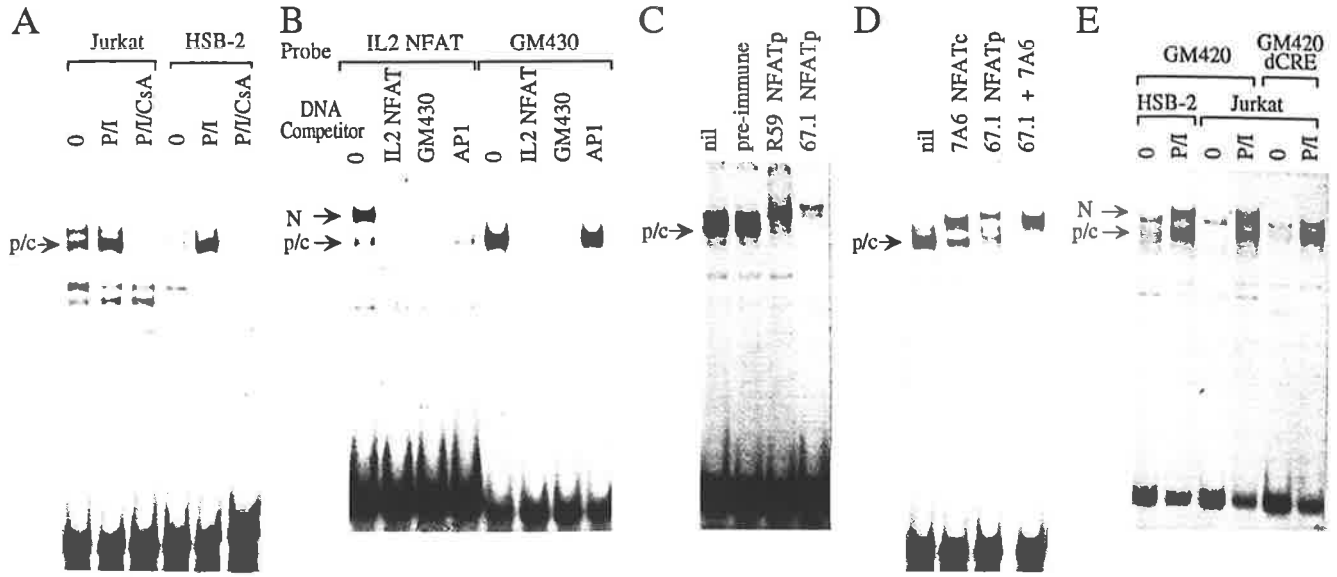

FIG. 4. Gel electrophoretic mobility shift assays of the GM420 NFAT site. (A) GM430 binding with nuclear extracts prepared from unstimulated cells (0) and cells stimulated with PMA and A23187 in the presence (P/I/CsA) and absence (P/I) of CsA. Bands migrating below the GM430 NFATp/c complex appear to represent nonspecific constitutive factors, while the complex migrating above NFATp/c is a factor unrelated to NFATp/c that often appears constitutively. (B) Assays of stimulated Jurkat cell nuclear extract binding to the GM430 and IL-2 NFAT probes in the presence and absence of 25 ng of duplex oligonucleotide competitors. (C) Assays of stimulated Jurkat cell nuclear extract binding to the GM430 probe in the presence and absence (nil) of preimmune serum, R59 antiserum directed against recombinant NFATp, and 67.1 antiserum directed against an NFATp peptide. Both NFATp antisera produced a supershifted complex characteristic of specific NFATp binding. (D) Assays of stimulated HSB-2 cell nuclear extract binding to the GM430 probe in the presence and absence of R59 and 67.1 NFATp and 7A6 NFATc antisera. (E) GM420 and GM420 dCRE binding with nuclear extracts prepared from unstimulated cells and cells stimulated with PMA and A23187 (P/I). Note that a constitutively expressed factor unrelated to NFAT binds to the GM420 probe and migrates between the NFATp/c and NFAT complexes. NFAT (N) and NFATp/c (p/c) complexes are indicated with arrows.

NFATp/c-like factor (p/c in Fig. 4A), which comigrated with the NFATp/c complex associated with the IL-2 NFAT site (Fig. 4B). Significantly, the GM430 sequence bound NFATp/c to approximately a 5- to 10-fold-greater extent than the IL-2 sequence.

The GM430 motif was formally identified as an NFATp/c-binding site on the basis of several operational criteria. The GM430 sequence efficiently inhibited NFAT and NFATp/c binding to the IL-2 NFAT site, and the IL-2 NFAT site inhibited binding of NFATp/c to the GM430 sequence (Fig. 4B). This inhibition was specific since an AP-1 competitor blocked the formation of just the upper IL-2 NFAT complex and did not affect NFATp/c binding to the GM430 probe. The GM430 NFATp/c complexes that formed with Jurkat cell extracts were also either blocked or supershifted by two different specific NFATp antibodies but not by preimmune serum (Fig. 4C) or by NFATc antibodies (data not shown). This suggested that our Jurkat NFATp/c complexes contained predominantly NFATp, since the 67.1 NFATp antibody was raised against a peptide not conserved between NFATp and NFATc. The GM430 probe did, however, have the capacity to associate with both NFATp and NFATc, since both complexes were detected when HSB-2 extracts were employed. Hence, NFATp/c complexes formed with HSB-2 extracts were partially supershifted by either NFATp or NFATc antibodies and were completely supershifted by a combination of the two antisera (Fig. 4D).

We anticipated that the full-length GM420 element would function as a composite site. Since other NFAT sites typically bind NFATp/c-like factors in conjunction with AP-1 and since CREs are closely related to AP-1 sites (5, 14, 29), we tested the ability of the full-length GM420 element to form NFAT-like complexes with nuclear extracts prepared from activated Jurkat and HSB-2 cells. As anticipated, the GM420 probe formed more-slowly migrating inducible NFAT-like complexes resembling those formed with the IL-2 NFAT site, in addition to forming NFATp/c complexes (Fig. 4E). The CRE was clearly required for the upper NFAT complex formation, since a mutation of the CRE from TGACATCA to TAGCACTA (GM420 dCRE; Fig. 4E) eliminated NFAT but not NFATp/c complex formation. We have not yet determined the composition of the upper complex, but since CREs are closely related to AP-1 sites, it could contain various combinations of AP-1 and CREB/ATF family proteins.

Like the GM420 element, the three previously identified GM-CSF enhancer NFAT sites all form complexes resembling the upper IL-2 NFAT complex (8). It was therefore of interest to determine if NFATp/c contributed to the formation of the upper NFAT complex with each site. Each of the four NFAT sites was tested in gel mobility shift assays with activated Jurkat cell nuclear extracts, which were shown above to contain predominantly NFATp rather than NFATc (Fig. 5). In each case the upper NFAT complex was specifically eliminated when the R59 antibody directed against recombinant NFATp was included, and a characteristic supershifted complex was seen with the 67.1 NFATp peptide antibody. Furthermore, the analysis of HSB-2 extracts with NFATc and NFATp antibodies indicated that each of the four NFAT sites could associate with either NFATp or NFATc (data not shown).

For each of the NFAT probes we also investigated the components of the inducible Jurkat extract complexes appearing below the NFAT band that comigrated with NFATp/c and AP-1 (Fig. 5). Previous studies suggested that these bands contained both AP-1 and NFATp/c complexes in the case of GM170 but just AP-1 in the cases of GM330 and GM550 (8). This view was confirmed in the present study, since the lower GM330 and GM550 complexes were unaffected by NFATp

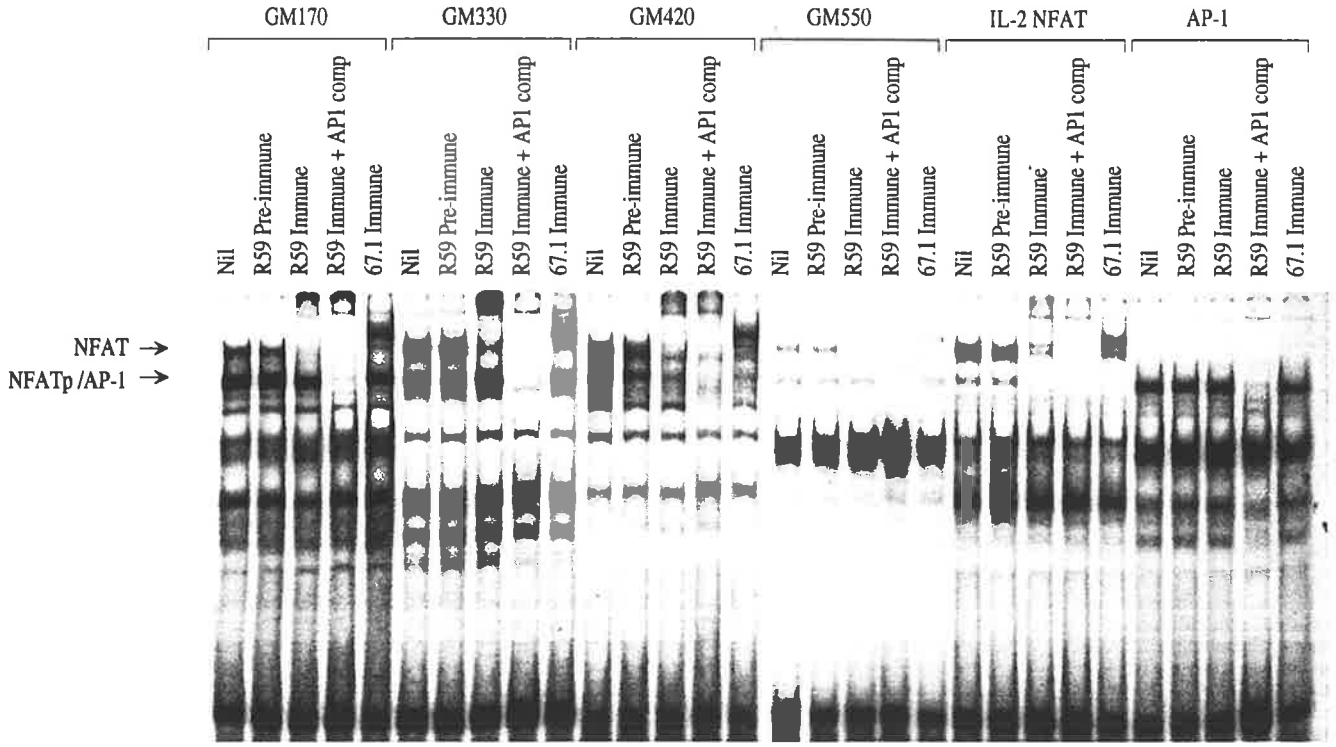

FIG. 5. Gel electrophoretic mobility shift assays of stimulated Jurkat cell nuclear extract binding to NFAT sites in the presence and absence (Nil) of preimmune antiserum, R59 antiserum raised against recombinant NFATp with or without additional AP-1 oligonucleotide competitor (AP1 comp), or 67.1 antiserum raised against an NFATp peptide. The upper arrow indicates the mobility of NFAT complexes containing NFATp/c and AP-1. The lower arrow indicates the mobility expected for individual NFATp, NFATc, and AP-1 complexes.

antibodies but were completely inhibited upon inclusion of an AP-1 oligonucleotide competitor (Fig. 5) (8). In the case of the GM170 probe, the lower complex was reduced in the presence of NFATp antibodies (Fig. 5) or competitor (8) and was completely inhibited when AP-1 competitor and NFATp/c antibodies were combined. The effects of the antibodies appeared to be specific, since they had no effect upon AP-1 binding to an AP-1 probe. The accumulated data therefore suggest that the GM170 element has roughly equal and moderately high affinities for both AP-1 and NFATp, while the GM330 and GM550 elements are very-low-affinity NFATp-binding sites. These studies also suggested that the GM420 probe was a weak AP-1-binding site, since a faint AP-1-like band remained after inclusion of NFATp antibodies and this band was eliminated by an AP-1 competitor. The ability of the four GM-CSF NFAT sites to form AP-1 complexes distinguished them from the IL-2 NFAT site, which independently associated with NFATp/c but not AP-1.

NFATp and AP-1 bind cooperatively to three of the four NFAT sites. Binding studies described above and elsewhere (8) suggest that AP-1 and NFATp/c bind together to form NFAT complexes with the GM-CSF enhancer. To verify this hypothesis and to determine whether the binding of AP-1 and NFATp to each site is cooperative, we examined the binding of bacterially expressed recombinant proteins to NFAT sites in the GM-CSF enhancer and IL-2 promoter (Fig. 6). Heterodimers of truncated cFos and cJun proteins containing the DNA-binding and leucine zipper domains were employed as the source of AP-1 (1) and assayed either in isolation or together with a truncated NFATp protein (22) corresponding to the conserved Rel domain (22, 25) in gel mobility shift assays.

The four GM-CSF enhancer NFAT sites showed widely

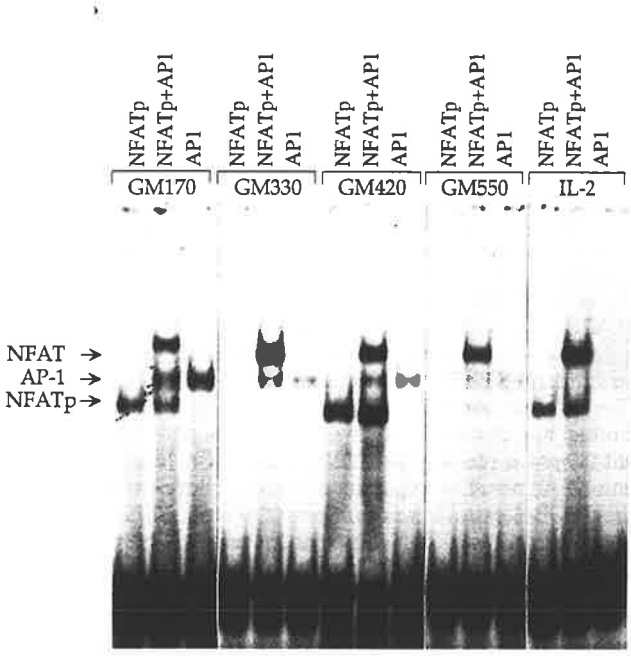

FIG. 6. Gel electrophoretic mobility shift assays of NFAT-like complexes formed with recombinant NFATp and AP-1. Binding reaction mixtures contained 8 ng of wbJun and 5 ng of wbFos (AP1) and/or 0.5 ng of truncated NFATp. Each reaction mixture contained 0.2 ng of probe and 100 ng of poly(dI-dC), and autoradiographs with different exposures have been matched to compensate for differences in specific activities of probes.

