THE INTERACTIONS OF INTERLEUKIN-3 AND GRANULOCYTE-MACROPHAGE COLONY-STIMULATING FACTOR WITH HUMAN MONOCYTES

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ABSTRACT

The Interactions of Interleukin-3 and Granulocyte-Macrophage Colony-Stimulating Factor with Human Monocytes

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Techniques were established for the purification of human monocytes from normal peripheral blood. Monocyte purities of greater than 95% were achieved by a combination of density centrifugation and countercurrent elutriation, with yields of up to $1.2x10^8$ monocytes from 500ml whole blood. The effects of interleukin-3 (IL-3) and granulocyte-macrophage colony-stimulating factor (GM-CSF) on monocyte number, size and antigen expression after *in vitro* culture were measured. Both cytokines enhanced monocyte survival between days 4 and 14 of culture at concentrations as low as 6pM. In contrast, stimulated ^{3}H-thymidine uptake was only seen at CSF concentrations of 60pM and above. Autoradiographic studies demonstrated a small subpopulation of cells (1-3%) with nuclear grains. IL-3 and GM-CSF also stimulated increased cell size, increased protein content, and enhanced surface expression of the complement receptor 3 and other adhesion-related antigens in 4 to 14 day cultures. These studies suggest that IL-3 and GM-CSF may help regulate monocyte numbers and maturation at inflammatory sites where CSF are produced.

The roles of IL-3 and GM-CSF in the regulation of monocyte function were studied, focusing on monocyte adhesion. Both cytokines stimulated adhesion to endothelial monolayers, extracellular matrix proteins and plastic in a biphasic manner. 'Early phase' stimulated adhesion was seen within 10 minutes of addition with both cytokines, but the rate

of increase was greater for GM-CSF than for IL-3. 'Late phase' adhesion was seen after 9 hours of culture, and showed an identical temporal profile for the two CSF. The two phases of adhesion could be further distinguished by the use of an inhibitor of protein synthesis, which abolished late- but not early-phase stimulated adhesion. Inhibition experiments with monoclonal antibodies (MAb) to antigens of the leukocyte functional antigen (LFA) family showed that the stimulated adhesion at both early and late time points was LFA dependent. Levels of surface expression of LFA antigens correlated poorly with functional status, however, suggesting that a change in the distribution or activation state of the relevant adhesion molecules was responsible for the stimulated adhesion. In contrast to IL-3 and GM-CSF, interleukin-4 was found to inhibit both basal and stimulated monocyte adhesion, suggesting a possible anti-inflammatory role for this cytokine *in vivo*.

Monocyte receptors for IL-3 and GM-CSF were studied by radio-iodinating the cytokines, and performing binding experiments. Scatchard analyses of equilibrium binding data showed the presence of two classes of receptor for each CSF: a small number of high affinity receptors (K_D 5-39pM) and a larger number of low affinity receptors (K_D 513-1120pM). High affinity binding for both ligands was subject to partial competitive inhibition by the other CSF, but not by a range of other cytokines, while the low affinity sites were non-competable. Despite the similarities in equilibrium binding for the two CSF, kinetic binding experiments revealed major differences. GM-CSF showed more rapid association to its receptors (t1/2=40 seconds) than did IL-3 (t1/2=2.5 minutes), correlating with the more rapid stimulation of monocyte adhesion by the former cytokine. Dissociation rates were also more rapid for GM-CSF than for IL-3, allowing the calculation of kinetically-derived K_D values which were similar for the two ligands and showed close agreement with those derived from Scatchard analysis. Internalisation of receptor-ligand complexes was a relatively slow process for both IL-3 and GM-CSF, with a t1/2 of 14-15 minutes.

The striking similarities between the actions of IL-3 and GM-CSF on human monocytes, together with their competition for binding to the monocyte membrane, suggested a common mode of cell activation. A model of interacting IL-3 and GM-CSF receptors was therefore proposed, and discussed in terms of both the biological and binding data.

DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University.

To the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

In accordance with the University of Adelaide regulations, I give my consent to this thesis being made available for photocopying and loan if accepted for the award of the degree.

Michael JH Elliott

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ABBREVIATIONS

ANL	Acute nonlymphocytic leukemia
APAAP	Alkaline phosphatase-anti-alkaline phosphatase
BMM	Bone marrow-derived macrophage(s)
BSA	Bovine serum albumin
CD	Cluster determinant
CFU	Colony-forming unit
CHO	Chinese hamster ovary
cpm	counts per minute
CSA	Colony-stimulating activity
CSF	Colony-stimulating factor(s)
CSL	Commonwealth Serum Laboratories
CTL	Cytotoxic T lymphocytes
DMEM	Dulbecco's modified eagle's medium
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EMP	Extracellular matrix protein
EPO	Erythropoietin
FACS	Fluorescence-activated cell sorter
FCS	Fetal calf serum
FIM	Factor increasing monocytopoiesis
G	Granulocyte
h	human
HBSS	Hanks balanced salt solution (without Ca^{++} or Mg^{++})
HIV	Human immunodeficiency virus
HUVEC	Human umbilical vein endothelial cells
ICAM-1	Intercellular adhesion molecule-1

IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
Ka	Association rate constant
Kd	Dissociation rate constant
K_D	Apparent dissociation constant
KD	Kilodalton
Ke	Internalisation rate constant
LAD	Leukocyte adhesion deficiency
LAK	Lymphokine-activated killer
LFA	Leukocyte functional antigen
LPS	Lipopolysaccharide
M	Monocyte/macrophage
m	murine
MAb	Monoclonal antibody(ies)
MAF	Macrophage activating factor
MBC	Maximal binding capacity
MHC	Major histocompatibility complex
MNC	Mononuclear cell(s)
Mr	Molecular weight
NK	Natural killer
NSE	Non-specific esterase/chloroacetate
PAF	Platelet activating factor
PAM	Pulmonary alveolar macrophage(s)
PBS	Phosphate-buffered saline
PDGF	Platelet-derived growth factor
PEM	Peritoneal exudate macrophage(s)

PHA	Phytohaemagglutinin
PMA	Phorbol myristate acetate
r	recombinant
R	Receptor
RA	Rheumatoid Arthritis
SD	Standard deviation
SDA	Self-displacement analysis
SEM	Standard error of the mean
SR	Specific radioactivity
t1/2	Half time
TGF	Transforming growth factor
TNF	Tumor necrosis factor

PUBLICATIONS ARISING

Publications arising from the work included in this thesis, and from related collaborative work, are:

1. AF Lopez, PG Dyson, LB To, MJ Elliott, SE Milton, JA Russell, CA Juttner, Y-C Yang, SC Clark and MA Vadas. Recombinant human interleukin-3 stimulation of haematopoiesis in humans: loss of responsiveness with differentiation in the neutrophilic myeloid series. Blood 72:1797-1804, 1988.

2. JR Gamble, MJ Elliott, E Jaipargas, AF Lopez and MA Vadas. Regulation of human monocyte adherence by granulocyte-macrophage CSF. Proceedings of the National Academy of Science USA 86:7169-7173, 1989.

3. MJ Elliott, MA Vadas, JM Eglinton, LS Park, LB To, LG Cleland, SC Clark and AF Lopez. Recombinant human interleukin 3 (IL-3) and granulocyte-macrophage colony stimulating factor (GM-CSF) show common biological effects and binding characteristics on human monocytes. Blood 74 (in press).

4. GW Krissansen, MJ Elliott, CM Lucas, FC Stomski, MC Berndt, DA Cheresh, AF Lopez and GF Burns. Identification of a novel integrin beta subunit expressed on cultured monocytes: evidence that one alpha subunit can associate with multiple beta subunits. Journal of Biological Chemistry (in press).

5. GW Krissansen, CM Lucas, FC Stomski, MJ Elliott, MC Berndt, AW Boyd, MA Horton, DA Cheresh, MA Vadas and GF Burns. Blood leukocytes bind platelet

glycoprotein (IIb-IIIa)' but do not express the vitronectin receptor. International Immunology (in press).

6. MJ Elliott, MA Vadas, LG Cleland, JR Gamble and AF Lopez. Interleukin-3 and granulocyte-macrophage colony-stimulating factor stimulate two distinct phases of adhesion in human monocytes. (Submitted)

CHAPTER 1

INTRODUCTION

1.1 Preface

The work described in this thesis was undertaken with the aim of establishing the role of two haemopoietic growth factors, granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin 3 (IL-3) in the regulation of human monocyte biology. Specifically, the work aimed to:

(1) investigate possible roles for IL-3 and GM-CSF in the regulation of monocyte **numbers** outside the bone marrow, and

(2) determine whether IL-3 or GM-CSF were involved in the regulation of monocyte **function**.

The fulfilment, at least in part, of these aims prompted investigations into the mechanism of action of the two factors on monocytes. Accordingly, the second half of this thesis explores the interactions of IL-3 and GM-CSF with their receptors on monocytes, and the findings have been used to explain similarities and differences between the two factors in their biological effects.

At the time of commencement of this work there was no evidence to show that either factor could influence human monocyte function, although the identification of GM-CSF as a neutrophil-activating factor, and the role of both cytokines in normal monocytopoiesis, suggested that this was likely. This prediction has proven correct, with the publication of more than thirty articles during the past three years describing CSF-monocyte interactions of one type or another. The current intense interest in this field may simply reflect an appreciation of the importance of CSF in general, and of their potential for clinical use. In addition, it may reflect the long standing interest of many investigators in mononuclear phagocyte biology, and in the central role of this cell type in immunological and inflammatory reactions.

1.2 The molecular control of monocytopoiesis

The control of myeloid cell production and differentiation has been recently reviewed (Cannistra et al, 1988B; Metcalf, 1989). The development of semi-solid culture systems for the growth of bone marrow cells *in vitro* (Bradley and Metcalf, 1966; Ichikawa et al, 1966) has allowed the factors regulating haemopoiesis, and their target cells, to be extensively characterised. The most primitive clonogenic cell in human marrow is the colony-forming unit (CFU)-blast, which gives rise to a colony of self-renewing undifferentiated blast cells. Upon replating, blast cells may give rise to more differentiated cells, which lack self-renewal or secondary plating potential, and which show commitment to a given haemopoietic lineage. The granulocytic and monocyte/macrophage lineages share a common progenitor, the CFU-granulocyte/monocyte (CFU-GM). The production of monocytes from CFU-GM proceeds via a process of proliferation and differentiation, through monoblast and promonocyte stages. Like mature peripheral blood monocytes, monoblasts and promonocytes are strongly adherent cells, stain with non-specific esterase (NSE) and peroxidase, express cell surface Fc receptors, and are capable of membrane ruffling and phagocytosis (Nathan and Cohn, 1985). In man, promonocytes represent approximately 3% of all bone marrow cells, with a pool size of $6x10^8$/kg.

At least three CSF are involved in the survival, proliferation and differentiation of monocyte progenitor cells *in vitro*. IL-3 stimulates a range of haemopoietic progenitor cells, giving rise to granulocytic, monocytic, erythroid, mast cell, megakaryocytic and mixed colonies (Metcalf, 1989), justifying its alternative name 'multi-CSF'. GM-CSF shows a somewhat more restricted spectrum, with monocytic colonies seen at low cytokine concentrations, followed by granulocytic-monocytic, eosinophil, megakaryocytic and mixed colonies with progressively increasing CSF concentrations. Macrophage CSF (M-CSF) is, in contrast, a lineage restricted haemopoietin, giving rise to monocyte/macrophage colonies only. Human

(h) M-CSF shows relatively weak colony-stimulating activity (CSA) in human bone marrow culture, but like other lineage restricted CSF (G-CSF, IL-5) is active across the species, and shows greater activity in murine cultures. The CSA of hM-CSF has been shown to be considerably enhanced with the use of sub-stimulatory concentrations of GM-CSF (Caracciolo et al, 1987), and similar potentiating effects of IL-3 on GM-CSF-stimulated haemopoiesis (Donahue et al, 1988) and of IL-3 on M-CSF-stimulated haemopoiesis (Williams et al, 1987) have been reported *in vivo*. Through some of these and other studies (Sonoda et al, 1988; Leary et al, 1987) it has become apparent that IL-3 acts on more primitive progenitor cells than GM-CSF, and that the potentiating effects of IL-3 may arise through expansion of an early cell population, which requires a later acting factor (GM-CSF, M-CSF) to complete its development (Donahue et al, 1988).

The *in vivo* administration of CSF leads to changes which are largely predictable from *in vitro* studies (reviewed by Nicola, 1989). Injection of GM-CSF causes an increase in blood monocyte and granulocyte counts, and a marked elevation of macrophage numbers in the peritoneal cavity and in other organs in mice. IL-3 induces similar tissue changes, but only modest and delayed increases in blood leukocyte counts. M-CSF administration in mice results in only small changes in blood counts, but its action, as described above, is synergistic with GM-CSF and IL-3. Insights into the effects of chronic administration of CSF have resulted from the use of murine models transgenic for GM-CSF (Lang et al, 1987) or transplanted with bone marrow harbouring a retrovirally activated GM-CSF gene (Nicola, 1989). High serum levels of GM-CSF are detected in these animals, and extensive macrophage-associated pathology in the eyes, muscles, liver and lungs develops, with a fatal outcome. Transplantation of bone marrow transfected with the IL-3 gene results in a somewhat different picture (Chang et al, 1989). High serum levels of IL-3 are seen, together with increases in blood and tissue granulocyte and tissue mast cell numbers. However, the changes in monocyte/macrophage biology seen in the GM-CSF-transfected

mice are not evident. While this may indicate differences between IL-3 and GM-CSF in their capacity to stimulate cells of the monocyte/macrophage lineage, differences in the number and pattern of transfected clones, and in CSF levels in the serum may also be of relevance (Chang et al, 1989).

In addition to the factors so far discussed, two other cytokines have been shown to modulate the process of monocytopoiesis *in vitro*. These are interleukin-4 (IL-4) which inhibits CSF-induced monocyte colony formation in man (Jansen et al, 1989) and induces the formation of multinucleate giant cells (McInnes et al, 1988) and IL-6, which synergises with M-CSF in macrophage colony formation (Bot et al, 1989).

1.3 Characteristics of IL-3 and GM-CSF

IL-3 and GM-CSF share a number of properties in addition to their roles in haemopoiesis. Both CSF have been molecularly cloned in the mouse and in man (Wong et al, 1985; Yang et al, 1986) and produced in a variety of expression systems. Recombinant human (rh) IL-3 and GM-CSF produced in yeast or mammalian cells are glycoproteins of molecular weight (Mr) 18-30KD, or approximately 15KD when non-glycosylated. They are of similar length (IL-3: 133 amino acids; GM-CSF: 127 amino acids) and show some similarities in their predicted tertiary structure (S. Leach, personal communication). Although they show little primary sequence homology, the close tandem linkage of their genes on the long arm of chromosome 5, together with some similarities in the pattern of exons in the respective genes, has led to the suggestion that they have evolved from a common ancestor (Yang et al, 1988).

As with many secreted factors, the two CSF are synthesised as precursor molecules with a hydrophobic leader sequence of 19-25 amino acids, which is cleaved from the mature

protein prior to secretion. While GM-CSF is produced by a number of cell types *in vitro*, including T cells, endothelial cells, fibroblasts and activated macrophages, the production of IL-3 is limited to T cells (Nicola, 1989). A recent publication described the detection of CSF mRNA in peripheral blood mononuclear cells (MNC) by *in situ* hybridisation (Wimperis et al, 1989). Unstimulated MNC showed no expression of IL-3 or GM-CSF, while stimulation with phytohaemagglutinin (PHA) and phorbol myristate acetate (PMA) or ionomycin, resulted in 5% of cells expressing GM-CSF mRNA, and only 1% expressing IL-3 message. Fractionation of the MNC into T lymphocytes, monocytes, natural killer (NK) cells, and T4+ and T8+ lymphocyte subsets allowed the presence of GM-CSF mRNA to be demonstrated in all cell types, while mRNA for IL-3 was detected only in the unfractionated lymphocytes. Further, while only a small fraction of freshly isolated MNC expressed IL-3 and GM-CSF mRNA, pretreatment with IL-2 for 1 week followed by stimulation with PMA and ionomycin resulted in 50% and 3% of the lymphocyte population expressing GM-CSF and IL-3 mRNA respectively. These results confirm the more restricted pattern of expression of IL-3 compared with GM-CSF, and also point to possible mechanisms for regulation of expression of both cytokines.

In contrast to M-CSF, which is found in normal serum, or can be purified from mouse and human urine (Bartocci et al, 1987) GM-CSF and IL-3 are not normally detectable in the blood. High concentrations of GM-CSF and IL-3 in the serum have been recorded in the CSF-transgenic and -transplanted murine models, however, and small rises in circulating GM-CSF are seen in mice injected with lipopolysaccharide (LPS) (Nicola, 1989). Colony-stimulating factors have also been detected at extravascular sites in man, usually in association with inflammatory disease. Colony-stimulating activity was detected in the blister fluids of patients with bullous pemphigoid (Varigos et al, 1982) and the synovial fluid of patients with rheumatoid arthritis (RA) (Williamson et al, 1988). The former activity was strongly eosinophilopoietic, while in the latter patients, CSA for both

granulocytes and monocytes was detected. More recently, a predominantly macrophage CSA was identified in synovial fluid from RA patients using the murine colony-forming assay. Since hIL-3 and hGM-CSF do not show activity on murine cells, the CSA is most likely to represent M-CSF. In addition, GM-CSF has been detected in rheumatoid synovial effusions and in the culture supernatants from rheumatoid synovial tissue cells by radio-immuno assay, confirming the presence of at least this CSF at the inflammatory site (Xu et al, 1989; Alvaro-Gracia et al, 1989).

1.4 Mononuclear phagocytes in inflammation

The characteristic histological feature of inflammation is the presence of a cellular infiltrate in the perivascular and interstitial spaces. While granulocytes predominate early in the course of an acute inflammatory insult, by 36 hours the major phagocytic cell type is the monocyte or monocyte-derived macrophage (Dieppe and Blake, 1986). In chronic inflammatory diseases, such as RA, mononuclear phagocytes predominate within the inflamed synovium, although granulocytes are found in the synovial fluid particularly during acute exacerbations of the disease.