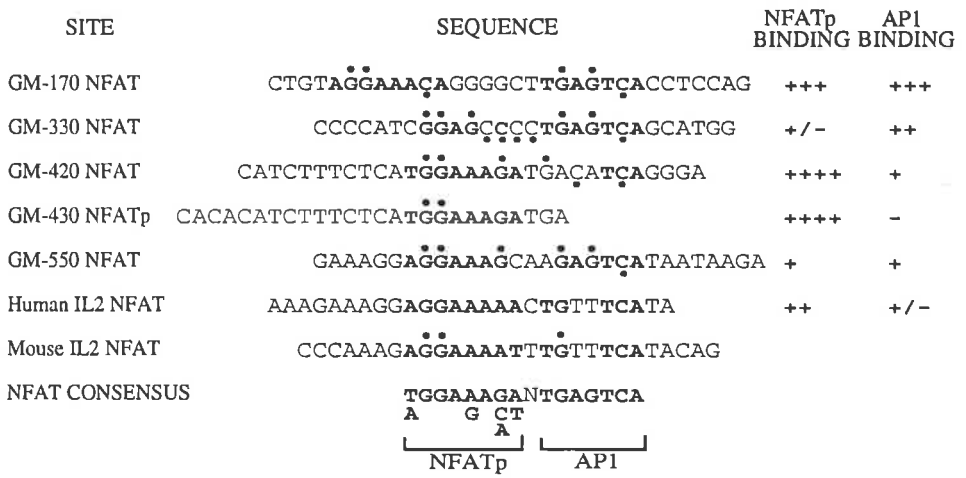

FIG. 7. Methylation interference assays of NFAT binding. Methylated DNA probes were prepared from plasmids containing the GM170, GM330, GM420, GM430, and GM550 oligonucleotides. Bound NFAT and NFATp/c complexes were recovered from gel mobility shift assays which employed 25 μg of heparin-Sepharose-purified nuclear extract prepared from Jurkat cells stimulated with 20 ng of PMA per ml and 2 μM A23187. DNA recovered from NFAT or NFATp/c complexes was electrophoresed alongside unbound probe recovered from the same gel. Assays were performed on both strands of each sequence, and G residues required for binding are indicated by circles above and below each sequence. Alongside each sequence is shown its relative binding affinity for AP-1 and NFATp, as estimated from accumulated binding and competition data. The consensus sequence derived from this subfamily of NFAT sites has taken into consideration the methylation interference data and the ability of each site to bind AP-1 and NFATp both cooperatively and independently. No methylation interference assays were performed with the human IL-2 NFAT site, and the mouse IL-2 NFAT data are taken from reference 28.

varying affinities for both NFATp and AP-1. As previously observed with nuclear extracts, the GM420 element was a high-affinity NFATp site and the GM170 and IL-2 NFAT sites were intermediate-strength NFATp sites, while the binding of NFATp to the GM330 and GM550 sites was with very low affinity. All four GM-CSF enhancer NFAT sites had the capacity to bind AP-1 in the absence of NFATp, while the IL-2 NFAT site did not. The GM170 element appeared to have the highest affinity for AP-1, in agreement with results of previous experiments that used nuclear extracts as the source of AP-1 (8) (Fig. 5).

The cooperativity of binding of AP-1 in combination with NFATp was very striking in the cases of the GM330, GM420, GM550, and IL-2 NFAT elements, where the binding of AP-1 as part of the NFAT complex was, respectively, 16-, 4-, 10-, and 400-fold greater than that obtained with AP-1 alone. Significantly, even though there was no independent binding of one of the two components for the GM330, GM550, and IL-2 sites, NFAT-like complex formation resulted in greatly enhanced binding of both components. Although recombinant AP-1 and NFATp also combined to form an NFAT-like complex with the GM170 element, there was no evidence for cooperativity in their binding, and the NFAT-like complex had a reduced mobility relative to the other four probes. This was unexpected, since the GM170 element was the only one of the sites examined that bound both the AP-1 and the NFATp components with moderate to high affinities.

To more directly compare the affinities of each of the NFAT sites for NFATp and AP-1, each element was also tested as an inhibitor of recombinant NFATp and AP-1 binding to high-affinity GM430 NFATp- and stromelysin AP-1-binding sites. The ability of each element to function as an NFATp or AP-1 competitor (data not shown) directly reflected the binding activities previously observed with nuclear extracts (Fig. 5) (8), and a summary of the relative affinities of each element for AP-1 and NFATp is included in Fig. 7. The GM430 and GM420 elements functioned equally well as high-affinity NFATp competitors, being about four times as efficient as the intermediate-affinity GM170 and IL-2 NFAT sites. The GM330 and GM550 sites, which supported no detectable NFATp/c binding, functioned poorly as NFATp competitors. As expected, the GM170 and GM330 elements functioned as efficient AP-1 competitors, while the GM420 and GM550 elements, which do not exactly match the AP-1 consensus, were about 50% as efficient as competitors.

NFAT binding requires a GGA core sequence and an AP-1 element. The most highly conserved features of the NFAT sites described here are a GGA core within a purine-rich segment and a downstream AP-1-like motif. The requirement for these regions in each of the four sites was tested by DNA methylation interference assays (3) of Jurkat cell NFAT complexes, and the data obtained from both strands are summarized in Fig. 7. These assays clearly demonstrated that both G residues in the conserved GGA core were required for NFAT binding to all four sites and for NFATp/c binding to the truncated GM430 element. The repeated GAAAGATG motif present on the opposite strands of the GM420 and GM430 probes did not appear to participate in binding. It was also evident that G residues in both halves of the AP-1-like motif contributed to NFAT complex formation in each case. Significantly, the CRE-like motif present in the GM420 probe gave a pattern similar to that obtained with the AP-1 elements present in the other NFAT sites. The finding that G methylation interfered with both NFATp/c and AP-1 binding indicated that these complexes must interact at least in part with the major groove of the DNA helix. A best-fit NFAT consensus sequence has also been derived here from the accumulated binding and methylation interference data obtained with both nuclear extracts and recombinant proteins.

Three of the four NFAT-like elements function efficiently as enhancer elements. The apparent redundancy of activation elements in the GM-CSF enhancer makes identification of individual functional elements very difficult. Therefore, to examine the potential of the NFAT-like motifs to function as enhancer elements, each site was tested as a multimer in isolation from the enhancer. Tandem arrays of three copies of each of the four individual NFAT sites were placed upstream of the GM-CSF promoter and tested for enhancer activity as

TABLE 1. Transient transfection assays of enhancer function of NFAT site multimers in Jurkat cells[a]

Construct	CAT activity[b]	Induction (fold)	CAT activity with CsA[c]
pHGMBB425	100 (8)	87	3
pGM170-3	11 ± 5 (7)	4	6
pGM330-3	79 ± 33 (7)	25	21
pGM400-3	5 ± 2 (4)	2	
pGM420-3	176 ± 55 (7)	160	3
pGM550-3	81 ± 63 (6)	37	6
pHGM	3 ± 3 (5)	3	

[a] Three head-to-tail copies of each oligonucleotide duplex were inserted into the appropriate *Bam*HI or *Bgl*II site upstream of the GM-CSF promoter in pHGM in their natural orientations relative to the promoter. Plasmids are named according to whether they contain three copies of the GM170, GM330, GM420, or GM550 NFAT site or the GM400 AP-1 site. CAT activities (relative to that of pBB425) and inductions (relative to that of an unstimulated plasmid control for each plasmid) obtained in response to activation by PMA and A23187 are expressed as in Fig. 1.

[b] Mean ± standard deviation. The number of times each plasmid was assayed is shown in parentheses.

[c] Activity obtained when stimulation was in the presence of 0.1 μM CsA.

described above (Table 1). Three of the DNA multimers (GM330, GM420, and GM550) supported inducible transcriptional activity almost as great or greater than that with the 425-bp *Bam*HI-*Bal*I fragment. The enhancer activity of each multimer was also suppressed by CsA, although this suppression was incomplete in the case of the GM330 element. Significantly, the most active element was the GM420 motif, which represented the highest-affinity NFATp/c site and formed part of the enhancer's essential core. In contrast, the GM170 element had very modest activity in this context, suggesting that a lack of cooperative binding between AP-1 and NFATp/c translates to a lack of independent function. The weak GM400 AP-1-binding site (8) had no activity when tested as an enhancer element.

DISCUSSION

The GM-CSF enhancer encompassed four NFAT sites, three of which (GM330, GM420, and GM550) were highly inducible by PMA and A23187 and were likely to account for much of the enhancer's CsA-sensitive activity. The inducible DH site within the enhancer encompassed the GM330 and GM420 elements and two overlapping CBF-like sites. The most significant of these sites was the GM420 element, which functioned as a high-affinity NFATp/c site and was the only NFAT site present in the functional enhancer core. Since NFATp is induced in the nucleus within 15 min of activation by Ca^{2+} (12), we speculate that high-affinity binding of NFATp/c to this site is one of the earliest events in the activation of the locus and one that leads to cooperative binding of AP-1 and triggers formation of the DH site. The induction of the DH site in this fashion would thus facilitate binding of other factors, such as CBF, to adjacent sites and increase the likelihood of NFATp/c gaining access to flanking lower-affinity NFATp/c sites. Since both PMA and A23187 are required for the induction of the DH site, its maintenance is likely to rely on cooperative binding of AP-1 and NFATp/c.

The NFAT sites listed in Fig. 7 that function as enhancer elements and bind NFATp/c and AP-1 in a cooperative fashion constitute a conserved family having the minimum consensus sequence GGA(N)$_5$TCA and an optimal consensus that might resemble TGGAAAAATTGAGTCAG. Most likely, just half of the palindromic AP-1 motif would be sufficient for function within an NFAT site, and this could be equally well provided by an AP-1 site or a CRE. While the NFATp/c- and AP-1-binding motifs are themselves in some cases poorly conserved, the spacing between the two is remarkably constant. In addition, both components have to contact the DNA to form the NFAT complex, and the lack of cooperativity seen for the GM170 element suggests that a particular spacing of the binding sites is required for a cooperative interaction to occur. Indeed, there is exactly one turn of the DNA helix separating the GG sequence from the TCA sequence in the consensus sequence GGA(N)$_5$TCA, which allows for direct contact between the NFATp/c and AP-1 complexes if both associate via the major groove. The observation that the GM170 motif was nonfunctional as an enhancer, even though it bound both AP-1 and NFATp/c with a high affinity, suggested that a cooperative interaction was essential for enhancer function. Conversely, each of the functional NFAT sites described here had the capacity to bind AP-1 and NFATp in a highly cooperative manner, and this cooperativity was enforced by either the AP-1- or the NFATp-binding site being a very weak binding site.

The ideal consensus binding sequence for NFATp/c remains to be determined, but high-affinity NFATp/c sites usually contain the sequence GGAAA. The sequence TGGAAA is likely to be favored over AGGAAA, since the GM420 motif is a much stronger NFATp/c-binding site than the GM550 motif and the sequence TGGA also occurs in IL-4 and tumor necrosis factor alpha gene NFAT sites that bind NFATp/c independently (28). A comparison of several high-affinity NFATp/c-binding sites (28) (Fig. 7) suggests TGGAAAAAT as an optimal NFATp/c-binding site. The 3′ T appears to be favored in the case of the mouse and human IL-2 NFAT sites (28), and a T also occurs in this position in the GM420 element.

The NFAT sites described here most likely represent a highly specific subfamily, as many NFAT sites that do not conform to the same rigidly conserved structure have now been identified. Several cytokine gene promoters have NFAT sites termed CLEO elements (19, 20), in which GGAAA core sequences directly abut weak AP-1 sites, but there is no strong cooperativity in the binding of AP-1 and NFAT to these sites (unpublished observations). In the IL-4 promoter, NFATp/c-binding sites are closely linked to Oct-1-binding sites rather than AP-1-binding sites (28). One other example of an NFATp/c site upstream of a CRE exists in the case of the tumor necrosis factor alpha promoter (12), but the spacing between the two sites is greater than that in the GM420 sequence, and AP-1 and NFATp/c do not appear to bind cooperatively to this site.

It is significant that NFAT sites can encompass either AP-1 sites or CREs. This raises the possibility that NFATp/c may bind to NFAT sites in conjunction with either AP-1 or CREB/ATF family proteins, and these two families of proteins may compete for NFAT-binding sites. The CRE consensus sequence TGACGTCA is remarkably similar to the AP-1 consensus sequence TGAGTCA, and the Fos/Jun family is closely related to the CREB/ATF family of leucine zipper proteins (5, 15). Consequently, Fos and Jun proteins can also associate with CREs, and Jun/ATF heterodimers can bind either CREs or AP-1 sites (5, 14, 29). Since both Jun/Jun and Jun/Fos dimers can form NFAT complexes, we speculate that Jun/ATF heterodimers may also associate with the CRE-like sequence in the GM420 element to form NFAT-like complexes. Although we have no evidence to date for CREB/ATF involvement in NFAT-like complex formation, we have data from gel mobility shift assays indicating that both AP-1 (Fig. 6) and CREB/ATF proteins (data not shown) can associate with the

GM420 sequence. It is also intriguing that the same GM420 CRE-like sequence TGACATCA arises in other genes expressed in T cells, such as the T-cell receptor, CD3, and CD8 genes (2, 11, 13).

These studies indicated that GM-CSF enhancer function relies on converging signalling pathways that activate an array of composite transcription factor-binding sites. The cooperative nature of factor binding to these sites ensures not only that the binding of both components of the NFAT complex is greatly enhanced but that activation of these sites is very tightly regulated. Since the two components of the NFAT complex require two distinct activation pathways, it follows that the GM-CSF enhancer is unlikely to be inappropriately activated via one pathway alone.

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The human granulocyte-macrophage colony-stimulating factor gene is autonomously regulated *in vivo* by an inducible tissue-specific enhancer

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The granulocyte-macrophage colony-stimulating factor (GM-CSF) gene is part of a cytokine gene cluster and is directly linked to a conserved upstream inducible enhancer. Here we examined the *in vitro* and *in vivo* functions of the human GM-CSF enhancer and found that it was required for the correctly regulated expression of the GM-CSF gene. An inducible DNase I-hypersensitive site appeared within the enhancer in cell types such as T cells, myeloid cells, and endothelial cells that express GM-CSF, but not in non-expressing cells. In a panel of transfected cells the human GM-CSF enhancer was activated in a tissue-specific manner in parallel with the endogenous gene. The *in vivo* function of the enhancer was examined in a transgenic mouse model that also addressed the issue of whether the GM-CSF locus was correctly regulated in isolation from other segments of the cytokine gene cluster. After correction for copy number the mean level of human GM-CSF expression in splenocytes from 11 lines of transgenic mice containing a 10.5-kb human GM-CSF transgene was indistinguishable from mouse GM-CSF expression (99% ± 56% SD). In contrast, a 9.8-kb transgene lacking just the enhancer had a significantly reduced ($P = 0.004$) and more variable level of activity (29% ± 89% SD). From these studies we conclude that the GM-CSF enhancer is required for the correct copy number-dependent expression of the human GM-CSF gene and that the GM-CSF gene is regulated independently from DNA elements associated with the closely linked IL-3 gene or other members of the cytokine gene cluster.

Granulocyte-macrophage colony-stimulating factor (GM-CSF) was first identified as a cytokine that stimulates the *in vitro* growth and differentiation of colonies of GM lineage cells from bone marrow progenitor cells (1). *In vivo*, GM-CSF functions predominantly in the recruitment and activation of myeloid lineage cells at sites of inflammation. In both humans and mice the GM-CSF gene is located just downstream of the IL-3 gene and these closely related genes appear to have arisen by gene duplication. The human GM-CSF and IL-3 genes reside just 10 kb apart in a compact locus that includes the two genes (2) and numerous associated regulatory elements (3–11), all within a span of about 30 kb. This pair forms part of a larger cytokine gene cluster that also includes the IL-4, IL-5, and IL-13 genes several hundred kb downstream of the GM-CSF gene (12).