Much interest has focussed on the origin of mononuclear phagocytes in inflammation, and the available evidence, gathered largely from animal studies, suggests two pathways. The majority of synovial macrophages are recently derived from blood monocytes, as shown by Volkman and Gowans (1965) and Loewi (1969). Monocytes normally circulate in the blood with a half time (t1/2) of 8-71 hours in man (Nathan and Cohn, 1985) and although the presence of a marginating pool has been demonstrated in mice (van Furth et al, 1986), an equivalent pool in man remains controversial (Johnston, 1988). In non-inflamed tissues, monocytes leave the circulation apparently at random by adhering to, and migrating through, the capillary endothelium. Once in the tissue space, they differentiate to become

tissue macrophages, and rarely re-enter the circulation. During inflammation, a number of changes in the life cycle of monocytes occur. The rate of monocyte production within the marrow may increase up to 4 fold, with increases seen within 12 hours of the inflammatory insult (Nathan and Cohn, 1985). This is achieved by an increase in the size of the promonocyte pool, an increase in the proportion of promonocytes actively cycling, a decrease in cycling time, and a more prompt release into the circulation. Sluiter et al (1987) have described and partially characterised a 'factor increasing monocytopoiesis' (FIM) which is seen in the serum of mice and rabbits at the onset of inflammation. The factor is a monokine, with Mr of 10-25KD, stimulates only monocyte production and is not M-CSF or IL-1. Whether FIM is a previously described CSF, or represents a new haemopoietic growth factor is not at present known.

In addition to increased monocytopoiesis, inflammation is characterised by the directed immigration of monocytes into the inflammatory site, such that up to 70% of the marrow production can localise to a single experimentally induced lesion (Nathan and Cohn, 1985). Immigration into defined anatomical sites may result from changes in the vessel wall, or from the action of chemotactic or pro-adhesive factors on blood monocytes. The potential for local production of CSF leading to enhanced monocyte-endothelial attachment at the inflammatory site, is explored in Chapter 4 of this thesis.

The second pathway leading to increased mononuclear phagocyte numbers in inflammation is through *in situ* proliferation of tissue macrophages (Loewi, 1969; North, 1969; Spector, 1979). Several *in vitro* studies support a role for CSF in the control of survival and proliferation of mononuclear phagocytes in the mouse (Tushinski et al, 1982, 1985; Chen et al, 1986, 1988A, 1988B; Lin et al, 1989), while information in the human system is scant (Becker et al, 1987; Koyanagi et al, 1988). The role of CSF in human granulocyte survival (Begley et al, 1986), together with the murine data referred to above, suggested a possible

role for IL-3 and GM-CSF in the local control of monocyte/macrophage numbers. This possibility is explored in Chapter 3 of this thesis.

1.5 Regulation of monocyte function by lymphokines

An important step in the understanding of the biology of mononuclear phagocytes came with the work of Mackaness and his colleagues during the 1960s (reviewed by Mackaness, 1970), who noted that macrophages from animals which had recovered from an infection with an intracellular parasite showed enhanced microbicidal activity against a variety of micro-organisms. This work had followed earlier observations, including those of Metchnikoff (1905) who noted that macrophages from infected hosts were more phagocytic than normal macrophages, and of Lune who showed that macrophages from tuberculous animals were larger and more phagocytic than those from controls (Nathan and Cohn 1985). A number of characteristics of this 'activated' macrophage phenotype have been described, and reviewed by Johnston (1988). Activated macrophages are larger than their non-activated counter-parts, show increased membrane ruffling and pseudopod formation, enhanced adhesive and spreading capability and increased numbers of pinocytic vesicles. In addition, they exhibit enhanced capacity to kill intracellular organisms or tumour cells, and an increase in the secretion of a wide range of products including monokines, reactive oxygen intermediates, and cytolytic proteinases.

While some investigators use the term 'macrophage activation' to refer to the whole range of stimulated functions detailed above, others restrict its usage to stimulated tumouricidal and microbicidal activity, leading to some difficulty in communication between different groups. Different opinions on what constitutes 'activation' partly result from the view that a gradual augmentation of function cannot be equated with a rapid functional response to a stimulus (Nathan and Cohn, 1985). For reasons of clarity, therefore, the term will be

avoided as far as possible in this thesis, and the concept of stimulation of monocyte/macrophage function will be employed instead.

The stimulation of macrophage function seen in late stages of infection, or after the host recovers, results from the secretion of lymphokines by antigen-stimulated T cells. The major macrophage stimulating lymphokine in concanavalin A-stimulated lymphocyte supernatants was identified as interferon γ (IFNγ) (Nathan et al, 1983, 1984), using macrophage oxidative metabolism and microbicidal activities as the functions of interest. Stimulation of macrophage function by IFNγ generally requires the presence of a second stimulus, usually in the form of a bacterial cell wall product such as LPS. The two stimuli act in a defined sequence, with IFNγ 'priming' the cell for subsequent stimulation (Hamilton and Adams, 1987). The existence of macrophage stimulating lymphokines other than IFNγ has been a topic of some interest. A recent paper described only low levels of IFNγ in synovial fluid and tissue in RA, despite the 'activated' phenotype of the synovial macrophages (Firestein and Zvaifler, 1987), suggesting the presence of other locally produced factors. The past three years have seen a number of publications describing the stimulation of monocyte/macrophage function by CSF and interleukins in both murine and human cells. These functions include antigen presentation (Morrisey et al, 1987), antimicrobial function (Reed et al, 1987; Weiser et al, 1987; Wang et al, 1989), tumouricidal activity (Grabstein et al, 1986; Cannistra et al, 1988A), monokine production (Cannistra et al, 1988A; Horiguchi et al, 1987; Vellenga et al, 1988; Oster et al, 1989) and secretion of prostaglandin E_2 (Heidenreich et al, 1989). In addition, CSF have been shown to regulate IL-2 receptor expression (Hancock et al, 1988) and the replication of human immunodeficiency virus (HIV) in human monocytes (Koyanagi et al, 1988; Perno et al, 1989). It is clear, therefore, that the CSF are 'macrophage activating factors' by whatever criteria one uses, and that they have fundamental influences on monocyte/macrophage biology.

1.6 Regulation of monocyte adhesion in inflammation

One of the more striking characteristics of mononuclear phagocytes is their propensity to adhere to glass or plastic surfaces, and this property has been used for many years to facilitate their purification from other leukocytes. The critical role of phagocyte adhesion in the development of inflammation was first recognised by Metchnikoff (1905) and is now well established. Like neutrophils, monocytes must first adhere to the endothelium before emigrating from the intravascular to the extravascular space (Migliorisi et al, 1987; Pawlowski et al, 1988). The migration of monocytes through the extravascular space may also depend on adhesion, if models developed for fibroblasts can be shown to apply. Such models generally describe the anchoring of fibroblasts to specific components of the extracellular matrix by means of plasma membrane receptors, while coupling of the receptors to the cytoskeleton allows cell motility (Bretcher, 1988; Hemler, 1988; Dunn et al, 1989). In addition, accumulation of monocytes at the inflammatory site may be facilitated by adhesion to fibronectin and other matrix proteins produced by endothelial cells (Clark et al, 1982; Dunn et al, 1989). Adhesion events may also determine the functional phenotype of monocytes (Dougherty et al, 1988; Parker et al, 1988; Haskill et al, 1988 and Eierman et al, 1989), and are essential for certain effector functions including antigen presentation (Dougherty et al, 1988) and monocyte-tumour cell conjugation (Te Velde et al, 1987).

Understanding of the molecular basis of leukocyte adhesion has shown significant advances over the past 10 years, with the identification of a number of separate families of adhesion receptors known as integrins. Each family comprises a beta chain which can associate with any of several different alpha chains, giving rise to the characteristic heterodimeric integrin complex. Although there has been no evidence to suggest the formation of inter-family heterodimers, recent evidence from this laboratory indicates that the alpha chain of the vitronectin receptor (a member of the beta 3 integrin family) can associate in addition with a

novel beta chain on cultured human monocytes (Krissansen et al, in press), indicating that current models may need to be revised. A series of observations by Springer and colleagues has emphasised the importance of the beta 2 integrins in leukocyte adhesion. This family, also known as the leukocyte functional antigen (LFA) family, comprises a 95KD β chain which can associate with any of three different alpha chains, giving rise to the three adhesion complexes MAC-1 (or the complement receptor 3), LFA-1 and p150/95 (reviewed by Gallin, 1985). Patients with a congenital deficiency or complete absence of expression of these molecules show a number of abnormalities of leukocyte function, including delayed separation of the umbilical cord, recurrent bacterial and fungal infections, difficulty in healing, severe gingivitis, and poor leukocyte adhesion (Gallin, 1985). Although most studies on patients with this leukocyte adhesion deficiency (LAD) syndrome have focussed on granulocytes, at least one study has shown the failure of migration of monocytes to skin windows or extravascular sites of infection in LAD patients, suggesting that the LFA family is also critical to monocyte adhesion (Bowen et al, 1982). The individual members of the LFA family, their component alpha and beta chains, their ligands (where known) and the monoclonal antibodies used to identify them are shown in Table 2.1 (Materials and Methods).

While the importance of monocyte adhesion in inflammation is clear, and the molecular mechanisms mediating adhesion have been partially defined, the regulation of this process in health and disease, remains poorly understood. Complement activation *in vivo* during haemodialysis leads to a transient monocytopenia as a result of margination of monocytes in the pulmonary vasculature (Craddock et al, 1977) and studies *in vitro* confirm that the complement product C5a stimulates monocyte adhesion to endothelium (Doherty et al, 1987). Similar stimulation *in vitro* is seen with the chemotactic peptide formyl-methionyl-leucyl-phenylalanine, with PMA, and with autologous plasma (Doherty et al, 1987; Wallis et al, 1985). Data on the regulation of monocyte adhesion by cytokines is rudimentary, but

two reports suggest a role for IFNγ in this capacity. The first demonstrated IFNγ induced homotypic adhesion in monocytes (Mentzer et al, 1986), a phenomenon which may be viewed as a model for monocyte-tumour cell conjugation (Mentzer et al, 1986). This may also be relevant to the induction of multinucleate giant cell formation by IFNγ in long term monocyte cultures (Weinberg et al, 1984), a process which is dependent on monocyte fusion. More recently, adhesion of murine peritoneal exudate macrophages (PEM) to basement membrane glycoproteins was shown to be stimulated with IFNγ and LPS (Shaw and Mercurio, 1989). No reports could be found, however, concerning the regulation of monocyte adhesion by CSF.

In Chapter 4 of this thesis, the stimulation of human monocyte adhesion to cultured human endothelium, basement-membrane and other extracellular proteins, and to plastic is shown with IL-3 and GM-CSF, and the mechanisms involved are explored. In Chapter 5, the capacity for inhibition of these phenomena by IL-4 is demonstrated, and a possible anti-inflammatory role for this cytokine is discussed.

1.7 CSF receptors

Discussion in the preceding sections has focussed on the functional effects of stimulating mononuclear phagocytes with CSF. The mechanisms by which such stimulation arises are also of great interest, and can be broadly grouped into receptor and post-receptor events. Several lines of evidence support the contention that specific CSF-receptor interactions are involved in functional stimulation of target cells. Firstly, the cellular distribution of receptors for the various CSF matches the distribution of response (reviewed by Nicola, 1987), with minor exceptions in leukaemic cells (Park et al, 1989B). Secondly, variations in receptor number or intracellular processing of ligand within a given lineage can, in general, be correlated with the biological response (Murthy et al, 1989; Guilbert et al, 1986). In

addition, the recent cloning of the receptor for human IL-6, a pleiotropic CSF, has allowed expression of the hIL-6 receptor in a murine cell line (Taga et al, 1989) leading to responsiveness to the human cytokine.

Receptors for IL-3 (IL-3-R) and for GM-CSF (GM-CSF-R) are found on bone marrow cells and on most mature cells of the granulocyte and monocyte/macrophage lineages in man (Park et al, 1989A; Park et al, 1986; Gasson et al, 1986; Di Persio et al, 1988). Differentiation in the neutrophilic granulocyte series, however, is associated with loss of IL-3-R (Lopez et al, 1989). Several other studies in murine cells, or on tumour cell lines add to the understanding of these receptors (reviewed by Nicola, 1989). In general, they are expressed at low levels (up to a few hundred per cell), show high affinity (dissociation constants of 200-300pM), and have been identified by cross-linking in several laboratories. Unfortunately, large differences in molecular weight and even in the number of chains identified have been reported from the different groups (Nicola, 1989). Most binding and cross-linking studies have been performed without corresponding functional experiments, leading to difficulty in interpretation of the differing data in the different cell types.

In contrast to the receptors for IL-3 and GM-CSF, the M-CSF-R is expressed only on cells of the mononuclear phagocyte lineage and on trophoblast, and is seen at concentrations of up to 50,000 per cell. The M-CSF-R has been well characterised as a single chain glycoprotein, which undergoes autophosphorylation on tyrosine upon the binding of M-CSF (Yeung et al, 1987). It is almost certainly the cellular fms oncogene product (Sherr et al, 1985; Sacca et al, 1986), shows extensive homology with the receptor for platelet derived growth factor (PDGF) (Roberts et al, 1988B), and has been molecularly cloned (Sherr et al, 1985).

One characteristic which has been common to all the CSF-R is their specificity of ligand binding, such that each receptor type binds only the cognate CSF. Recently, however, Walker et al (1985A,B) have demonstrated in murine bone marrow cells the capacity of IL-3 to down-regulate (or 'trans-downmodulate') receptors for GM-CSF and M-CSF, and of GM-CSF to down-regulate M-CSF-R but not IL-3-R. This hierarchical model of receptor interactions has been proposed as an explanation for the combined proliferative and differentiative effects of the CSF in haemopoiesis, but has not so far been confirmed in human cells. In Chapters 6 and 7 of this thesis, equilibrium and kinetic binding data for the interaction of IL-3 and GM-CSF with their receptors on human monocytes is presented. The binding data are correlated with the functional effects of these cytokines described in Chapters 3 and 4, and an interaction between IL-3-R and GM-CSF-R is revealed. The nature of this interaction, however, is shown to be quite different from that previously described in the mouse. A new model of interacting IL-3 and GM-CSF receptors is therefore proposed, and discussed in terms of both the biological and binding data.

CHAPTER 2

MATERIALS AND METHODS

2.1 Media

2.1.1 *Media for monocyte preparation and culture*

Hank's balanced salt solution without Ca^{++} or Mg^{++} (HBSS): a 10x concentrate was prepared with 4g KC1; 0.6g KH_2PO_4; 80g NaC1; 3.5g $NaHCO_3$; 0.475g Na_2HPO_4 in 1L distilled water, and was sterilised by autoclaving. 1x HBSS was prepared on the day of use by diluting the concentrate, and adding 2.5ml per litre of sterile-filtered 40% glucose in water.

Elutriation medium: HBSS with 0.1% heat-inactivated fetal calf serum (FCS; Flow Laboratories, North Ryde, New South Wales) or pooled human type AB serum, plus 0.02% ethylenediaminetetraacetic acid (EDTA).

Detachment medium: HBSS with 0.02% EDTA.

Culture media: RPMI 1640 was purchased from Commonwealth Serum Laboratories (CSL; Parkville, Victoria) and supplemented with 0.2% $NaHCO_3$, penicillin and gentamicin and 10% heat-inactivated serum. Pooled human type AB serum was obtained from the Adelaide Red Cross transfusion service, and different batches were tested to exclude those with a high inherent capacity to support monocyte cultures *in vitro*. The AB serum was used in monocyte survival and proliferation experiments. For all other experiments FCS was used.

: Dulbecco's modified eagle's medium (DMEM) was purchased from MultiCel (Castle Hill, New South Wales) and supplemented as for RPMI. This medium was used for L-cell culture only.

2.1.2 *Media for radioligand binding*

Binding medium consisted of RPMI supplemented with 20mM HEPES and 0.5% bovine serum albumin (BSA). pH was adjusted to 7.2 by addition of NaOH solution.

For detachment of radioligand from cell surface receptors, a low pH medium was used. This consisted of 0.5M NaC1; 0.5M acetic acid in distilled water, with pH adjusted to 2 by dropwise addition of concentrated NaOH solution.

2.2 Cytokines

Recombinant human (rh) IL-3 from *Escherichia coli* and rhGM-CSF from monkey COS cells were used for the biological experiments. These cytokines were >99% pure by SDS-PAGE. For some experiments involving long term monocyte culture, rhIL-3 in the form of an unpurified COS cell supernatant was used, with supernatant from mock-transfected COS cells used as a control. These cytokines were gifts from Dr S Clark (Genetics Institute, Cambridge, MA).

For radioligand binding experiments, several CSF preparations were used. Because of difficulties in iodinating the *E. coli*-derived rhIL-3 to high specific radioactivity, presumably due to the presence of only one tyrosine residue, a modified rhIL-3 was used. This cytokine contained an N-terminal octapeptide with an extra tyrosine residue, and was expressed in yeast as described (Hopp et al, 1988; Park et al, 1989A). The addition of the octapeptide has been shown not to alter the biological activity of IL-3 (Park et al, 1989A). For equilibrium binding experiments, COS-derived rhGM-CSF was used, but for the kinetic studies, an N-terminal-modified rhGM-CSF was used, in order to increase the specific

activity. The modified cytokines were gifts from Dr L Park (Immunex Corporation, Seattle, WA).

Purified COS-derived rhGM-CSF was used as a competitor cytokine in all binding experiments. Purified *E. coli* derived rhIL-3 was used as a competitor in the equilibrium experiments. However, in the kinetic binding experiments, a further Chinese Hamster Ovary (CHO)-derived purified rhIL-3 preparation was used (Genetics Institute).

Other cytokines used and their sources were *E. coli* derived rhIL-1β, yeast-derived rhIL-4 (Dr L Park, Immunex); *E. coli*-derived rh tumour-necrosis factor α (TNFα), *E. coli*-derived rhIFNγ (Genentech, San Francisco, CA); *E. coli*-derived rhG-CSF (Dr L Souza, Amgen, Thousand Oaks, CA). All cytokines were specified by the suppliers as >99% pure. Purified synthetic peptides comprising amino acids 1-53, 54-127 and 14-127 of the GM-CSF molecule were gifts from Dr I Clark-Lewis (Vancouver, Canada).

2.3 Antibody preparations

2.3.1 *Anti-CSF antisera*

Rabbit antiserum raised to rhIL-3 (R165 10/21/87) and sheep antiserum to rhGM-CSF (S7, 5/8/87) were obtained from Dr S Clark (Genetics Institute). Anti-IL-3 was able to neutralise 4 units/ml IL-3 at 1/1000 dilution and anti-GM-CSF was able to neutralise 5 units/ml GM-CSF at 1/500 dilution in the colony forming assay.