IL-3 and GM-CSF expression is tightly regulated in a tissue-specific manner (1, 3, 7, 13). In T cells the two genes are coregulated as in most cases they both are expressed on activation of T cell receptor (TCR) signaling pathways. Although they respond to similar signaling pathways, the IL-3 and GM-CSF genes also can be differentially regulated. Although IL-3 expression is restricted primarily to T cells, GM-CSF is produced in response to proinflammatory agents such as bacterial lipopolysaccharide and tumor necrosis factor α by a wide range of additional cell types that includes monocytes, endothelial cells, and fibroblasts (1).

It is of general interest to determine whether conserved gene clusters use shared regulatory elements, as is clearly the case in the globin loci, or whether the regulatory apparatus is replicated along with the genes in conserved gene clusters. In the α and β globin loci, clusters of conserved genes are expressed stepwise during development and are regulated by upstream locus control regions that exist as DNase I-hypersensitive (DH) sites in erythroid cells (14–16). Our previous studies of the human IL-3/GM-CSF gene cluster revealed the existence of an extensive array of DH sites that encompasses DNA elements likely to be required for the correct inducible, tissue-specific, and differential regulation of the IL-3 and GM-CSF genes (3–6, 17) (summarized in ref. 6). The best-studied of these elements are two inducible DH sites located 3 kb upstream of the GM-CSF gene (3–5, 17) and 14 kb upstream of the IL-3 gene (6), which exist within inducible enhancers that respond to activation of TCR signaling pathways. Each enhancer encompasses an array of binding sites for the Ca^{2+}-inducible transcription factor NFAT (nuclear factor of activated T cells) and is suppressed by CsA that inhibits induction of NFAT (3, 4, 6, 13).

In this study we explored the *in vitro* and *in vivo* functions of the GM-CSF enhancer. We determined that the GM-CSF enhancer functions in essentially all cell types that express GM-CSF and that the GM-CSF locus can be correctly regulated *in vivo* independently of DNA elements in the IL-3 locus.

Materials and Methods

DH Site Analyses. DH sites in a wide range of cell lines were assayed as described (3, 6). Briefly, for each cell line a DNase I titration was performed and samples that had optimal extents of DNase I digestion were selected for Southern blot hybridization analysis of DH sites. DH sites up to 6.8 kb from the GM-CSF gene were mapped within a 9.4-kb *Eco*RI GM-CSF gene fragment by using a 1.5-kb *Bam*HI fragment as described (3). The region downstream of the IL-3 gene was mapped by using *Bam*HI-digested DNA and a 1-kb *Bgl*II/*Bam*HI fragment of λJ1–16 DNA (2).

Plasmid Construction. The plasmid pGM contains the 627-bp human GM-CSF promoter upstream of a luciferase reporter gene. pGM-GME and pGM-SV40E consist of a 717-bp *Bgl*II

Abbreviations: GM-CSF, granulocyte-macrophage colony-stimulating factor; TCR, T cell receptor; DH, DNase I-hypersensitive; NFAT, nuclear factor of activated T cells; SV40, simian virus 40; HUVEC, human umbilical vein endothelial cell; PMA, phorbol 12-myristate 13-acetate.

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fragment containing the GM-CSF enhancer or a 158-bp fragment of the simian virus 40 (SV40) enhancer, respectively cloned upstream of the GM-CSF promoter of pGM (5, 6). Note that the 717-bp GM-CSF enhancer contains an extra G at position 118 from the sequence published in ref. 3.

Cell Culture. The Jurkat, CEM, and HSB2 T cell lines, KG1a and U937 myeloid cell lines, K562 pro-erythroid cell line, human embryonic lung fibroblasts, HeLa cervical carcinoma cell line, HepG2 hepatic cell line, and Raji B and Ball-1 B cell lines were cultured in RPMI medium containing 10% FBS. The 5637 bladder carcinoma epithelial cell line was cultured in RPMI containing 7% FBS. Human umbilical vein endothelial cells (HUVECs) were cultured as described (17). Human peripheral blood T cells were prepared by isolating mononuclear cells from whole blood and depleting B cells by adhering them to nylon wool.

Transient Transfections and Luciferase Assays. All cells, with the exception of HUVECs, were transfected with 5 μg CsCl-purified plasmid DNA by electroporation, cultured for 20–24 hr, left either unstimulated or stimulated with 20 ng/ml phorbol 12-myristate 13-acetate (PMA) and 1 μM calcium ionophore A23187 for 9 hr, and assayed for luciferase reporter gene activities as described (6). HUVECs were transfected with 15 μg CsCl-purified plasmid DNA by the DEAE-dextran procedure, cultured for 24 hr, either unstimulated or stimulated with 20 ng/ml PMA and 2 μM calcium ionophore A23187 (PMA/I) for 9 hr, and assayed for luciferase reporter gene activities as described (17).

Generation and Identification of Transgenic Mice. The human transgene contains a 10.5-kb *Xho*I/*Hin*dIII region encompassing the human GM-CSF gene and extends 5.9 kb upstream of the gene and 2.4 kb downstream of the gene. This transgene was made by fusing the *Hin*dIII fragment of pCH5.2 (18) to a *Hin*dIII segment of λJ1–16 (2). The 9.8-kb transgene lacking the enhancer was prepared by excising a 717-bp *Bgl*II fragment encompassing the enhancer. Transgenic mice were generated by microinjection of DNA into the pronuclei of fertilized mouse eggs, which then were cultured overnight to the two-cell stage and transferred into the oviducts of pseudopregnant females. Transgenic founders were identified and their copy numbers of integrated transgenes were estimated by Southern blot analysis of transgenic mouse DNA. Founders then were bred to establish lines used for GM-CSF expression analyses.

GM-CSF Expression Assay. Whole spleens were isolated from transgenic mice and squeezed between two glass slides to extract lymphoid cells, and cells were plated out to 1×10^7 lymphocytes per ml in Iscove's modified Dulbecco's medium containing 10% FBS and 5×10^{-5} M β-mercaptoethanol. Cells were either left unstimulated (−) or stimulated with 20 ng/ml PMA and 1 μM calcium ionophore A23187 (PMA/I) for 15 hr (+). Supernatants were harvested and assayed for mouse and human GM-CSF levels by ELISA (R&D Systems).

Results

Chromatin Remodeling Occurs in the GM-CSF Enhancer in All Cells Where It Is Active. To determine whether chromatin remodeling of the GM-CSF enhancer was a consistent feature of GM-CSF-expressing cells we mapped DH sites in a wide variety of human cell types where we previously had assayed induction of GM-CSF mRNA expression (unpublished data). These cells included T cells that coexpress IL-3 and GM-CSF, myeloid and nonhaemopoietic adherent cell types that express GM-CSF but not IL-3, and cell lines that do not express either gene. Based on these expression patterns the collection of cells was divided into the three groups shown in Fig. 1*A*. As this panel of cells normally would require a diverse range of agents to activate the GM-CSF locus via cell surface receptors (1), we chose a combination of inducing agents that directly triggers the intracellular signaling pathways that lead to GM-CSF expression. GM-CSF enhancer activation and DH site induction in T cells and endothelial cells are known to involve both Ca^{2+} and kinase-dependent pathways, which in T cells are linked to the TCR (3–8, 13). Hence, all of the cells used in this study were activated with the combination of the calcium ionophore A23187 and the phorbol ester PMA, which directly activates Ca^{2+} and kinase pathways.

To induce DH sites cells were stimulated for 4–6 hr with A23187 and PMA. We mapped DH sites from an *Eco*RI site 6.8 kb upstream of the GM-CSF gene and detected up to three DH sites (Fig. 1 *A* and *C*). We observed a striking correlation between the induction of DH sites within the enhancer and the ability of cells to express GM-CSF on activation. Accordingly, the DH site was induced in the enhancer in peripheral blood T cells and in the Jurkat, CEM, and HSB2 T cell lines, all of which express both GM-CSF and IL-3. Within the myeloid lineage the DH site appeared in the cell lines KG1a and K562, which express GM-CSF (Fig. 1*A*), but not in activated U937 cells that do not express GM-CSF (data not shown). Among nonhaemopoietic cells the enhancer DH site appeared in HUVECs, human embryonic lung fibroblasts, HeLa cells, and HepG2 cells in parallel with induction of GM-CSF expression. We also analyzed the two B cell lines, Raji and Ball-1, that do not express GM-CSF, and no significant DH sites were detected upstream of the GM-CSF gene. The only cells that expressed GM-CSF without a DH site appearing in the enhancer were the 5637 cells. In most cases the DH site spanned a 250-bp region defined by *Apa*I and *Pst*I sites (4) but in pro-erythroid K562 cells the DH site appeared as a doublet and extended approximately 150 bp further upstream to include additional conserved sequences that include GATA and Ets elements (5). This doublet is seen more clearly in Fig. 1*D* where the patterns in Jurkat and K562 cells are compared at higher resolution.

We also detected a DH site in the GM-CSF promoter in most cells expressing GM-CSF, but there was no strict correlation between the presence of this site and the state of activity of the GM-CSF gene (Fig. 1*A*). Furthermore, this site was absent from activated Jurkat cells that express GM-CSF but present in U937 cells that do not express GM-CSF. Interestingly, we detected a strong constitutive DH site in the promoter in 5637 cells, suggesting that the locus is activated via promoter rather than enhancer-dependent mechanisms in these cells. In addition to the promoter and enhancer a third weak pre-existing DH site was detected in some cell lines in the vicinity of the *Hin*dIII site 1 kb upstream of the enhancer (M, Fig. 1 *A* and *C*). This site was most pronounced in the myeloid cell lines KG1a and U937.

Additional DH sites exist just downstream of the IL-3 gene (3) so we probed the same DNaseI-digested samples for DH sites within a 4.6-kb *Bam*HI fragment that overlaps the *Eco*RI fragment (Fig. 1 *B* and *C*). Mapping of these DH sites identified three ubiquitous pre-existing sites plus one previously unidentified inducible site within 2.4 kb of the IL-3 gene. It was apparent that there was no link between the appearance of these DH sites and the activity of the locus. As previous studies of this region had failed to find any regulatory elements in this region (3) these sites were not considered further. These mapping studies effectively conclude the analysis of DH sites in the GM-CSF locus as no sites were found in either the 12-kb region downstream of the GM-CSF gene or the region where the *Eco*RI and *Bam*HI fragments mapped in Fig. 1 overlap (data not shown). These data now can be used as a blueprint to develop hypotheses on the regulation of this locus.

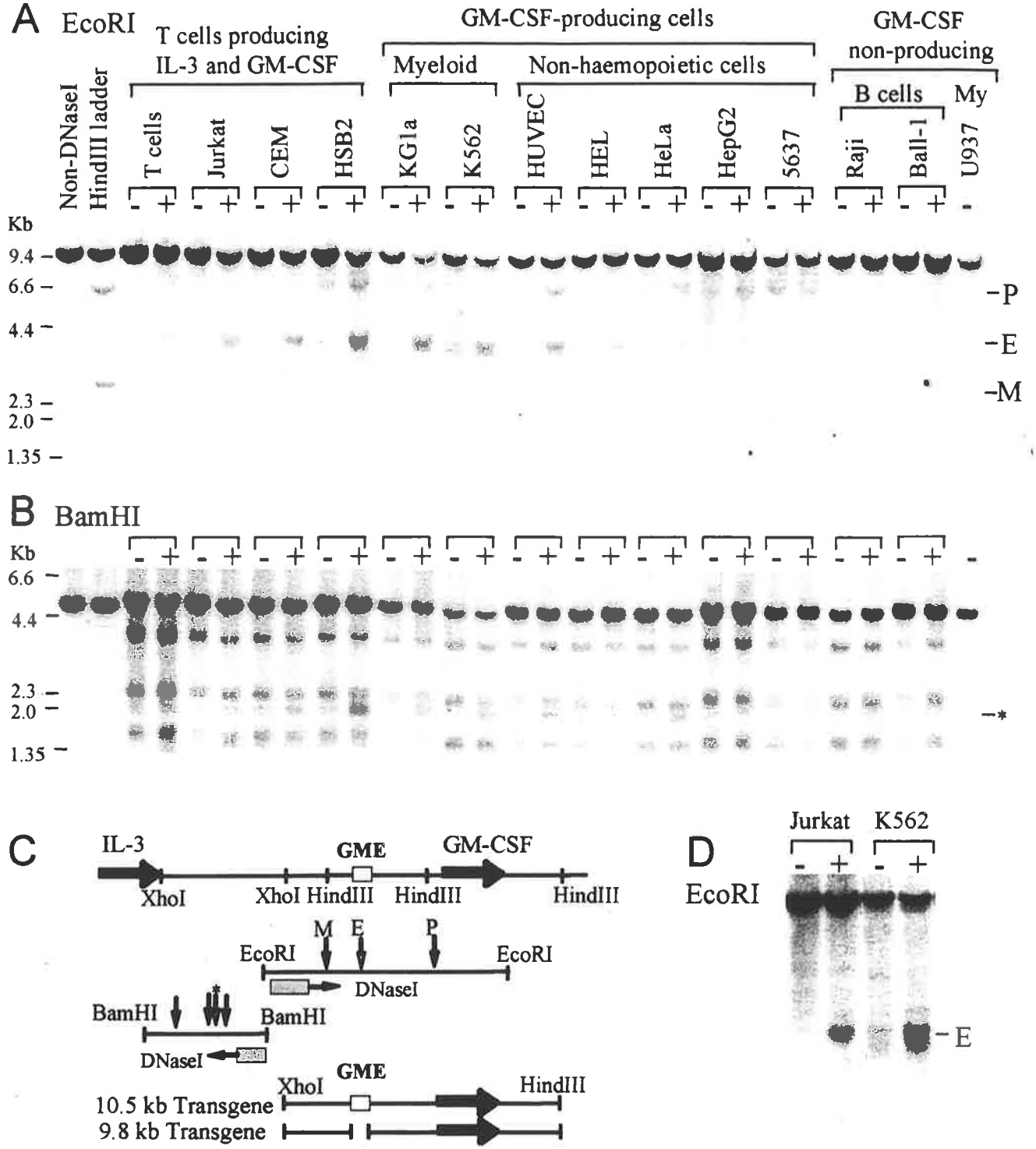

Fig. 1. Mapping of DH sites between the IL-3 and GM-CSF genes. (*A*) Mapping of GM-CSF enhancer and promoter DH sites in an *Eco*RI fragment in DNase I-digested nuclei isolated from either unstimulated cells (−) or cells stimulated for 4–6 hr with 20 ng/ml PMA and 2 μM A23187 (+). On the right, the letters indicate DH sites at the GM-CSF promoter (P), GM-CSF enhancer (E), and a site at −4.4 kb present predominantly in myeloid cells (M). (*A* and *B*) The first three lanes from the left hand side are a *Hin*dIII digest of λ DNA (molecular weight marker), total non-DNaseI-digested Jurkat DNA, and a partial *Hin*dIII digest of Jurkat DNA, respectively. The lanes labeled T cells are human peripheral blood T cells. (*B*) Mapping of DH sites in the *Bam*HI fragment downstream of the IL-3 gene as in *A*. * marks an inducible DH site present in some cell lines. (*C*) Map of identified DH sites together with the probes used to identify them. The transgene constructs used to make transgenic mice also are shown. (*D*) Mapping of DH sites in Jurkat and K562 cells as in *A* but at higher resolution.