2.3.2 *Anti-LFA MAb*

The individual polypeptide chains and the adhesion complexes of the LFA family are shown in Table 2.1, together with the chain-specific monoclonal antibodies (MAb) employed in this thesis. Purified mouse MAb 60.3 to the LFA family common β chain (CD18) and 60.1 to the α chain of the MAC-1 complex (CD11b) were kind gifts from Dr P Beatty (Fred Hutchinson Cancer Research Center, Seattle, WA) (Vedder et al, 1988). MAb TS 1/22 (α chain of the LFA-1 complex, CD11a) and TS 1/18 (common β chain) were gifts from Dr T Springer (Harvard Medical School, Boston, MA) (Sanchez-Madrid et al, 1983). MAb IH-4 (ICAM-1) was a gift from Dr A Boyd (Walter and Eliza Hall Institute, Melbourne, Victoria) (Boyd et al, 1988). MAb MO-1 (α chain of MAC-1 complex, CD11b) and 150/95 (α chain of p150/95 complex, CD11c) were purchased from Coulter Immunology (Hialeah, Florida). Isotype-specific control antibodies for 60.1 (IgG_1; Hy5.19) and for 60.3 (IgG_{2a}; Hy1.2) were used in functional inhibition experiments. These antibodies were prepared as previously described (Lopez et al, 1983). MAb MO-2, used for monocyte identification purposes, was purchased from Coulter Immunology.

2.4 Radioisotopes

^{51}Cr was purchased in the form of sodium chromate in 0.9% NaCl from Amersham Int. (Bucks, England). Specific radioactivity of the preparation was approximately 350-600mCi/mg.

^{3}H-(methyl)-thymidine (6.7Ci/mmol) was purchased from New England Nuclear (Billerica, MA).

Table 2.1 LFA family adhesion molecules

α chain	β chain	complex	Ligand
CD11a	CD18	LFA-1	ICAM-1, ICAM-2
(TS1/22)*	(TS1/18, 60.3)		(IH-4)
CD11b (MO-1, 60.1)	CD18 (TS1/18, 60.3)	MAC-1 (MO-1, CR3, C3biR)**	C3bi, Fibrinogen Factor X
CD11c (150/95)	CD18 (TS1/18, 60.3)	p150/95	C3bi

Individual α and β chains are indicated by their respective CD numbers, with

* chain-specific MAb shown in brackets beneath the relevant chain;

** alternative names for the MAC-1 complex. Fibrinogen and factor X form ligands for MAC-1 only in special circumstances (Altieri et al, 1988A,B).

Carrier-free $Na^{125}I$ in NaOH solution (pH 10) (approximately 17.4Ci/mg) was purchased from New England Nuclear.

2.5 Maintenance of LPS-free conditions

All laboratory glassware was washed using E-toxa-clean (Sigma, St. Louis, MO) and heated to 160°C for 3 hours to reduce LPS contamination. Plastic disposable laboratory-ware was used where possible. RPMI medium was specially purchased from CSL as 'low endotoxin' batches, and contained <10pg/ml LPS by Limulus amoebocyte lysate assay (Whittaker Bioproducts Inc., Walkersville, Maryland). Batches of fetal calf serum were screened by Limulus assay, and selected for low endotoxin levels. At the maximum concentration of FCS used in these experiments (10%), the media contained <40pg/ml LPS. All cytokines used were also tested by Limulus assay and contained undetectable levels of LPS at the maximum concentrations used.

2.6 Cell counting and viability

Cell counts were done using a Coulter counter (Coulter Electronics, Luton, England) after addition of Zap-o-globin (Coulter Electronics) to lyse contaminating red cells. Parallel counts using a hemacytometer yielded comparable results in several experiments. Cell viability was tested by mixing equal volumes of filtered trypan blue in phosphate-buffered saline (PBS) and the cell suspension. After 5 minutes at room temperature, the cells were examined by microscopy and those stained were scored as dead cells.

2.7 Cell staining

Monocyte suspesions were first sedimented onto glass microscope slides using a cytocentrifuge (Shandon Scientific Company, England), and were air dried. Three separate staining procedures were used in order to assess monocyte purity.

2.7.1 *Giemsa*

Cell morphology was assessed after staining with a modified Wright's-Giemsa stain using an automated method (Hema-tek, Ames, IN).

2.7.2 *Non-specific esterase/chloroacetate (NSE)*

NSE staining was performed using the following protocol.

Solutions: Fixative: Na_2HPO_4, 100mg; KH_2PO_4, 500mg; acetone, 225ml; formaldehyde, 125ml; distilled H_2O, 150mls. (stored at 4°C).

Pararosanalin solution: pararosonalin, 4g; distilled H_2O, 80ml; concentrated HC1, 30ml; the solution was heated to 56°C for 30 minutes, then cooled and filtered). (stored at room temperature).

Phosphate buffer pH 7.4: KH_2PO_4, 0.87g; Na_2HPO_4, 3.84g; distilled H_20 to 500mls. (stored at 4°C).

Phosphate buffer pH 6.3: KH_2PO_4, 3.5g; Na_2HPO_4, 1.1g; distilled H_20 to 500mls. (stored at 4°C).

Method: Cell preparations were fixed for 30 seconds at 4°C, and washed three times, before air drying. Staining solution was prepared by premixing 2ml ethylene glycol monomethyl ether with 2 drops α-naphthylbutyrate (Sigma, St Louis MO), and adding 38ml phosphate buffer pH 6.3. 0.25ml pararosanilin solution and 0.25ml 4% sodium nitrite in water were then mixed, allowed to react for 1 minute, and added to the staining solution. pH was adjusted to 6.1 by dropwise addition of NaOH, and slides were stained for 45 minutes at room temperature. After washing 3 times, the slides were subjected to a second staining in 38ml phosphate buffer, pH 7.4, 20mg fast blue BB (Sigma), 4mg AS-D chloroacetate and 2ml N-N dimethyl formamide (Sigma) for 1 hour at room temperature. After washing, the slides were counterstained with 2% methyl green (Sigma) for 10 minutes, washed and mounted. Using this procedure, monocytes stain a red-brown colour, granulocytes show bright blue cytoplasm, and lymphocytes and cell nuclei take up the counterstain only and therefore appear pale green.

2.7.3 *MO-2 Expression*

MO-2 is a human myeloid antigen expressed on monocytes and macrophages derived from peripheral blood, and also on blast cells in myelomonocytic leukemia (Todd and Schlossman, 1982). Expression of MO-2 on cytocentrifuge specimens was performed using the alkaline phosphate-anti-alkaline phosphatase technique (APAAP) as previously described (Cordell et al, 1984).

2.8 Cell preparation

2.8.1 *Mononuclear cell and neutrophil preparation*

Blood was obtained from the Adelaide Red Cross transfusion service in the form of packed cells and was used on the day of collection only. Cells were diluted 1:1 with HBSS, and 25ml aliquots were centrifuged through a 15ml cushion of Lymphoprep (Nyegaard, Oslo, Norway) for 30 minutes at room temperature. Mononuclear cells were collected from the interface, washed twice in HBSS, and purified further as detailed below. Neutrophils were obtained from the red cell pellet after sedimentation in 5% dextran, followed by hypotonic lysis of residual red cells, as described (Lopez et al, 1986). Polypropylene tubes (Falcon, Lincoln Park, N.J.) were used for all centrifugation steps in order to minimise leukocyte losses by adhesion.

2.8.2 *Purification of monocytes by adherence*

For cell survival experiments, monocytes were purified from mononuclear cells by adherence to serum-coated 35mm tissue culture dishes (LUX, Naperville, IL) in RPMI 1640 with 10% heat-inactivated pooled human AB serum. After 1 hour incubation at 37°C, non-adherent cells were washed off with at least three changes of medium, and adherent cells further cultured *in situ*. For ^{3}H-thymidine experiments, adherent cells prepared as above were detached using a teflon policeman at 4°C in detachment medium, washed twice in RPMI 1640, and aliquotted into 96 well plates (Nunc, Kamstrup, Denmark). Monocyte purity using these methods was 88.8±1.2% (mean ± SEM in five experiments) by staining with

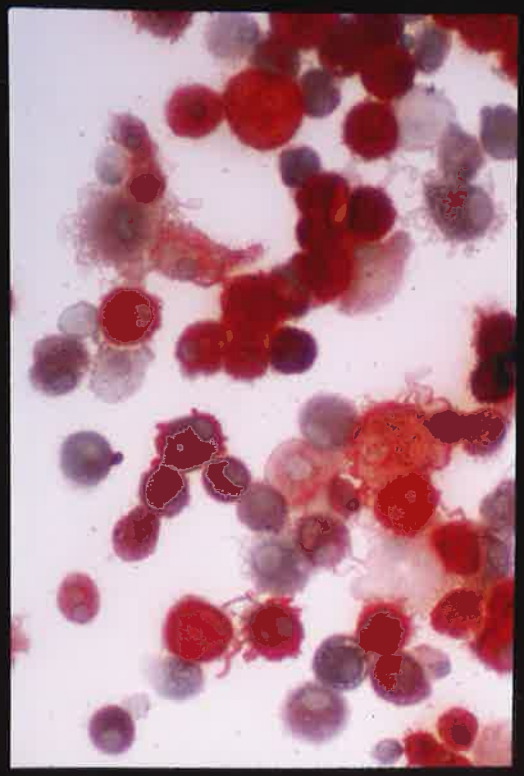
Wright's-Giemsa. The contaminating cells comprised approximately 6% lymphocytes and 5% granulocytes.