The GM-CSF Enhancer Functions in Transfected Cells Only When the Endogenous Enhancer Can Form a DH Site. The consistent appearance of the DH site in the enhancer in GM-CSF-expressing cells suggested a strong link between chromatin remodeling, enhancer function, and GM-CSF gene activation. To further explore this link we tested the GM-CSF enhancer for function in transfection

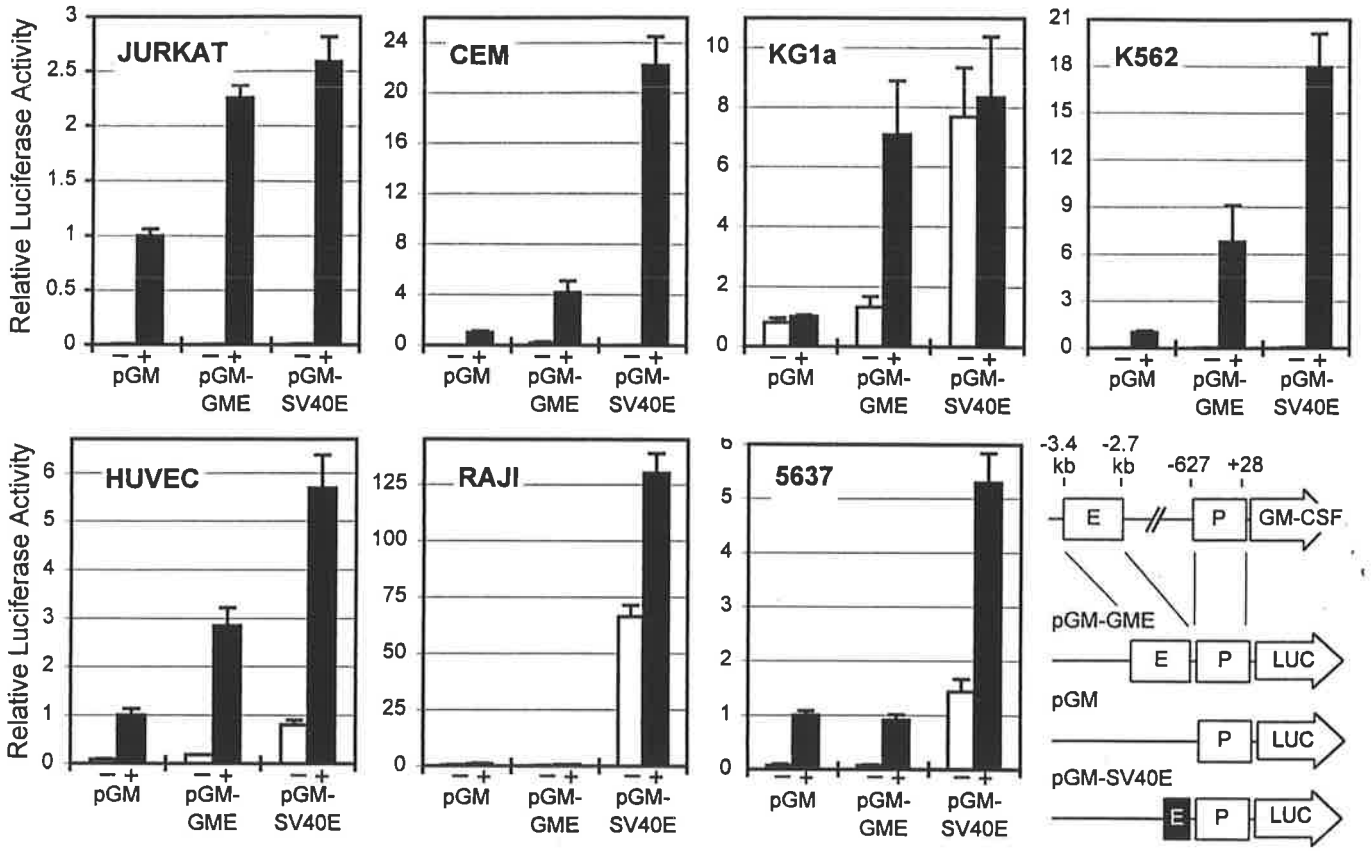

Fig. 2. Tissue-specific regulation of the GM-CSF enhancer. DNA fragments encompassing either the promoter alone (pGM), promoter plus the GM-CSF enhancer (pGM-GME), or promoter plus the SV40 enhancer (pGM-SV40E) were tested for enhancer function in cells transiently transfected with luciferase reporter gene plasmids. Luciferase activity was determined for both unstimulated (−) cells and after stimulation of the cells for 9 hr with 20 ng/ml PMA and 1 μM A23187 (2 μM A23187 for HUVEC) (+). Error bars represent the SEM of 2–11 transfections. All data points are expressed relative to stimulated pGM = 1. A schematic diagram showing the constructs used also is shown.

assays in seven of the cell lines illustrated above (Fig. 2). A 717-bp *Bgl*II fragment of DNA containing all of the defined GM-CSF enhancer elements (3–5, 19) was inserted upstream of the full-length GM-CSF promoter (−627 to +28) in a luciferase reporter gene plasmid (pGM-GME). This plasmid was assayed in parallel with the luciferase plasmid pGM containing the GM-CSF promoter. The plasmid pGM-SV40 containing the GM-CSF promoter plus the SV40 enhancer also was included to provide a comparison with a powerful enhancer that should function in most cell types.

Cells were transiently transfected with each plasmid and after 20–24 hr were stimulated for 9 hr with PMA and A23187. The luciferase plasmid pGM containing the GM-CSF promoter alone functioned in a highly inducible manner in Jurkat and CEM T cells, HUVECs, K562 cells, and 5637 cells, all of which express GM-CSF, and was essentially inactive in Raji B cells, which do not express GM-CSF. In KG1a cells, however, pGM was constitutively active even though the endogenous GM-CSF gene is inducible.

The inclusion of the GM-CSF enhancer significantly increased the inducible activity of the GM-CSF promoter plasmid in each of the cell types where the enhancer formed a DH site (Jurkat, CEM, HUVECs, KG1a, and K562). In the five cell lines where it was active the GM-CSF enhancer increased the activity of the promoter by 2- to 7-fold. The GM-CSF enhancer had an activity similar to that of the SV40 enhancer in Jurkat and KG1a cells and was 20–50% as active as the SV40 enhancer in CEM, HUVECs, and K562 cells. The enhancer was inactive, however, in Raji and 5637 cells where no DH site induction occurs in the endogenous enhancer. The analyses in 5637 cells highlighted distinct differences in the activities of the GM-CSF promoter and enhancer as it was clear that the GM-CSF promoter and the SV40 enhancer were active but the GM-CSF enhancer was nonfunctional. Most importantly, these studies highlighted the fact that the GM-CSF enhancer only functions in those cells where a DH site can form in the endogenous enhancer.

The GM-CSF Enhancer Supports Correctly Regulated Expression of the GM-CSF Gene *in Vivo*. The *in vitro* studies above identified seven DH sites upstream of the GM-CSF gene and highlighted the GM-CSF promoter and enhancer as the elements most likely to be required for correct regulation of the GM-CSF locus *in vivo*. To determine the minimum requirements for a correctly regulated GM-CSF locus we generated transgenic mice containing a 10.5-kb *Xho*I/*Hin*dIII fragment of the human GM-CSF locus that extends from 5.8 kb upstream of the GM-CSF gene to 2.3 kb downstream of the gene (Fig. 1*C*). In addition to the promoter and enhancer this gene fragment includes the predominantly myeloid DH site 4.4 kb upstream of the GM-CSF gene (M, Fig. 1 *A* and *C*). A similar series of transgenic mice was generated in which the 717-bp *Bgl*II fragment containing the enhancer was excised from the 10.5-kb *Xho*I/*Hin*dIII fragment to generate a 9.8-kb transgene (Fig. 1*C*). An additional aim of this study was to determine whether the GM-CSF gene was regulated independently of elements linked to the IL-3 gene or, alternatively, whether the IL-3/GM-CSF gene cluster was coordinately regulated by shared enhancer elements.

Eleven lines of GM-CSF transgenic mice containing the

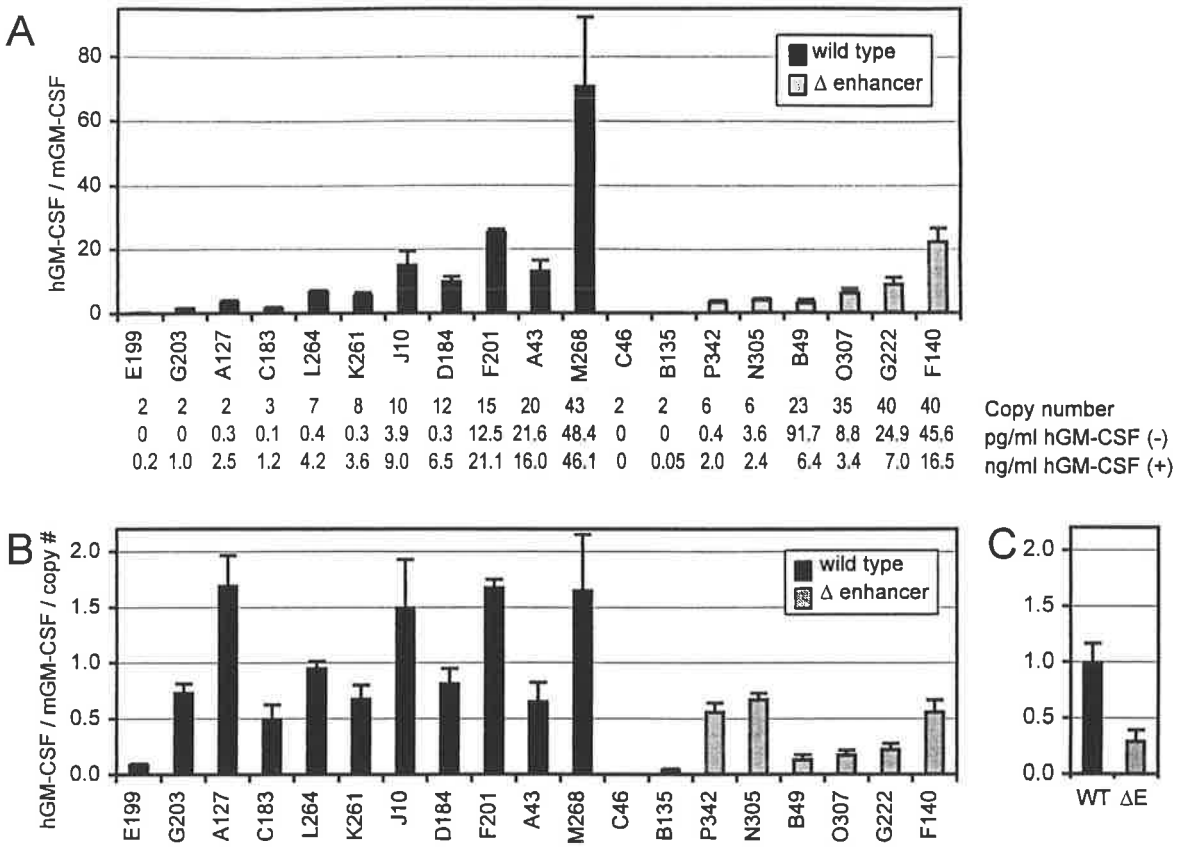

Fig. 3. GM-CSF expression in transgenic mouse lines. Human and mouse GM-CSF protein expressed by activated splenocytes. Error bars represent the SEM of 3–5 mice from each line. (*A*) Relative transgene activity expressed as a ratio of human GM-CSF/mouse GM-CSF from PMA/A23187-stimulated splenocytes (not adjusted for copy number). The copy number for each line is indicated below the graph. Levels of human GM-CSF in unstimulated and PMA/A23187-stimulated splenocytes also are shown. The average levels of mouse GM-CSF produced from all lines was 1 pg/ml from unstimulated cells and 828 pg/ml from PMA/I-stimulated cells. (*B*) Ratio of human GM-CSF/mouse GM-CSF adjusted for copy number. (*C*) Average level of transgene relative activity in either the wild-type (WT) mice (SD = 56%, SEM = 18%) or Δ enhancer transgenic (ΔE) mice (SD = 89%, SEM = 34%).

wild-type human locus and eight lines of GM-CSF transgenic mice lacking the enhancer were created. Transgene function was assessed by stimulating freshly isolated splenocytes for 15 hr with PMA and A23187. In these studies it was assumed that GM-CSF production could be largely attributed to the T cell population as these represent the predominant GM-CSF producers among splenocytes. Transgene activity was assessed by performing ELISAs of both mouse and human GM-CSF produced by activated splenocytes and expressed as a simple ratio of the transgene relative to the endogenous GM-CSF gene.

The wild-type human transgenes appeared to be correctly regulated in a copy number-dependent manner as activity increased progressively with copy number, and the average relative activity of the transgenes was indistinguishable from the endogenous mouse GM-CSF gene in stimulated splenocytes (Fig. 3). When GM-CSF expression levels were expressed per gene copy number, 10 of the 11 lines containing the wild-type gene had an activity within 2-fold of that observed for the mouse GM-CSF gene (Fig. 3*B*), and the average activity came to 99% of the endogenous GM-CSF gene (Fig. 3*C*). Linear regression analysis of the copy number dependency showed that it was highly significant with a coefficient of variance (R^2) of 0.91. The transgenes were highly inducible and increased in activity by an average of 7,800-fold on stimulation. GM-CSF transgenic mice lacking the GM-CSF enhancer supported a different pattern of GM-CSF gene activity in splenocytes. The average activity after correction for copy number was only 29% of that of the wild-type human transgene, and copy number dependency of expression was reduced (R^2 = 0.57). The transgenes lacking the enhancer remained highly inducible but had a lower average fold induction of 900-fold.

Discussion

The structural studies presented in this and our previous reports have identified key regulatory elements in the IL-3/GM-CSF locus. The summary of the locations and tissue-specific distributions of the DH sites in this locus (Fig. 4) is most likely a guide to all of the DNA elements required for the correct inducible and tissue-specific regulation of the human IL-3 and GM-CSF genes. This study focused on the GM-CSF enhancer and found that activation of the endogenous gene was almost always accompanied by the appearance of a DH site in the enhancer. In every instance where a DH site was detected within the enhancer, the enhancer had the capacity to function in these cells. Based on these observations we hypothesize that the GM-CSF enhancer is involved in most situations where GM-CSF expression is induced.

In this study we investigated the role of the enhancer in Ca^{2+}-dependent activation of gene expression because the enhancer encompasses an array of binding sites for the Ca^{2+}-inducible transcription factor NFAT (3, 5, 13). The most obvious function for the GM-CSF enhancer is as a TCR-response element driven by the array of composite NFAT/AP-1 elements. However, in K562 cells, which express little or no NFAT (unpublished data), the enhancer still functioned efficiently and we found evidence for chromatin remodeling upstream of the

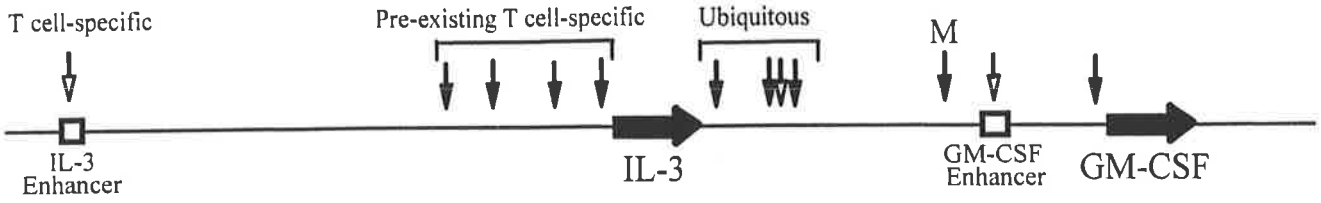

Fig. 4. Summary of DH sites and enhancers in the IL-3/GM-CSF locus. Constitutive DH sites are marked as a solid arrow, and inducible DH sites are indicated by hollow arrows. M indicates the DH site present predominantly in myeloid cells.

array of NFAT sites. It will be interesting to determine in future studies whether the enhancer also is involved in Ca^{2+}-independent activation of GM-CSF by agents such as lipopolysaccharide and tumor necrosis factor α (TNF-α). In a previous study in endothelial cells we observed that Ca^{2+}-dependent activation of GM-CSF gene expression was CsA-sensitive but induction by TNF-α was CsA-resistant (17). This observation raises the possibility that TNF-α works selectively on the promoter by pathways that may be similar to those operating in cells such as 5637 cells where the enhancer does not function.

We also examined the *in vivo* role of the GM-CSF enhancer in transgenic mice. Because the enhancer was required for efficient activation of the locus in transgenic mice we suggest that the GM-CSF enhancer plus the promoter constitute the minimum amount of DNA required for the correctly regulated activation of the GM-CSF gene. These elements were contained within a 10.5-kb fragment that extended 5.7 kb upstream of the GM-CSF gene and also contained a third DH site 4.4 kb upstream that may have a role in GM-CSF expression in myeloid cells. Remarkably, this short segment of DNA had the capacity to support a level of GM-CSF expression in transgenic mice essentially identical to the endogenous mouse GM-CSF gene. We previously found in transfection studies that the mouse GM-CSF promoter plus enhancer supported the same level of inducible activity as the human GM-CSF promoter plus enhancer (5). This body of work now suggests that the mouse and human GM-CSF genes are indeed expressed at similar levels. This work further implies that the GM-CSF gene does not require any additional regulatory information from either the IL-3 locus or other regions of the cytokine gene cluster. We also identified ubiquitous DH sites downstream of the IL-3 gene but they did not appear to be required for GM-CSF gene activation. They could, however, represent a structural element within chromatin that insulates the IL-3 and GM-CSF genes from each other.

The IL-3 gene likewise may be regulated by a distinct set of elements (summarized in Fig. 4). The IL-3 gene has its own upstream enhancer that resembles the GM-CSF enhancer in its organization (6) and an additional array of at least four DH sites exists immediately upstream of the IL-3 gene (3). As all of the DH sites upstream of the IL-3 gene are for the most part restricted to T cells (unpublished data) we suggest that the 14-kb region extending upstream of the IL-3 gene constitutes a distinct regulatory cassette required for the correct regulation of the IL-3 locus in T cells. Hence, in contrast to most other conserved gene clusters the IL-3 and GM-CSF genes may be independently regulated at the gene level even though they are coregulated at the level of TCR signaling.

This study provided a unique example of a gene that supports normal levels of gene expression when isolated from a conserved gene cluster. In other gene clusters, such as the α and β globin loci, normal expression of individual genes is governed by a shared locus control region (14–16). The regulation of the IL-3/GM-CSF locus appears to be markedly different, perhaps because unlike the globin genes they are not designed to be expressed in a step-wise manner. Hence, the duplication of the regulatory apparatus may have arisen from the requirements to be able to express the GM-CSF and IL-3 genes simultaneously in T cells but regulate them independently in other cell types.

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A T Cell–Specific Enhancer in the Interleukin-3 Locus Is Activated Cooperatively by Oct and NFAT Elements within a DNase I–Hypersensitive Site

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Summary

Interleukin-3 (IL-3) is a cytokine that is expressed primarily in activated T cells. Here we identified an inducible T cell–specific enhancer 14 kb upstream of the IL-3 gene that responded to activation of T cell receptor signaling pathways. The IL-3 enhancer spanned an inducible cyclosporin A–sensitive DNase I–hypersensitive site found only in T cells. Four NFAT-like elements exist within the enhancer. The two most active NFAT-like elements were located at the center of the DNase I–hypersensitive site. One of these NFAT-like elements encompassed overlapping Oct- and NFATp/c-binding sites, which functioned in a highly synergistic manner. We suggest that the T cell–specific expression of the IL-3 gene is partly controlled through the enhancer by cooperation between Oct and NFAT family proteins.

Introduction

Immune and inflammatory responses are orchestrated by a large network of inducible cytokines (Nicola, 1989). Specific responses rely on both the site and nature of cytokine gene expression. To approach the subject of cytokine gene regulation, we chose to study the closely linked interleukin-3 (IL-3) and granulocyte/macrophage colony-stimulating factor (GM-CSF) genes (Gasson, 1991; Masuda et al., 1993; Yang et al., 1988). This presents a valuable model for studying inducible cytokine gene expression as these related genes are induced via similar signaling pathways, yet in some cell types they are differentially regulated. As our previous studies (Cockerill et al., 1993) have not revealed the basis for this differential regulation, we chose here to seek additional mechanisms for IL-3 gene regulation.

IL-3 is a cytokine that regulates the growth and differentiation of hematopoietic progenitor cells (Nicola, 1989). IL-3 acts on both early stem cells and progenitors committed to distinct lineages that include monocytes, granulocytes, megakaryocytes, erythrocytes, eosinophils, and mast cells. The human IL-3 gene is tightly regulated and is expressed primarily in activated T cells and natural killer cells. The IL-3 gene is induced in T cells predominantly in response to T cell receptor (TCR) activation (Masuda et al., 1993). Like IL-2, IL-4, IL-5, and GM-CSF, IL-3 gene expression in activated T cells is repressed in the presence of the immunosuppressant cyclosporin A (CsA) (Masuda et al., 1993; Rao, 1994). CsA is known to function by inhibiting the Ca^{2+}-dependent phosphatase calcineurin, and one of the principle targets of CsA is the NFATp/c family of transcription factors (Clipstone and Crabtree, 1992; Jain et al., 1993; O'Keefe et al., 1992; Rao, 1994; Schreiber and Crabtree, 1992).

The human GM-CSF gene lies just 10 kb downstream of the IL-3 gene (Yang et al., 1988) and is activated by an inducible CsA-sensitive enhancer located between the two genes (Cockerill et al., 1993, 1995b). Although the GM-CSF enhancer has the potential to activate both the IL-3 and the GM-CSF promoters (Cockerill et al., 1993; Osborne et al., 1995), it is likely that this enhancer functions predominantly to regulate GM-CSF gene expression. The GM-CSF enhancer resides just upstream of the GM-CSF gene and is active not only in T cells but also in cell types such as endothelial cells, which express GM-CSF but not IL-3 (Cockerill et al., 1995a).

Although several inducible transcription factor–binding sites exist in the human IL-3 promoter, the basis for the cell specificity of IL-3 gene expression remains unclear. In common with many other cytokine genes, the human IL-3 promoter encompasses binding sites for transcription factors such as AP-1, Elf-1, Oct-1, and CBF, and a CK-1 element (Cameron et al., 1994; Davies et al., 1993; Gottschalk et al., 1993; Mathey-Prevot et al., 1990; Park et al., 1993; Shannon et al., 1988; Shoemaker et al., 1990). None of these factors alone is responsible for T cell–specific gene expression, as all of these factors can function in cells other than T cells. It has been suggested, however, that closely linked AP-1 and Elf-1 binding sites in the IL-3 promoter can direct T cell–specific expression (Gottschalk et al., 1993). Other factors that may activate the IL-3 promoter include factors that bind the NF-IL3A element (Davies et al., 1993; Zhang et al., 1995) and zinc finger proteins that bind the GC-rich region (Koyano-Nakagawa et al., 1994).

The NFATp/c family of transcription factors represent candidates for factors that might also be implicated in IL-3 gene regulation (Rao, 1994). The IL-3 gene has a pattern of Ca^{2+} inducibility and CsA sensitivity in common with other cytokines expressed in T cells, such as IL-2, IL-4, IL-5, and GM-CSF, that each utilize NFATp/c sites for their activation (Cockerill et al., 1995b; Masuda et al., 1993; Rao, 1994; Tsuruta et al., 1995). In the case of the IL-2 promoter and the GM-CSF enhancer, NFATp/c binds to DNA in close association with the AP-1 family of transcription factors (Curran and Franza, 1988) to form a higher order complex termed NFAT (Boise et al., 1993; Cockerill et al., 1993, 1995b; Jain et al., 1992; Nolan, 1994; Northrop et al., 1993). At these sites, and at sites in the IL-4 promoter, NFATp/c associates with the consensus sequence T/AGGAA/GAA/GA/T (Cockerill et al., 1995b; Masuda et al., 1993; Rao, 1994). In the GM-CSF enhancer and the IL-2 promoter, T/AGG AA/GAA/GA/TNTGAGTCA defines an arrangement of NFATp/c- and AP-1-like motifs able to support cooperative binding of NFATp/c and AP-1 (Cockerill et al., 1995b). NFATp/c was initially described as a lymphoid-specific factor (Shaw et al., 1988), but is now known to

comprise a large family of proteins, the best characterized of which are NFATp and NFATc (Hoey et al., 1995; Masuda et al., 1995; McCaffrey et al., 1993; Northrop et al., 1994). Although NFATc is largely lymphoid specific, NFATp is more widely expressed (Northrop et al., 1994). The utilization of NFATp and AP-1 by the GM-CSF enhancer may partly account for the activity of the GM-CSF locus not only in T cells but also in other cell types that express these factors (Cockerill et al., 1995a).

To identify DNA elements that regulate IL-3 expression, we have previously mapped DNase I–hypersensitive (DH) sites across the IL-3/GM-CSF locus (Cockerill et al., 1993; see Figure 7). We located a cluster of four DH sites spanning a 4 kb region immediately upstream of the IL-3 gene and another cluster of three DH sites just downstream from the IL-3 gene. However, these DH sites are constitutively present in T cells and do not appear to respond to TCR activation.

To identify additional DNA elements that control the regulation of IL-3 gene expression, we sought DH sites further upstream of the IL-3 gene. Here we report the identification of an inducible T cell–specific DH site 14 kb upstream of the IL-3 gene that functions as an inducible enhancer. This enhancer may cooperate with other upstream and downstream elements in the control of the T cell–specific expression of the IL-3 gene.

Results

A T Cell–Specific DH Site Resides Upstream of the IL-3 Gene

In a previous study we mapped DH sites located within 12 kb of the human IL-3 gene (Cockerill et al., 1993). Here we sought additional regulatory elements by mapping DH sites within a 25 kb region extending upstream from a BamHI site that exists 10 kb upstream of the IL-3 gene (Frolova et al., 1991). As a control we used the same DNase I–digested DNA samples to probe for the inducible DH site that we previously identified within the GM-CSF enhancer (Cockerill et al., 1993).

In Jurkat T cells we identified a DH site 14 kb upstream of the IL-3 gene that in many ways resembled the DH site located within the GM-CSF enhancer (arrows in Figure 1). Both DH sites were induced by the combination of phorbol ester and calcium ionophore A23187 (PMA/I), signals that mimic TCR activation pathways. As previously shown for the GM-CSF enhancer DH site (Cockerill et al., 1993), the IL-3 DH site first appeared about 1–2 hr after stimulation, just before the onset of IL-3 and GM-CSF transcription (data not shown). The induction of each DH site was inhibited in the presence of either the immunosuppressant CsA or the protein synthesis inhibitor cycloheximide (Figure 1). Significantly, however, the IL-3 DH site exhibited the properties of a T cell–specific DH site. While Jurkat cells had both DH sites, the GM-CSF enhancer DH site, but not the IL-3 DH site, was induced by PMA/I in cell lines that express GM-CSF but not IL-3 (human embryonic lung [HEL] fibroblasts and HeLa cells). No inducible DH sites were detected in the Ball-1 B cell line, which expresses neither IL-3 or GM-CSF. By including restriction enzyme–digested control samples, we determined that the DH

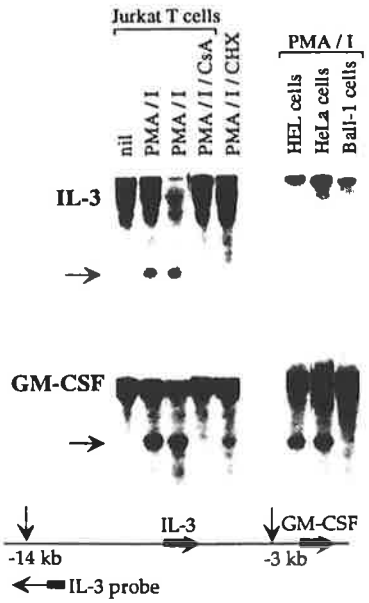


Figure 1. A T Cell–Specific DH Site Exists Upstream of the IL-3 Gene

DH sites were mapped in DNase I–digested nuclei isolated from Jurkat cells, HEL fibroblasts, HeLa cells, and Ball-1 B cells. Where indicated, cells were either untreated (nil) or treated for 4 hr with combinations of PMA/I, 0.1 μM CsA, and 20 μg/ml cycloheximide (CHX) as previously described (Cockerill et al., 1993). The upper panel is a Southern blot of BamHI-digested DNA samples probed upstream from a BamHI site located 10 kb 5′ of the IL-3 gene. The lower panel is a Southern blot of the same DNA samples digested with EcoRI and probed under conditions used previously to map a DH site upstream of the GM-CSF gene (Cockerill et al., 1993). The arrows indicate the locations of inducible DH sites 14 kb upstream of the IL-3 gene and within the GM-CSF enhancer. The IL-3 DH site was located 3.8 kb upstream of the BamHI site that delimits the probe.

site existed as a broad region approximately 200 bp across that was centered midway between SphI and XbaI sites located 300 bp apart (data not shown; Frolova et al., 1991).

At least two additional DH sites were detected in the IL-3 locus further upstream of the DH site located at −14 kb, but the bands representing these sites existed in a poorly resolved upper region of the Southern blot (Figure 1). These DH sites resided approximately 20 and 25 kb upstream of the IL-3 gene and were more visible with increased DNase I digestion (Figure 1, lane 3).

The Upstream DH Site Is an Inducible T Cell–Specific Enhancer

A 1.2 kb BglII fragment of DNA encompassing the inducible DH site at −14 kb was tested for enhancer function in Jurkat T cells. This fragment extended from 0.6 kb upstream to 0.3 kb downstream of the 300 bp SphI–XbaI segment that spans the DH site. Enhancer function was assessed by inserting the 1.2 kb BglII fragment in front of the IL-3 promoter in a luciferase reporter gene plasmid. Plasmids were transiently transfected into Jurkat cells, cultured for 20 hr, and then stimulated for 9 hr in the presence of PMA/I before cell extracts were harvested for luciferase activity.