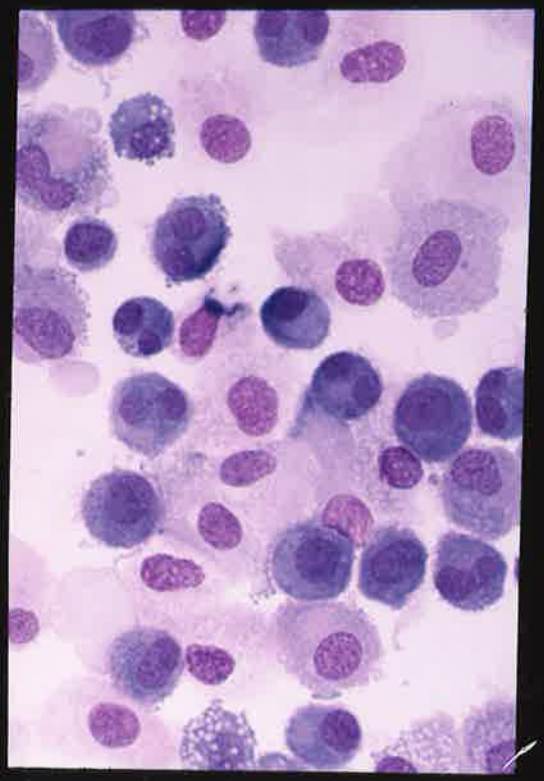
2.8.3 *Purification of monocytes by elutriation*

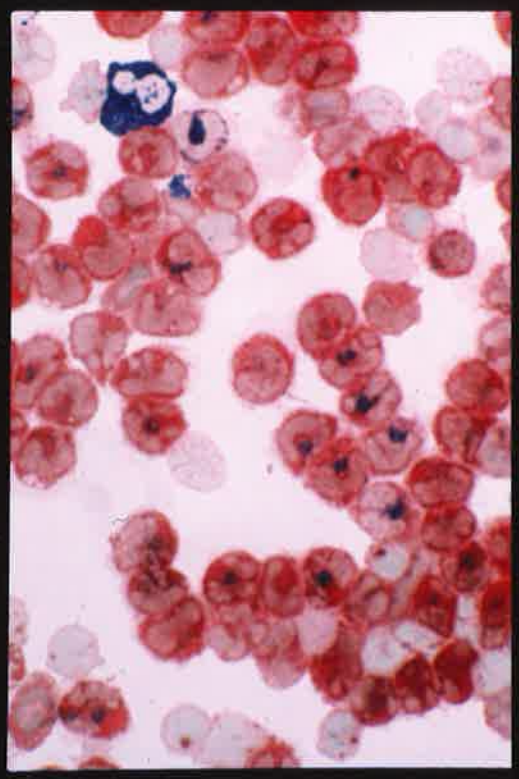
For experiments involving monocyte function, antigen expression or radioligand binding, cells were purified by countercurrent elutriation using a modification of the protocol of Sanderson (1977). Briefly, mononuclear cells were washed twice in RPMI 1640 at 100g to reduce platelet contamination, and resuspended in elutriation medium. The cells were separated in a Beckman JE-6B elutriator using the Sanderson chamber, with a rotor speed of 2050rpm and a flow rate of 11.8ml/min. Cells remaining in the chamber after 30 minutes were collected, washed twice in RPMI, and used immediately. These methods routinely yielded between 5 and $12x10^7$ monocytes from 500mls of whole blood. However, for some radioligand-binding experiments, elutriated monocytes from several donors were pooled to achieve the required numbers. Elutriation resulted in a monocyte purity by Wright's-Giemsa staining of 91±3.4% (mean ± SEM in five experiments). In some experiments, however, purities exceeded 95%. In addition, >90% of elutriated cells were phagocytic for opsonised zymosan. The contaminating cells comprised approximately 4% lymphocytes and 5% granulocytes, principally basophils.

Photomicrographs of Giemsa and NSE-stained monocytes purified by elutriation are shown in Fig. 2.1. Both staining methods indicate high monocyte purity for this cell preparation. A contaminating granulocyte (blue cell, right hand panel) illustrates the capacity of NSE staining to differentiate different myeloid lineages.

Figure 2.1 Photomicrographs of fresh monocytes prepared by countercurrent elutriation (top panels, x400 magnification) and of 7 day-old adherent-cultured monocytes (bottom panels, x200 magnification). The left-hand panels show staining with Wright's-Giemsa; the right hand panels show staining with NSE. In NSE preparations, monocytes and macrophages stain red-brown, granulocytes stain bright blue, and lymphocytes and cell nuclei stain pale green.







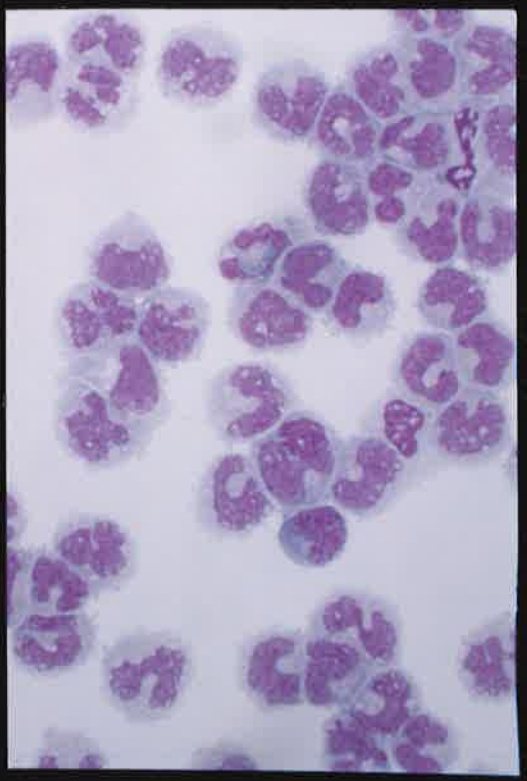


Table 2.2 Purity of day 7 cultured monocytes (percent)

Staining Method		
Giemsa	NSE	MO-2
97.1±0.8 (8)*	95.4±1.3 (8)	93.6±2.5 (5)

Monocytes were purified by adherence and cultured using the adherent protocol. Cells were harvested at day 7 and stained with Giemsa, NSE or MO-2. Results are expressed as the percentage of cells showing macrophage morphology (Giemsa) or staining positively (NSE, MO-2).

* number of experiments.

2.9 Culture methods

2.9.1 *Adherent culture*

Monocytes purified by adherence were cultured ***in situ*** in 35mm tissue culture dishes (LUX) for up to 14 days. Culture medium (2ml, 10% human AB serum) was changed every 3-4 days with readdition of any cells in suspension to the fresh medium. CSF were added at day 0 and replaced with each change of medium. Incubations were performed in a 5% CO_2 fully humidified incubator at 37°C.

Long term culture of monocytes using these methods led to the development of cells with the morphological features typical of tissue macrophages (Geissler et al, 1989). These include the presence of round, eccentrically placed nuclei, abundant cytoplasm, and a consequent increase in the cytoplasmic to nuclear ratio. In addition, many cells showed cytoplasmic processes and some showed multinuclear formation (Figure 2.1). The cells were strongly positive for NSE (Figure 2.1, and Table 2.2) and the majority expressed MO-2 by APAAP (Table 2.2).

For experiments involving monocyte ^{3}H-thymidine uptake, cells prepared by adherence were aliquotted into microtitre wells at day 0 ($0.5x10^5$/well) and incubated under the conditions described above.

2.9.2 *Suspension Culture*

Monocytes purified by elutriation were washed 3 times in sterile medium, and resuspended in culture medium (10% FCS, without $NaHCO_3$) at concentrations of 1-5x10^6/ml. Cell suspensions were aliquotted into 10ml polypropylene tubes

(Disposable Products, Adelaide, South Australia) and rotated at 37°C for up to 5 days.

With this procedure less than 10% reduction in cell numbers was observed after overnight culture with or without CSF. The recovered cells were >99% viable as judged by trypan blue exclusion.

2.10 Preparation of Endothelial Monolayers

Human umbilical vein endothelial cells (HUVEC) were obtained by collagenase treatment of umbilical veins. The cells were plated onto gelatin-coated 25cm^2 flasks (Costar, Cambridge, MA), maintained as previously described (Wall et al, 1978) and used between 4 and 8 days after establishment of cultures. For use in adherence assays, cells were harvested using 0.05% trypsin with 0.02% EDTA (Flow Laboratories) and replated into the central 60 wells of flat-bottomed gelatin coated microtitre trays (Nunc, Kamstrup, Denmark) at 2x10^4 cells per well and grown to confluence overnight. Immediately prior to use, monolayers were washed twice with RPMI 1640.

2.11 Preparation of L cell monolayers

The murine L cell line, and the L cell line transfected with the gene for human intercellular adhesion molecule-1 (ICAM-1), were gifts from Dr S Wawryk (WEHI, Melbourne, Victoria). The transfected cells have been previously shown to stably express high levels of surface ICAM-1 and to bind a variety of cell types in an ICAM-1-specific fashion (Wawryk et al, in press). Both cell lines were grown in 500ml Costar tissue culture flasks in DMEM with 10% FCS and antibiotics. For use in adherence assays, cells were harvested using trypsin-EDTA and replated into flat bottomed microtitre trays at 2x10^4 cells per well and

grown to confluence overnight. Immediately prior to use, monolayers were washed twice with RPMI.