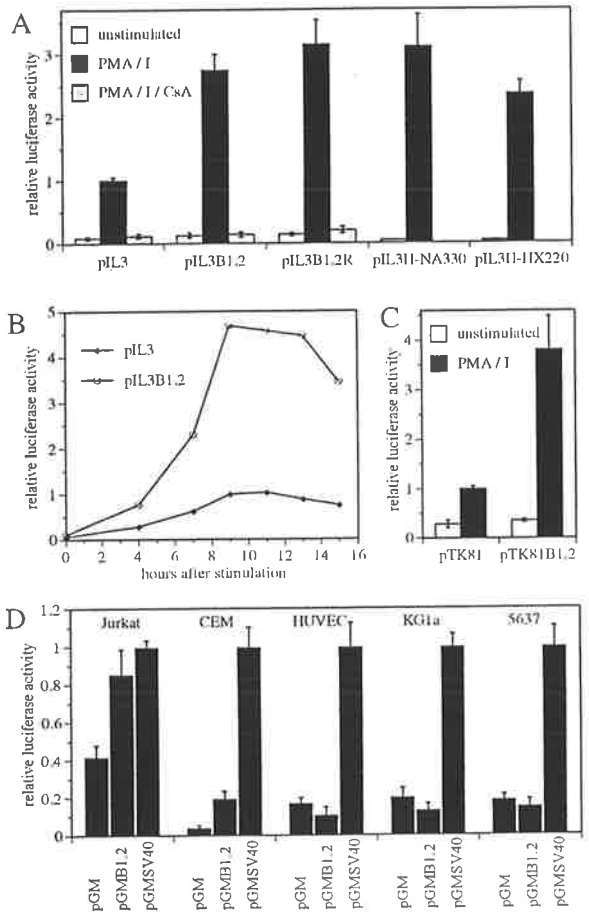


Figure 2. An Inducible Enhancer Spans the Inducible DH Site Upstream of the IL-3 Gene

DNA fragments encompassing the upstream IL-3 DH site were tested for enhancer function in cells transiently transfected with luciferase reporter gene plasmids.

(A) IL-3 enhancer function upstream of the IL-3 promoter in Jurkat cells stimulated with PMA/I for 9 hr in the presence or absence of 0.1 μM CsA.

(B) Time course of IL-3 promoter (pIL3) and IL-3 promoter/enhancer (pIL3B1.2) induction in Jurkat cells stimulated with PMA/I.

(C) IL-3 enhancer function upstream of the thymidine kinase promoter in Jurkat cells stimulated with PMA/I for 9 hr.

(D) Activities of the IL-3 and SV40 enhancers upstream of the GM-CSF promoter/luciferase gene plasmid pGM in the indicated cell types stimulated for 9 hr with PMA/I.

(A), (C), and (D) all include standard error of the mean and represent 4 to 11 independent transfections for each plasmid. Note that the plasmid pIL3 has inducible CsA-sensitive properties different from those of the CsA-resistant IL-3 promoter/CAT reporter plasmid pHIL3 (Cockerill et al., 1993) used in our earlier studies.

The inducible activity of the IL-3 promoter was increased by 2- to 3-fold in the presence of the enhancer regardless of whether the enhancer was inserted in its natural or reverse orientation relative to the promoter (Figure 2A). The inducible activity of each of the IL-3 promoter/enhancer constructs was essentially eliminated in the presence of 0.1 μM CsA. Functional assays of segments of the 1.2 kb enhancer indicated that enhancer activity was concentrated within the region of the DH site. A 330 bp NsiI–AccI fragment that encompassed the DH site had an enhancer activity essentially the same as the 1.2 kb BglII fragment (pIL3H-NA330; Figures 2A and 3A). Further deletions at the edges of the enhancer generated a 220 bp HinfI–XbaI fragment (see Figure 3A), which was only marginally less active than the 330 bp and 1.2 kb enhancer fragments (pIL3H-HX220; Figure 2A).

The kinetics of IL-3 enhancer and promoter activation (Figure 2B) mirrored the previously described kinetics of IL-3 mRNA induction in Jurkat cells stimulated with PMA/I (Cockerill et al., 1993). In the presence of the enhancer, IL-3 promoter activity was increased by 4- to 5-fold at each timepoint between 7 and 15 hr, with peak activation occurring after 9 hr of stimulation (Figure 2B).

The IL-3 enhancer also activated heterologous promoters in stimulated Jurkat cells. When placed in front of the thymidine kinase promoter, the 1.2 kb IL-3 enhancer fragment increased luciferase reporter gene expression by 4-fold (Figure 2C). When placed in front of the GM-CSF promoter, the IL-3 enhancer increased luciferase gene activity by 2- to 3-fold (Figure 2D).

As the GM-CSF gene is active in numerous cell types, the GM-CSF promoter/luciferase plasmid was used as a vehicle to test the cell specificity of the IL-3 enhancer, with a simian virus 40 (SV40) enhancer/GM-CSF promoter plasmid also included as a control. The SV40 enhancer is highly active in a range of cell types, and the 158 bp SV40 enhancer fragment used here is also known to support the formation of a DH site (Jongstra et al., 1984). Each enhancer/promoter plasmid was transfected into Jurkat T cells, CEM T cells, endothelial cells (HUVEC), KG1a myeloid leukemic cells, and 5637 bladder carcinoma cells and activated with PMA/I. Each of these cell types can express GM-CSF, but only Jurkat and CEM cells express IL-3. In stimulated Jurkat cells the activity of the IL-3 enhancer was similar to that of the SV40 enhancer (Figure 2D). In stimulated CEM T cells, the IL-3 enhancer elevated promoter activity by 5- to 10-fold, but was not as active as the SV40 enhancer. In marked contrast with the SV40 enhancer, the IL-3 enhancer was inactive in stimulated HUVEC, KG1a, and 5637 cells (Figure 2D).

The IL-3 Enhancer Encompasses Four NFATp/c Sites

To aid identification of DNA elements that mediate the inducible CsA-sensitive activity of the IL-3 enhancer, we determined the DNA sequence in the region of the DH site (Figure 3A) and scanned it for NFATp/c consensus binding sites (Cockerill et al., 1995b) and other transcription factor–binding sites recorded in the Signal Scan data base (Prestridge, 1991). The enhancer was found to contain four sites that were either identical to or closely resembled the NFATp/c consensus sequence T/AG GAA/GAA/GA/T (Figure 3). These sites, in accordance with their approximate locations in the sequence, were termed the IL70, IL140, IL190, and IL280 elements. The IL140 and IL190 elements bore the closest match to the NFATp/c consensus and were located at the center of the DH site. Interestingly, the IL190 and IL280 elements both appeared to be composite sites. The IL190 NFATp/c site overlapped an octamer element (Staudt and Lenardo, 1991), and the IL280 NFATp/c site was

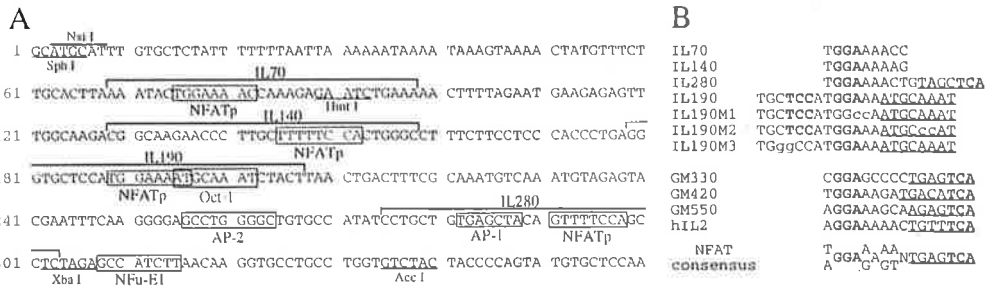


Figure 3. Sequences of the IL-3 Enhancer and NFAT-like Elements

(A) DNA sequence of the SphI–XbaI fragment of DNA encompassing the IL-3 enhancer core and DH site. Consensus transcription factor–binding sites (boxed) were identified with the aid of a computer data base (Prestridge, 1991) and the NFATp/c consensus derived from the GM-CSF enhancer and IL-2 promoter NFAT motifs (Cockerill et al., 1995b). Brackets encompass DNA sequences used to construct the oligonucleotides used in subsequent binding assays and construction of luciferase reporter plasmids.

(B) Sequences of NFAT-like elements that exist in the IL-3 enhancer (IL70, IL140, IL190, and IL280), the GM-CSF enhancer (GM330, GM420, and GM550), and the IL-2 promoter (hIL2). Highlighted are conserved GGA sequences that act as NFATp/c sites and TCA sequences that form part of AP-1-binding sites. Altered bases in the IL190 mutants are shown in lowercase. Below is the NFAT consensus sequence derived from the IL-2 and GM-CSF elements (Cockerill et al., 1993, 1995b).

linked to an AP-1-like element. In addition, the enhancer core also encompassed a consensus AP-2 motif, and a NF-μE1 motif (Staudt and Lenardo, 1991) lies just downstream of the XbaI site.

The inducible NFATp/c binding potential of each of the four IL-3 enhancer NFATp/c-like motifs was examined in gel electrophoretic mobility shift assays using either nuclear extracts prepared from Jurkat cells stimulated with PMA/I (Figure 4) or purified recombinant NFATp (Figure 5A). As controls we included the distal NFAT site from the human IL-2 promoter and the GM430 NFATp/c site from the human GM-CSF enhancer (Cockerill et al., 1995b). The GM430 probe encompasses the sequence TGGAAAGA, representing just the NFATp/c segment of the GM420 NFAT site, and is sufficient to support high affinity NFATp/c binding even in the absence of AP-1 (Cockerill et al., 1995b). The IL-2 probe is a moderate affinity NFATp site, which binds NFATp and AP-1 in a highly cooperative fashion.

Each of the four IL-3 enhancer NFATp/c-like elements formed inducible CsA-sensitive NFATp/c-like complexes with nuclear extracts (Figure 4A), and NFATp/c complex formation was specifically inhibited in the presence of the GM430 oligonucleotide competitor (Figure 4B). The IL190 and IL280 probes also formed additional higher order complexes that appeared to contain NFATp/c and either Oct- or AP-1-like factors. The NFATp/c-like complexes appeared to contain primarily NFATp, as all of the NFATp/c-like complexes were shifted by specific NFATp antisera (Figure 5C; data not shown). The IL140 motif functioned as a very high affinity site, binding NFATp/c-like complexes at least as efficiently as the GM430 element.

Assays using purified recombinant NFATp confirmed that each NFAT-like element associated with NFATp at least as efficiently as the IL-2 NFAT probe (Figure 5A). Unexpectedly, the IL190 probe also formed an additional higher order NFATp complex, suggesting that two NFATp-binding sites exist within the IL190 element (Figure 5A). Each element was also tested as a competitor of purified NFATp binding to the GM430 probe so as to estimate their relative affinities for NFATp (Figure 5D). These assays indicated that the IL140 and IL190 elements both had NFATp affinities even higher than the GM430 probe, while the IL70 and IL280 elements had affinities intermediate between those of the IL-2 and GM430 NFATp elements.

Additional gel shift assays of substituted IL190 probes (IL190M1 and IL190M3 in Figure 3B) with purified NFATp confirmed the existence of two independent NFATp sites within the IL190 probe (data not shown). Assays employing these elements as competitors indicated that the sequence TGGAAAAT functioned as a high affinity NFATp site and the sequence TGGAGCAC functioned as a low affinity NFATp site (IL190M1 and IL190M3 in Figure 5D). In assays using nuclear extracts, only the high affinity NFATp site made a significant contribution to the formation of NFATp-containing complexes (Figure 5B).

The IL280 Probe Supports Cooperative Binding of AP-1 and NFATp

The IL280 element resembled other composite NFAT sites that bind NFATp/c and AP-1 cooperatively (Figure 3B). Like the IL-2 NFAT probe, the IL280 probe formed inducible higher order complexes that included both NFATp/c- and AP-1-like factors and appeared unable to bind AP-1 independently (Figures 4A, 4B, and 5A). The IL280 probe was, however, considerably less efficient than the IL-2 NFAT probe at forming these higher order NFAT complexes with nuclear extracts (Figure 4A).

Since the IL-2 and IL280 NFAT sites can recruit AP-1 even in the absence of intact AP-1 sites, the IL70, IL140, and IL190 probes were also examined for their ability to bind AP-1 as part of an NFAT complex. In contrast with the IL280 element, the IL70, IL140, and IL190 elements did not appear to form any AP-1-containing complexes, as an AP-1 oligonucleotide competitor had no significant effect on nuclear protein binding (Figure 4B). Assays with purified recombinant proteins further suggested that there was no significant degree of cooperative binding of AP-1 with NFATp to the IL70, IL140, or IL190 probes (Figure 5A). We did, however, detect some

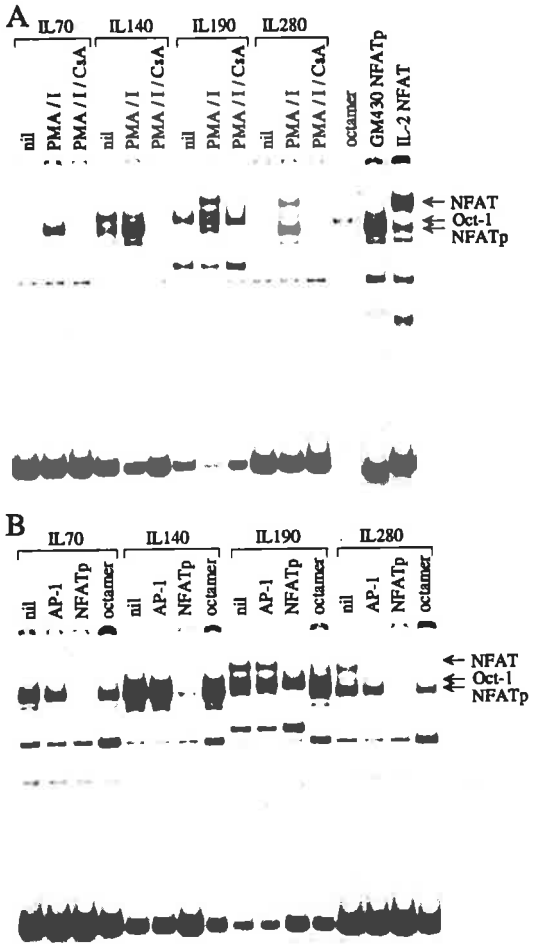


Figure 4. The IL-3 Enhancer Contains Four NFATp-Binding Elements

Consensus NFATp/c sequences in the IL-3 enhancer were assayed for NFAT-like complex formation with Jurkat cell nuclear extracts in gel electrophoretic mobility shift assays.

(A) Induction of CsA-sensitive NFATp/c and NFAT-like complexes with Jurkat cell nuclear extracts. Cells were either untreated (nil) or treated with PMA/I or PMA/I in combination with 0.1 μM CsA. The probes employed are indicated above each assay.

(B) Inhibition of specific complex formation in the presence of oligonucleotide competitors. Assays include PMA/I-activated Jurkat cell nuclear extract and 25 ng of the indicated AP-1, NFATp/c, or Oct competitor. Probes used are indicated above each set of assays. Oligonucleotide competitors used were the stromolysin gene AP-1 site, the GM430 NFATp site, and an ideal Oct consensus sequence. All probes except the weak octamer probe were labeled to approximately the same specific activity.