2.12 ^{3}H-thymidine Incorporation

For ^{3}H-thymidine incorporation experiments, 0.5-1x10^{5} monocytes prepared by adherence were aliquotted to each well in microtitre plates, and supplemented with medium plus CSF or control to a total volume of 200ul. Cultures were pulsed on day 4 with 1uCi/ml of ^{3}H-thymidine (6.7Ci/mmol, New England Nuclear) and harvested 18 hours later onto glass filters using an automated cell harvester (Titertek, UK). Harvesting was performed under hypotonic conditions and following initial lysis of the cells with 0.02% Triton X (Labchem, Australia) in water. These procedures were designed to ensure that only thymidine incorporated into DNA was subsequently counted. Filter discs were dried and counted in a liquid scintillation counter (Beckman Instruments).

2.13 Autoradiography

Four day old cells were pulsed for 18 hours with ^{3}H-thymidine and then detached and used to prepare cytocentrifuge slides. The slides were fixed in methanol:acetic acid (3:1), gelatin-coated, air-dried and dipped in Ilford K2 emulsion. Slides were developed after 24 hours exposure and the labelling index determined by counting the percentage of cells with >15 grains per nucleus. A minimum of 400 cells were counted per slide and results expressed as the mean ± SD of duplicate slides per stimulus.

2.14 Protein assays

2.14.1 *Cell protein content*

Adherent monocytes were detached from the culture dishes using detachment medium at 4°C, together with mechanical scraping. The cell pellets were washed twice in HBSS, and lysed by 3 consecutive freeze-thaw cycles. Protein content was measured by Coomassie-blue G250 binding (Biorad, Richmond, CA) with the results read spectrophotometrically. Absorbance values were compared with a standard curve to yield protein content per culture.

2.14.2 *Measurement of Protein Synthesis*

Monocytes were prepared by elutriation and suspended at a concentration of $4x10^6$/ml in methionine-free RPMI 1640 (Selectamine, Gibco, New York) with 1% FCS. The cells were aliquotted into 2ml polypropylene tubes, warmed to 37°C and control medium or CSF were added to give a final concentration of 600pM. Control medium or cycloheximide was then added (final concentration 5ug/ml) and 30 second later each tube was pulsed with 10uCi ^{35}S-methionine (Amersham Int., Bucks, England). The monocytes were incubated for 30 minutes at 37°C with gentle agitation followed by transfer of aliquots of cells to paper filters. Incorporated ^{35}S-methionine was precipitated by treatment with 10 per cent trichloro-acetic acid and subsequent washing steps removed free methionine, as described (Oleinick et al, 1976). Filters were counted in a Beckman liquid scintillation counter.

2.15 Colony forming assay

The CFU-GM assay was performed using adherent and non-adherent fractions of mononuclear cells, prepared as described above. Cells were cultured at a concentration of 0.5 to $1x10^5$/ml in Iscove's modified Dulbecco's medium (IMDM, Gibco, Grand Island, NY) containing 0.33% agar (Difco, Detroit, MI), 25% FCS (CSL) and 20uM 2 mercaptoethanol. GM-CSF was added to the plates in different concentrations at the onset of culture. Plates were prepared in triplicate and scored after incubation at 37°C in 5% CO_2 in a humidified incubator after 7 and 14 days. Clones containing >10 cells (clusters) and >20 cells (colonies) were counted at day 7 and 14 respectively.

2.16 Adhesion assays

Adhesion was measured using either of two different protocols. The co-incubation assay was used for all 'early phase' adhesion experiments, and for 'late phase' adhesion experiments on plastic or extracellular matrix proteins. The suspension culture system was used for all 'late phase' experiments on endothelium.

2.16.1 *^{51}Cr-labelling*

Adhesion was measured following isotopic labelling of monocytes essentially as described (Wallis et al, 1985). In brief, purified monocytes ($0.5\text{-}1x10^8$) were resuspended in 1ml RPMI with 0.1% FCS and antibiotics and incubated for 30 minutes at 37°C with 500uCi Cr^{51} in the form of sodium chromate (Amersham Int., Buckinghamshire, England). Cells were washed thrice in RPMI 1640 and resuspended in culture medium consisting of RPMI, 10% FCS, antibiotics and 0.2% sodium bicarbonate. Attempts to label monocytes using periods of incubation longer

than 30 minutes, or with higher concentrations of FCS resulted in significantly higher levels of background adhesion.

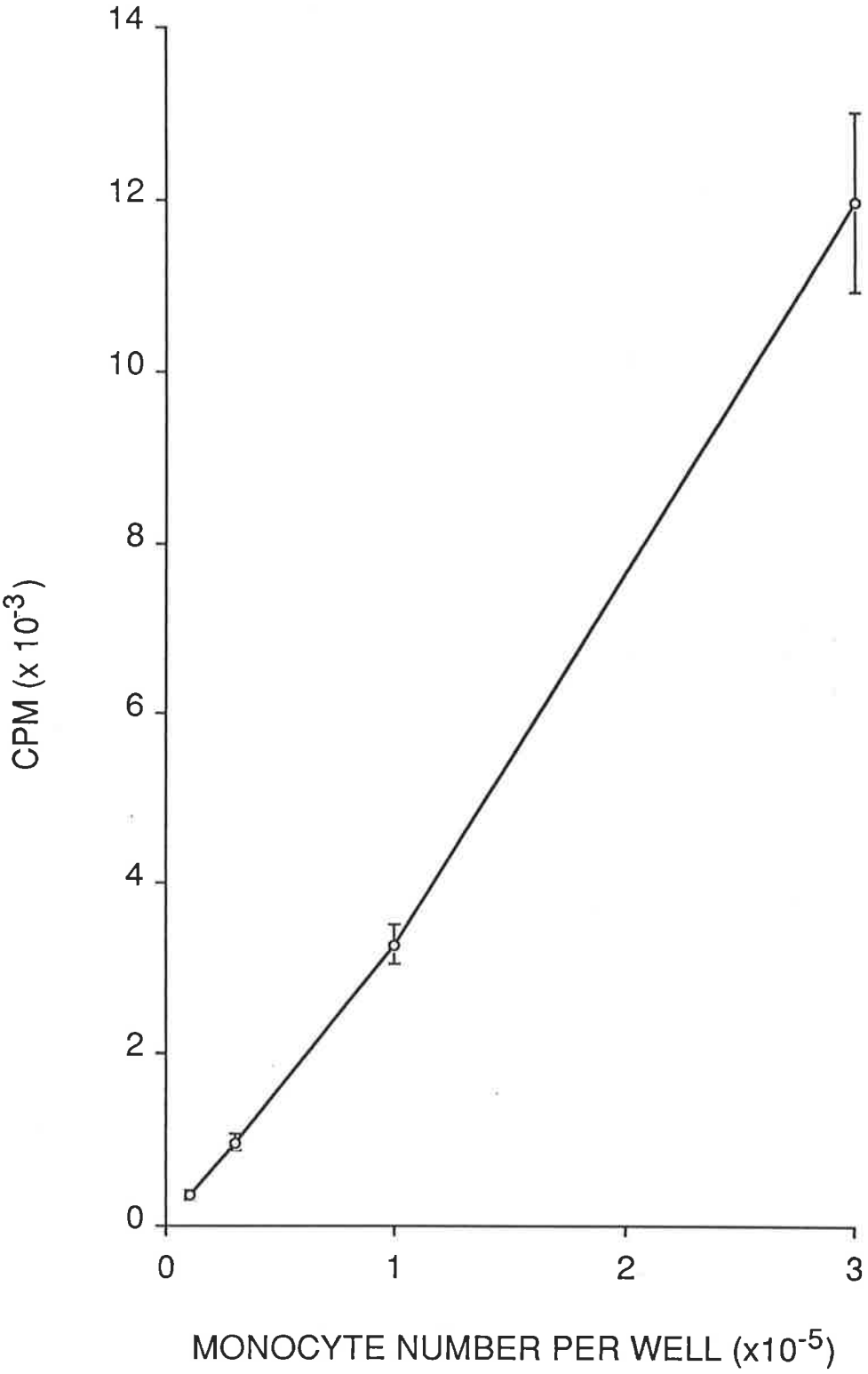
2.16.2 *Co-incubation adhesion assay*

1-2.5x10^5 monocytes were aliquotted per well in flat-bottomed 96 well microtitre plates (Nunc) together with stimuli or control medium to a total volume of 100ul, and incubated at 37°C in a 5% CO_2 incubator for the indicated periods. Monocyte settling under these conditions was observed to be complete within 10 minutes. For antibody inhibition experiments, MAb was included in the wells for the duration of the assay. In some experiments, cycloheximide (Sigma, St Louis, MO) at a final concentration of 5ug/ml was included in the medium for the indicated times. At harvest, samples of supernatant were taken to assess spontaneous Cr^{51} release (usually <10% of cell-associated radioactivity), wells were washed thrice with RPMI 1640 at 37°C, and residual adherent cells lysed in 10mM Tris-hydrochloride, 0.15M sodium chloride, and 1% Nonidet p40 detergent (Sigma, St Louis, MO). Lysates were transferred to tubes and counted in a Packard auto-gamma 5650. Percent adhesion was calculated according to the formula:

$$\% \text{ adhesion} = \frac{\text{residual adherent cpm} \times 100}{\text{total cpm added - cpm spontaneously released.}}$$

The experiment shown in Fig. 2.2 indicates that adhesion to plastic at 90 minutes of culture is essentially linear with differing cell numbers of between 0.1 and 3x10^5 per well, validating the choice of cell numbers used in subsequent experiments.