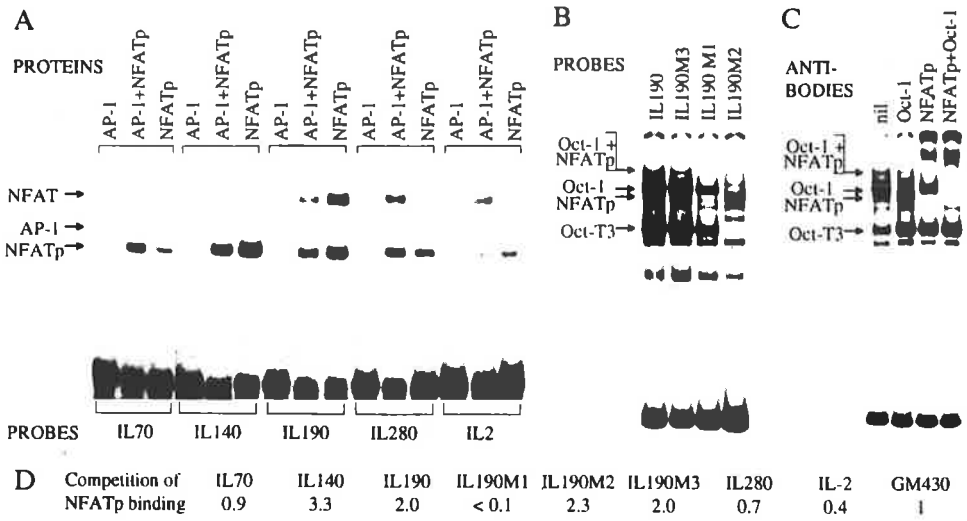
enhancement of NFATp binding to IL70 probe in the presence of AP-1 (Figure 5A), and a faint higher order NFAT complex became visible in longer exposures of this assay. We cannot rule out weak AP-1 binding to the IL190 probe, as the higher order IL190 NFATp complex has the same mobility as the NFAT-like complex in Figure 5A.

The IL190 Element Assembles Oct-1/NFATp Complexes

The IL190 binding pattern was especially interesting as it provided direct evidence for the existence of higher order complexes containing both NFATp/c and Oct family proteins binding to overlapping sites. A total of at least five specific complexes were detected with the IL190 probe. Two constitutive Oct-like complexes were detected (Figure 4A) that were specifically inhibited by Oct DNA competitor (Figure 4B). Above the Oct-1-like complex, we detected two inducible, CsA-sensitive NFATp/Oct-like complexes (Figure 4A) that were eliminated in the presence of either NFATp/c or Oct DNA competitors (Figure 4B). The lower of these two NFATp/Oct-like complexes appeared to migrate just above another NFATp/c-like band that became visible in the presence of Oct competitor DNA (Figure 4B), which we believe represents NFATx (Masuda et al., 1995) (this NFATx-like band was also visible with the IL140 and GM430 probes [Figure 4A] and the IL190M2 probe [Figure 5B]).

The formation of the two NFATp/Oct-like higher order complexes was absolutely dependent upon the integrity of the high affinity NFATp/c site and the Oct site, as a mutation within either site eliminated both of the upper complexes (IL190M1 and IL190M2 in Figure 5B). The use of the IL190 probes bearing single site mutations (IL190M1 and IL190M2 in Figure 5B) also indicated that the NFATp/c and Oct elements both functioned independently as high affinity binding sites. Furthermore, there was no evidence for cooperativity in the binding of Oct and NFAT family proteins to the IL190 element. In the presence of DNA competitors, the individual Oct and NFATp/c complexes are roughly equal to the sum of the individual and higher order complexes seen in the absence of competitor (Figure 4B). The increased intensity seen with the single complexes may even indicate that there is an element of competition in the binding of Oct and NFATp/c factors to the IL190 probe. A mutation within the low affinity IL190 NFATp/c site (IL190M3 in Figure 5B) had no effect on the formation of any of the complexes detected with Jurkat cell extracts. These results imply that the higher order complexes involve primarily the high affinity NFATp site that overlaps the Oct site and not the more distal low affinity NFATp site.

The identities of the two IL190 NFATp/Oct-like complexes were further investigated with the aid of specific antisera. The addition of NFATp antisera eliminated the formation of not only the NFATp-like complex but also both NFATp/Oct-like complexes (Figure 5C). The uppermost band was identified as a NFATp/Oct-1 complex on the basis that it was eliminated in the presence of either Oct-1 or NFATp antisera (Figure 5C) or DNA competitors (Figure 4B). The identity of the Oct-1 complex just above the NFATp complex was confirmed as it was eliminated by Oct-1 antisera (Figure 5C). The Oct-like complex below the NFATp band probably represents Oct-T3, which is abundant in Jurkat cells (Bhargava et al., 1993), as it was eliminated by Oct DNA competitor (Figure 4B) but not by Oct-1 or Oct-2 antisera (Figure 5C; data not shown). In the presence of both NFATp and Oct-1 antisera, the only specific complexes remaining were the Oct-T3-like band and a very weak NFATx-like band (Figure 5C). On the basis of the data described above, we suggest that the NFATp/Oct-like complex just above the Oct-1 complex contains predominantly NFATp and Oct-T3.



D

Competition of NFATp binding	IL70	IL140	IL190	IL190M1	IL190M2	IL190M3	IL280	IL-2	GM430
	0.9	3.3	2.0	< 0.1	2.3	2.0	0.7	0.4	1

Figure 5. Characterization of Transcription Factor Binding to IL-3 Enhancer NFAT-like Elements

(A) Gel electrophoretic mobility shift assays of purified recombinant NFATp and AP-1 binding to NFAT-like elements in the IL-3 enhancer. Assays include just NFATp, just AP-1, or NFATp plus AP-1. The IL-2 NFAT site is included as a control. The recombinant proteins used here encompass just the DNA-binding domains and amino acids that support cooperative AP-1/NFATp binding to DNA.

(B) Analysis of NFATp/c and Oct binding to mutated IL190 oligonucleotides in gel electrophoretic mobility shift assays with PMA/I-activated Jurkat cell nuclear extracts.

(C) Analysis of NFATp/c and Oct binding to the IL190 element using PMA/I-activated Jurkat cell nuclear extracts in the presence and absence of 1 μl of Oct-1 and 0.2 μl of NFATp antisera.

(D) Relative affinities of NFATp/c motifs for recombinant NFATp. Assays of purified recombinant NFATp binding to the GM430 probe were performed in the presence or absence of 1, 2, 4, or 8 ng of unlabeled DNA competitors. The relative affinities of the NFATp/c sites were expressed as their potencies as competitors relative to the GM430 element.

IL-3 Enhancer NFAT-like Elements Activate Transcription

The four IL-3 enhancer probes that form NFATp complexes were tested for inducible enhancer function by placing three head-to-tail copies of each oligonucleotide upstream of a truncated GM-CSF promoter fragment (pCLE). This minimal promoter segment (nucleotides −55 to +28) encompasses a conserved lymphokine element (CLE0) that appears in the proximal promoters of several cytokines expressed in T cells and supports only modest inducible activity in the absence of upstream elements (Cockerill et al., 1996; Masuda et al., 1993). As with the weakly active thymidine kinase promoter (Figure 2C), the full-length IL-3 enhancer increased the activity of pCLE by 4-fold in Jurkat cells (Figure 6).

Three of the multimerized NFAT-like elements had enhancer capabilities equal to or greater than the full-length IL-3 enhancer when placed directly upstream of the minimal GM-CSF promoter (Figure 6). The IL280 NFAT trimer increased the activity of the promoter by 4-fold and had an activity similar to the full-length enhancer. The trimer of the IL190 NFATp/Oct element was three times as active as the full-length enhancer, increasing the activity of the promoter by 13-fold. Surprisingly, the IL140 high affinity NFATp element functioned as an even more powerful enhancer element than the IL190 element, even in the absence of additional transcription factor sites. Not all NFATp motifs can function in isolation, however, as the moderate affinity IL70 NFATp motif had no enhancer activity under these conditions.

Synergistic Activation of Oct-1 and NFATp Motifs in the IL190 Element

To determine whether the IL190 NFATp and Oct sites functioned independently or as a discrete unit, we assayed various mutants of the IL190 motif in Jurkat cell transfection assays (Figure 6). The IL190, IL190M1, and IL190M2 elements were each assayed for enhancer

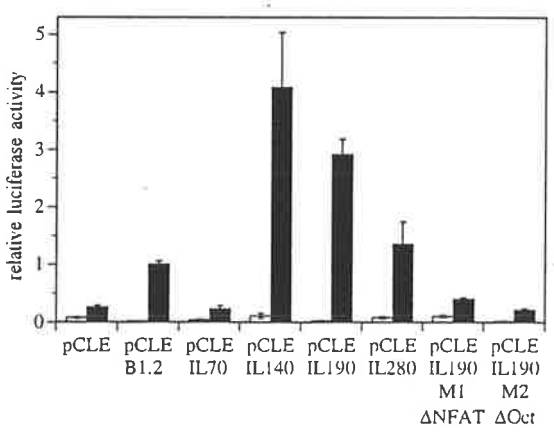


Figure 6. Three NFAT-like Elements in the IL-3 Enhancer Function as Transcriptional Activators

Three copies of each NFAT-like element were placed upstream of the GM-CSF CLE0 element in the luciferase reporter plasmid pCLE. Plasmids were transfected into Jurkat cells and assayed for PMA/I-inducible enhancer activity, using the full-length IL-3 enhancer (B1.2) as a control. Error bars represent the standard error of the mean of at least four transfections.

function as trimers in front of the pCLE promoter (Figure 6). A mutation in the Oct element (IL190M2) abolished enhancer activity, indicating that the NFATp site could not function on its own as a transcriptional activator. A mutation within the high affinity NFATp motif (IL190M1) led to a 6-fold decrease in activity, indicating that the Oct site is also inefficient as an isolated enhancer element. These assays suggested that despite an absence of cooperative binding, there is a high degree of synergy in the functions of Oct and NFAT family proteins in the activation of the IL190 element.

Discussion

The IL-3 Promoter Is Activated by a T Cell–Specific Enhancer

The human IL-3 gene is expressed almost exclusively in T cells, in contrast with the closely linked GM-CSF gene, which is expressed by a number of cell types at sites of inflammation. In this study, we identified a T cell–specific enhancer 14 kb upstream of the human IL-3 gene that is likely to play a role in the T cell–specific activation of the IL-3 gene. The IL-3 enhancer responded to activation by a combination of inducing agents (PMA/I) that activate the Ca^{2+} and protein kinase C signaling pathways that normally originate from the TCR. The IL-3 promoter and enhancer both appeared to be repressed in the presence of CsA, which blocks the Ca^{2+} signaling pathways by targeting the Ca^{2+}-dependent phosphatase calcineurin. In many ways the pattern of induction of the IL-3 enhancer mirrored that of the IL-3 gene as well as several other cytokine genes such as IL-2 and GM-CSF.

IL-3 enhancer activation was T cell specific at the levels of both chromatin structure and transcriptional activation. The DH site within the IL-3 enhancer most likely represents a nucleosome-free region, and this altered chromatin structure appeared in activated Jurkat T cells, but not in fibroblasts, HeLa cells, or a B cell line (Figure 1). We have also recently detected the DH site in activated CEM T cells, but not in endothelial cells (P. N. C., unpublished data). In contrast, the DH site within the GM-CSF enhancer is inducible in every cell type examined that has the capacity to express GM-CSF (Figure 1; Cockerill et al., 1995a; P. N. C., unpublished data).

In transfection assays, the IL-3 enhancer increased the inducible levels of reporter gene activity by 2- to 10-fold in Jurkat and CEM T cells, but was entirely inactive in other cell types that normally express GM-CSF but not IL-3. In the context of the IL-3, GM-CSF, or thymidine kinase promoters, the IL-3 enhancer had a level of activity in T cell lines that was indistinguishable from that of the human GM-CSF enhancer (Osborne et al., 1995; K. N. D., A. G. B., J. R. Burrows, and P. N. C., unpublished data).

The IL-3 Enhancer Resembles IL-2, IL-4, and GM-CSF Gene Regulatory Elements

The IL-3 enhancer appeared to be activated principally by an array of NFATp/c elements operating in cooperation with either Oct or AP-1 elements. In this regard the IL-3 enhancer resembled the GM-CSF enhancer and the IL-2 and IL-4 promoters. The GM-CSF enhancer has three functional NFATp/c sites linked to AP-1 sites (Cockerill et al., 1995b), and the IL-2 and IL-4 promoters each have up to four NFATp/c sites that are linked to AP-1 or Oct sites (or both) (Chuvpilo et al., 1993; Masuda et al., 1993; Pfeuffer et al., 1994; Rao, 1994; Rooney et al., 1995a, 1995b; Schreiber and Crabtree, 1992; Szabo et al., 1993; Todd et al., 1993). The IL-3 enhancer encompassed three functional NFATp/c elements that potentially account for most of the activity of the enhancer. These three elements were all located within the 220 bp HinfI–XbaI core region, which was almost as active as 1.2 kb BglII enhancer fragment. The two most active IL-3 enhancer elements (IL140 and IL190) were also the highest affinity NFATp sites and appeared to be located at the center of the DH site. The IL70 moderate affinity NFATp site and the NFμ-E1 motif did not appear to be essential since they could be deleted without significant loss of enhancer activity.

The Role of NFATp/c Motifs in the IL-3 Enhancer

The mechanisms by which NFATp/c family proteins function as transcriptional activators remain poorly defined. NFATp/c sites do, however, usually function in cooperation with other transcription factor–binding sites. NFATp/c may play a role in the modification of chromatin structure, as NFATp/c sites are closely associated with inducible CsA-sensitive DH sites in the GM-CSF enhancer and IL-2 promoter (Cockerill et al., 1995b; Durand et al., 1987). As the closely linked IL140 and IL190 elements also existed at the center of an inducible DH site, they may function partly by disrupting the interactions between histone proteins and the enhancer to form the DH site. The IL140 element had nuclear protein binding properties resembling those of the GM430 probe (Figure 4A), which represents just the high affinity NFATp/c-binding region of the GM420 element (Cockerill et al., 1995b). We have preliminary evidence that the GM430 element is sufficient to support the induction of a DH site even in the absence of the closely linked AP-1-binding site (P. N. C., unpublished data). These findings may, therefore, implicate the IL140 and IL190 NFATp/c sites as the DNA elements that mediate the induction of the DH site within the IL-3 enhancer.

From the evidence obtained with the IL70 and IL190M2 constructs (Figure 6), it was apparent that some NFATp/c sites are poor transcriptional activators in the absence of other closely linked transcription factor–binding sites. In contrast, the IL140 high affinity NFATp/c site did function as a powerful enhancer element even in the absence of any obvious adjacent transcription factor–binding sites. The IL140 NFATp complex was clearly unable to recruit AP-1, even when purified recombinant proteins were employed. However, if the IL140 element has the ability to create a DH site that encompasses nearby elements, it may still function in part by cooperating with other transcription factor–binding sites. In the native enhancer, it may cooperate with the IL190 element. In the pCLE-IL140 transfection experiments, where it functioned as a powerful enhancer (Figure 6), it may be acting to maintain the nearby the CLE0

promoter element in a nucleosome-free state. An alternative explanation for the high activity of the IL140 NFATp/c site could be the existence of another, as yet unidentified factor-binding site within the IL140 element.

Cooperation between NFATp/c and Oct Elements in T Cell–Specific Genes

Although we have yet to define the basis for the T cell specificity of the IL-3 enhancer, we have narrowed our focus to the IL190 composite NFATp/Oct element. This study provides evidence that Oct and NFATp/c factors can bind simultaneously to overlapping sites and that closely linked Oct and NFATp/c sites can function in a highly synergistic fashion. Preliminary studies suggest that the IL190 element can function as an enhancer element in T cells but not in endothelial cells, while the GM-CSF enhancer GM420 element can function as an enhancer in both cell types (J. R. Burrows and P. N. C., unpublished data).

NFATp/c and Oct elements are known to play central roles in directing the lymphoid cell–specific activation of other cytokine genes, such as IL-2 and IL-4 (Chuvpilo et al., 1993; Masuda et al., 1993; Pfeuffer et al., 1994; Rao, 1994; Rooney et al., 1995a, 1995b; Schreiber and Crabtree, 1992; Szabo et al., 1993; Todd et al., 1993). Furthermore, the pairing of an NFATp/c site with an Oct site is the most striking difference between the IL-3 and GM-CSF enhancers, which clearly have different functions despite their overall similarities. In the IL-2 promoter, there are five NFATp/c sites and two Oct sites (Rooney et al., 1995b; Schreiber and Crabtree, 1992). One of the IL-2 promoter NFATp/c sites is immediately upstream from closely linked AP-1 and Oct sites, and there is evidence for the formation of DNA complexes containing all three of these factors (Rooney et al., 1995b). In the IL-4 promoter, there exist at least four NFATp/c sites, three of which are linked to weak Oct-binding sites. However, unlike the IL190 element, the IL-4 promoter elements appear to be unable to bind NFATp/c and Oct factors simultaneously (Pfeuffer et al., 1994). The IL-3 promoter also encompasses an Oct site (Davies et al., 1993), and we have found that the nearby CK1 element also has the capacity to bind NFATp (K. N. D. and P. N. C., unpublished data).

We are currently exploring mechanisms by which composite NFATp/Oct elements such as the IL190 element might function as T cell–specific enhancer or promoter elements. Significantly, it is known that cell-specific transcription factors exist within both the Oct and NFATp/c families of proteins. The activation of the IL190 element might, therefore, be dependent on the coexpression of specific NFATp/c and Oct family proteins. We have detected Oct-1 and an Oct-T3-like factor as the predominant Oct family proteins in Jurkat, CEM, and Molt4 T cells, and Oct-1 and Oct-2 as the predominant Oct proteins in peripheral blood T cells (Figure 5; A. G. B., J. B. Burrows, and P. N. C., unpublished data). The specific combination of Oct-2 and NFATc or NFATp occurs in lymphoid cells, and this could relate to the ability of T cells to express IL-2, IL-3, and IL-4. NFATp is implicated in IL-3, IL-4, and GM-CSF gene expression since NFATp-deficient mice express reduced levels of all three cytokines (Hodge et al., 1996). The role of Oct-T3 in cytokine gene regulation is unclear as this remains an ill-defined Oct-like factor. The IL190 Oct element may also require a lymphoid-specific coactivator. In B cells, for example, Oct sites play an important role in regulating immunoglobulin gene expression (Staudt and Lenardo, 1991), and the B cell–specific coactivator Bob1 contributes to the B cell–specific and promoter-specific utilization of Oct elements (Gstaiger et al., 1996). Hence, the IL-3 enhancer may only function in a highly restricted population of cells that express specific combinations of Oct and NFATp/c factors and other coactivators.

If, as suggested above, NFATp/c functions inefficiently on its own, then the specific pairing of NFATp/c with other factors is a powerful means of directing tightly controlled cell-specific gene activation. As the IL-3 promoter also requires AP-1, the combination of at least AP-1, NFATp/c, and Oct factors may be required for IL-3 gene activation. The GM-CSF enhancer, on the other hand, is not reliant on Oct factors and is known to function in endothelial cells which express NFATp and AP-1. The dual requirement for AP-1 and NFATp/c in IL-2, IL-3, and GM-CSF gene expression ensures that at least two distinct signaling pathways must be activated before gene activation can occur.

It is likely that more detailed future studies will discover roles for additional transcription factors in the regulation of the IL-3 enhancer. This initial study is not a comprehensive survey and has focused on elements that are conserved among several cytokine genes. The NFATp sites in the IL-3 enhancer may, for example, cooperate with architectural proteins such as the HMG I/Y proteins (Chuvpilo et al., 1993). The T cell–specific regulation of the IL-3 gene could involve additional tissue-specific factors analogous to c-*maf*, which plays a central role in directing the T helper type 2 T cell–specific expression of the IL-4 gene (Ho et al., 1996).

Regulation of the IL-3/GM-CSF Locus

On the basis of the above findings, we suggest that the IL-3 and GM-CSF genes are independently regulated rather than coactivated by the intergenic GM-CSF enhancer. Fitzpatrick and Kelso (1995) have demonstrated that, even among T cell clones, the IL-3 and GM-CSF genes can be differentially expressed. Paradoxically, however, the IL-3 and GM-CSF promoters can be activated equally well by an upstream IL-3 or GM-CSF enhancer. The IL-3 gene may, therefore, also rely on additional flanking DH sites for its correct regulation in vivo (Figure 7). These DNA elements might help to partition the GM-CSF/IL-3 locus into distinct chromatin domains that could then be activated independently in a tissue-specific manner. The cluster of ubiquitous DH sites downstream of the IL-3 gene may represent a boundary element that segregates the IL-3 gene from the effects of GM-CSF enhancer activation in cell types that express GM-CSF and NFAT but not IL-3. Boundary elements were first identified in Drosophila and have the capacity to shield genes from adjacent regulatory elements (Kellum and Schedl, 1991). In addition, the IL-3 enhancer might cooperate in vivo with the cluster of DH

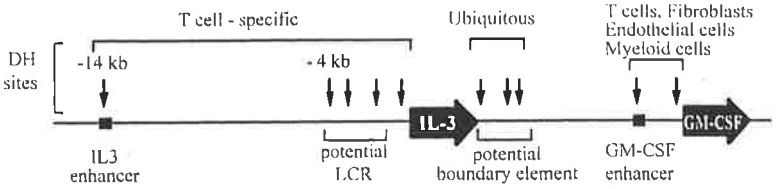


Figure 7. Regulatory Elements within the IL-3/GM-CSF Locus

Summary of the properties of DH sites identified in this and previous studies (Cockerill et al., 1993, 1995a).

sites located just upstream of the IL-3 gene. These DH sites, extending up to 4 kb upstream of the gene, are both constitutive and T cell specific. One of these sites corresponds to the promoter that associates with at least one T cell–specific factor (Davies et al., 1993).

Some cell-specific genes, such as the globin genes (Dillon and Grosveld, 1993) and the TCR genes (Diaz et al., 1994), are regulated by locus control regions (LCRs), which can usually be detected as clusters of constitutive cell-specific DH sites. LCRs typically cooperate with enhancers and promoters to maintain chromatin domains in an active conformation. The DH sites just upstream of the IL-3 gene have some of the properties expected of LCRs. Hence, we suggest that the IL-3 enhancer cooperates with other upstream and downstream elements to activate the IL-3 gene in a T cell–specific manner.

Experimental Procedures

DH Site Analyses

DH sites in Jurkat cells, CEM cells, human umbilical vein endothelial cells (HUVECs), Ball-1 cells, and human embryonic lung fibroblasts (HEL cells) were assayed as described previously (Cockerill et al., 1993). In brief, nuclei were isolated from cells by lysis in 0.1% Nonidet P-40 and resuspended to ~0.4 mg/ml nucleic acid (optical density at 260 nm of an aliquot in 1 M NaOH = 10 absorbance units/milliliter). Aliquots of nuclei were digested for 3 min at 22°C with DNase I (Worthington) at 2–20 U/ml in nuclei isolation buffer containing 1 mM $CaCl_2$. DNA was purified, digested with restriction enzymes, electrophoresed on a 0.8% agarose gel, blotted onto a Hybond N membrane (Amersham), and hybridized to ^{32}P-labeled probes. The IL-3 DH site was mapped using BamHI-digested DNA and a 0.7 kb EcoRI–BamHI fragment of λ-66 DNA (Cockerill et al., 1993; a gift of S. Clark and Y.-C. Yang, Genetics Institute, Boston, MA) that extends upstream from the BamHI site located 10 kb upstream of the human IL-3 gene. The GM-CSF enhancer DH site was mapped as described previously (Cockerill et al., 1993).

Plasmid Construction

pIL3 was made by cloning a 613 bp fragment of the human IL-3 promoter (−559 to +50) into a HindIII site of the pXP1 (Nordeen, 1988) luciferase reporter plasmid. The IL-3 promoter fragment was obtained as a HindIII fragment of a PCR product generated using the oligonucleotide primers GTGTCAAGGAGAAGCTTCCCGAAG CCC (−559) and CGGGCAGGCGGCTCAAGCTTGGATCGGCAGG (+50) and the DNA clone λ-66 (Cockerill et al., 1993). pIL3B1.2 and pIL3B1.2R were made by cloning the 1.2 BglII fragment encompassing the IL-3 enhancer into a BglII site upstream of the IL-3 promoter in pIL3 in either the normal or opposite orientation relative to the promoter, respectively. The 1.2 kb BglII fragment containing the enhancer was isolated from the plasmid pG2 (a gift of E. Frolova, Shemyakin Institute of Organic Chemistry, Moscow, Russia) and is normally located approximately 13.5–14.7 kb upstream of the human IL-3 gene (Frolova et al., 1991). pIL3NA330 and pIL3HX220 were made by cloning either a 330 bp NruI–AccI fragment of the IL-3 enhancer or a 220 bp HinfI–XbaI fragment of the IL-3 enhancer into EcI136-cut pIL3. pTK81 is the plasmid pT81luc (Nordeen, 1988) consisting of a herpes simplex thymidine kinase (TK) promoter (−81 to +52) in a luciferase reporter gene plasmid. pTK81B1.2 was made by cloning the 1.2 kb BglII fragment encompassing the human IL-3 enhancer upstream of the TK promoter in the BamHI site of pTK81. The plasmid pGM contains the human GM-CSF promoter upstream of the luciferase gene (Osborne et al., 1995). pGMB1.2 and pGMSV40 were made by cloning either a 1.2 kb BglII fragment encompassing the IL-3 enhancer or a 158 bp fragment of the SV40 enhancer (113 to 270), respectively, into a BglII site upstream of the GM-CSF promoter in pGM. The plasmid pCLE contains nucleotides −55 to +28 of the human GM-CSF promoter and is the same as pGM55 (Cockerill et al., 1996). pCLEB1.2 had a 1.2 kb BglII fragment of the human IL-3 enhancer placed upstream of the −55 GM-CSF promoter in the BglII site of pCLE. All other pCLE constructs were made by inserting three head-to-tail copies of the appropriate oligonucleotides in the BglII site of pCLE. All DNA fragments (unless otherwise indicated) were inserted in the same orientation relative to the promoters as they exist in the GM-CSF/IL-3 locus.

Oligonucleotides

Oligonucleotide duplexes used as probes and competitors and in the construction of reporter plasmids had the sequences listed below. Each strand of these duplexes was synthesized with a 5′ GATC extension for cloning purposes. The sequence is depicted for one strand only.

IL70, gatccAAATACTGGAAAACCAAAGAGAATCTGAAA; IL140, gatctGCCCAGTGGAAAAAGCAAGGGTTCTTGCCG; IL190, gatccGG GTGCTCCATGGAAAATGCAAATCTACTa; IL190 M1, gatccGGGTGC TCCATGGccAATGCAAATCTACTa; IL190 M2, gatccGGGTGCTCCA TGGAAAATGCccATCTACTa; IL190 M3, gatccGGGTGggCCATGGA AAATGCAAATCTACTa; IL280, gaTCTAGAGGCTGGAAAACTGTAG CTCACAGCAGG; IL-3 CK-1, agcTTTTCTATGGAGGTTCCATGTCAG ATA; IL-2 NFAT, gatcCGAAAGGAGGAAAAACTGTTTCATACAGAAG; octamer, gatcCCTAATTTGCATG; AP-1, gatcTGGATCACCCGCAGC TTGACTCATCCTTGCA; GM430, gatcTCACACATCTTTCTCATGGA AAGATGA.

All nucleotides that differ from the natural sequence are depicted in lowercase. The AP-1-binding sequence is derived from the stromelysin gene, and the GM430 NFATp/c site is derived from the GM-CSF enhancer (Cockerill et al., 1995b). The octamer is derived from an immunoglobulin gene enhancer (Staudt and Lenardo, 1991) and is used as a competitor of Oct family proteins.

Cell Culture

The Jurkat T leukemic cell line, Ball-1 B leukemic cell line, KG1a myeloid leukemic cell line, HeLa cervical carcinoma cell line, 5637 bladder carcinoma epithelial cell line, and HEL cells were cultured in RPMI containing 10% FCS. The CEM leukemic T cell line was cultured in RPMI containing 15% FCS. HUVECs were cultured as previously described (Cockerill et al., 1995a).

Transfections and Luciferase Assays

All cells except HUVECs were transfected with 5 μg of CsCl-purified plasmid DNA by electroporation, cultured for 20–24 hr, stimulated with 20 ng/ml PMA and 1 μM calcium ionophore A23187 (PMA/I) for 9 hr, and assayed for luciferase reporter gene activities as previously described for Jurkat cells (Osborne et al., 1995). HUVECs were transfected with 20 μg of CsCl-purified plasmid DNA by the DEAE–dextran procedure, cultured for 36 hr, stimulated with 20 ng/ml PMA and 2 μM calcium ionophore A23187 for 9 hr, and assayed for luciferase reporter gene activities as previously described (Cockerill et al., 1995a).

Gel Electrophoretic Mobility Shift Assays

Gel shift assays and preparation of nuclear extracts were as previously described (Cockerill et al., 1995b), except that assays employed 5 μg of nuclear extract and 2 μg of poly(dI–dC) in a 15 μl volume. In some instances, nuclear extracts were enriched by binding to heparin Sepharose, washing with 0.15 M KCl, and eluting with 0.45 M KCl before the final dialysis. Assays of nuclear proteins used nuclear extracts prepared from unstimulated cells and cells stimulated for 2–3 hr with 20 ng/ml PMA and 2 μM A23187 in the presence and absence of 0.1 μM CsA. Some assays were performed in the presence of specific antisera. The R59 antiserum raised against murine NFATp was used as described previously (Cockerill et al., 1995b). The Oct-1 antiserum was a gift from R. Sturm and was raised against human Oct-1 (Sturm et al., 1988). The Oct-2 antiserum was raised against murine Oct-2 and was a gift from L. Corcoran (Walter and Eliza Hall Institute, Melbourne, Australia). To reconstitute NFAT-like complexes from purified recombinant proteins, we used 4 ng of wbJun and 2.5 ng of wbFos as the source of AP-1 and 0.1 ng of truncated NFATp with 100 ng of poly(dI–dC) and 0.2 ng of probe, as previously described (Cockerill et al., 1995b). To estimate the relative affinities of NFATp/c elements, we assayed 0.3 ng of purified recombinant NFATp with 0.2 ng of GM430 probe and included 1, 2, 4, or 8 ng of each element as unlabeled competitor DNA. For each NFATp/c element, the relative affinity for NFATp, or potency as a competitor, was expressed as the magnitude of inhibition of NFATp binding afforded by each element relative to the GM430 element.

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