Figure 2.2 Adhesion of different numbers of ^{51}Cr-labelled monocytes to plastic at 90 minutes. Between 0.1 and $3x10^5$ monocytes were aliquotted to microtitre wells in 200ul medium, and allowed to adhere at 37°C in 5% CO_2 for 90 minutes. Non-adherent cells were removed by washing and residual adherent radioactivity determined as described in Section 2.16.2. Each point represents the mean ± SD of 6 replicates.


14
12
10
8
6
4
2
0
CPM (x 10-3)
0
1
2
3
MONOCYTE NUMBER PER WELL (x10-5)

2.16.3 *Suspension culture adhesion assay*

For 'late phase' adhesion experiments on HUVEC, the co-incubation technique resulted in very high levels of adhesion in both stimulated and unstimulated wells. While the reasons for this are not clear, the time involved (21 hours) may have allowed for the development of immunological reactions between HLA-incompatible HUVEC and monocyte donors, with subsequent liberation of stimulatory cytokines. For these experiments, therefore, ^{51}Cr-labeled monocytes were incubated in suspension with or without CSF, centrifuged at 100g to minimise cell activation induced by physical handling, and resuspended in prewarmed culture medium without CSF. The cells were then allowed to adhere to HUVEC (or plastic, done in parallel) for 20-30 minutes at 37°C, prior to washing away the nonadherent cells. This method allowed the stimulatory effects of CSF on monocyte adhesion to HUVEC to be observed during the late phase. This was not the optimal method for measuring late phase adhesion to plastic, however, as background levels of adhesion to this surface were higher than when using the co-incubation technique.

2.16.4 *Preparation of extra-cellular matrices*

For some experiments, adhesion was measured in flat bottomed microtitre wells which had been pre-coated with purified extracellular matrix proteins (EMP). Human collagens type I and IV were purchased from Telios Pharmaceuticals (San Diego, CA), human fibronectin and laminin from Collaborative Research (C/o Integrated Sciences, Sydney, New South Wales), and human fibrinogen from Kabi (Stockholm, Sweden). Purified human vitronectin was a gift from C Lucas (IMVS, Adelaide, Sth Aust), and human thrombospondin, purified from platelet concentrates, was a gift from S Dawe (IMVS, Adelaide, Sth Aust). Culture wells

were coated with EMP according to established methods (Shaw et al, 1989). Proteins were suspended in HBSS at concentrations of 10-50ug/ml, and 100ul aliquots placed in each well. Binding of protein was allowed to proceed for 2 hours at 22°C, or overnight at 4°C, followed by aspiration of the buffer and 3 washing steps with sterile HBSS. Matrices were used within 2 days of preparation.

2.17 Immunofluorescence

Monocytes were labeled with a variety of MAb (see Section 2.3.2) after variable periods in suspension culture, and analysed by flow cytometry with an Epics V instrument (Coulter Electronics). The labelling protocol was as follows: monocytes were centrifuged from suspension at 100g for 5 minutes, and resuspended in RPMI with 0.2% sodium azide (NaN_3) and 5% FCS. Cell suspensions were cooled to 4°C and all subsequent steps were performed at this temperature, in 5ml polypropylene tubes (Disposable Products). 50ul aliquots of monocyte suspensions were added to 50ul of a 1:20 dilution of normal rabbit serum (CSL), in order to block Fc-mediated binding of MAb. Following a 15 minutes incubation, a further 50ul of medium containing the primary staining MAb at appropriate dilution was added and allowed to bind for 20 minutes. The cells were then washed at 100g for 5 minutes in washing medium consisting of PBS with 0.2% NaN_3 and 5% FCS. The cell pellets were resuspended in 50ul of medium containing a 1:40 dilution of a fluoresceine-isothiocyanate-labeled sheep anti-mouse antibody (DDAF, $F(ab')_2$ fragments; Silenus Laboratories, Hawthorn, Victoria) and incubated for 20 minutes. After a final wash, the cells were fixed in a solution of PBS with 0.2% NaN_3, 2% glucose and 1% formaldehyde and subjected to flow cytometry.

Cell size was assessed by forward light scatter, and antibody binding was measured by green fluorescence, with at least 10,000 cells counted per point. The co-efficient of variation for

the flow cytometer was 2-3%. Results for forward scatter are expressed on a linear scale, while fluorescence is expressed on a logarithmic scale in arbitrary units.

2.18 Radioidination of CSF

Iodination of IL-3 was achieved using the N-terminal-modified rhIL-3 expressed in yeast (see Section 2.2). Two preparations of GM-CSF were used: rhGM-CSF from COS cells, and an N-terminal-modified rhGM-CSF from yeast. The N-terminal-modified preparation yielded ^{125}I-GM-CSF samples of higher specific radioactivity than the unmodified cytokine.

The CSF were iodinated by reaction with IC1, as described by Contreras et al (1983). The process occurs in two steps:

(1) ICl + NaOH → HOI + NaCl

(2) HOI + tyrosine residue → iodinated tyrosine residue + H_2O.

This method has the advantage of speed and of generally leading to less oxidative damage of the protein than other methods, e.g. utilising chloramine-T. The protocol used was as follows: 2ug of CSF was diluted in 90ul phosphate buffered saline (PBS), pH 7, and 1mCi ^{125}I-Na in 10ul was added. 2ul ICl solution (0.2mM in 2M NaCl) was added to the CSF solution and vortexed, followed by incubation at 24^{o}C for 1 minute. A further 5ul of ICl solution was added, incubated for 1 minute, and the reaction mixture immediately applied to a Sephadex G-25 PD10 column (Pharmacia, Uppsala, Sweden) which had been pre-equilibrated in PBS with 0.02% Tween 20. The column was then washed with PBS with 0.02% Tween, and 30x0.5ml fractions collected. An aliquot of each fraction was counted in order to detect the radio-protein peak (usually between fractions 4 and 8) and the free radioactivity peak (fractions 10 to 20). An approximate specific radioactivity could then be

calculated from the known mass of protein used, and the number of cpm in the radio-protein peak. The radioligands were stored in siliconised glass tubes at 4°C for up to 4 weeks.

2.19 Purification of radioligands

Prior to use, the radioligands were purified from Tween and non-protein-associated radioactivity by cation-exchange chromatography. 100ul of radioligand was diluted 1:15 in 10mM NaH_2PO_4 buffer (pH 2.6 with citric acid) and loaded onto a 0.3ml CM-Sepharose CL-6B column (Pharmacia) which had been pre-equilibrated with the same buffer. The column was washed with 5ml of the low pH buffer to remove Tween and free ^{125}I, and the radioligand was then eluted with binding medium, pH 7.2 (see Section 2.1.2). 10x0.3ml fractions were collected, and the early acidic fractions brought to pH 7 by dropwise addition of 1M HEPES:concentrated NaOH, 9:1.

^{125}I-IL-3 and ^{125}I-GM-CSF prepared in this way retained >90% biological activity compared with non-labeled cytokines. ^{125}I-IL-3 was tested in an eosinophil polarisation assay and ^{125}I-GM-CSF in a neutrophil superoxide assay, as described (Lopez et al, 1986). Purified radioligands were stored for up to 7 days at 4°C in siliconised glass tubes.

2.20 Characterisation of radioligands

2.20.1 *Maximal binding capacity*

The maximal binding capacity (MBC, or bindability) of a radioligand is a measure of that proportion of the ligand capable of binding specifically to its receptor. Determination of MBC therefore gives a measure of possible degradation or other change in the ligand resulting from the iodination procedure or subsequent storage

(Bowen-Pope and Ross, 1982). MBC was measured by sequentially incubating a known amount of radioligand (usually 0.25-0.35ng) with 3 aliquots of fresh monocytes. The ratio of radioligand concentration to cell concentration was kept constant by transferring successively smaller aliquots of ligand to successively smaller cell numbers. Specific binding was measured at each point, as described in Section 2.21. Fig. 2.3 shows the results of such an experiment, performed to determine the MBC of a preparation of ^{125}I-IL-3. By plotting the number of cpm added at each point as a percentage of the initial cpm added (x axis) and the number of cpm bound as a percentage of initial cpm bound (y axis) a straight line graph is obtained. The x intercept is the proportion of ^{125}I-IL-3 which cannot bind even at (extrapolated) infinite numbers of sequential incubations. The MBC for this preparation is therefore 100-15 = 85%.

2.20.2 *Self-displacement analysis (SDA)*

Although an approximate value for the specific radioactivity (SR) of the radioligand can be calculated as previously described (see Section 2.18), a more accurate measure is given by self-displacement analysis (Calvo et al, 1983).

SDA was performed for each radioligand preparation by incubating a constant number of monocytes with a defined amount of radioligand, and either increasing amounts of radioligand or unlabeled CSF. Specific binding for each point was determined as described in Section 2.21. The results were plotted as the ratio of specifically bound/bindable free radioactivity versus amount of labeled or unlabeled CSF added, on a logarithmic scale. An example of self-displacement analysis for a sample of ^{125}I-IL-3 is shown in Fig. 2.4. The parallel lines indicate that the radio-ligand and unlabeled CSF bound to the cells with equal affinity. Assuming that at a

Figure 2.3 Determination of maximal binding capacity for a preparation of ^{125}I-IL-3. The radioligand was sequentially incubated with three different sets of monocytes, with preservation of the ligand:cell ratio for each incubation. Specific binding for each incubation is expressed as a percentage of specific binding for the first incubation, and plotted against added radioactivity, also expressed as a percentage. The non-bindable fraction of ^{125}I-IL-3 at an (extrapolated) infinite number of incubations is given by the intercept of the regression line with the horizontal axis. Each point is the mean ± SD of 2 replicates.

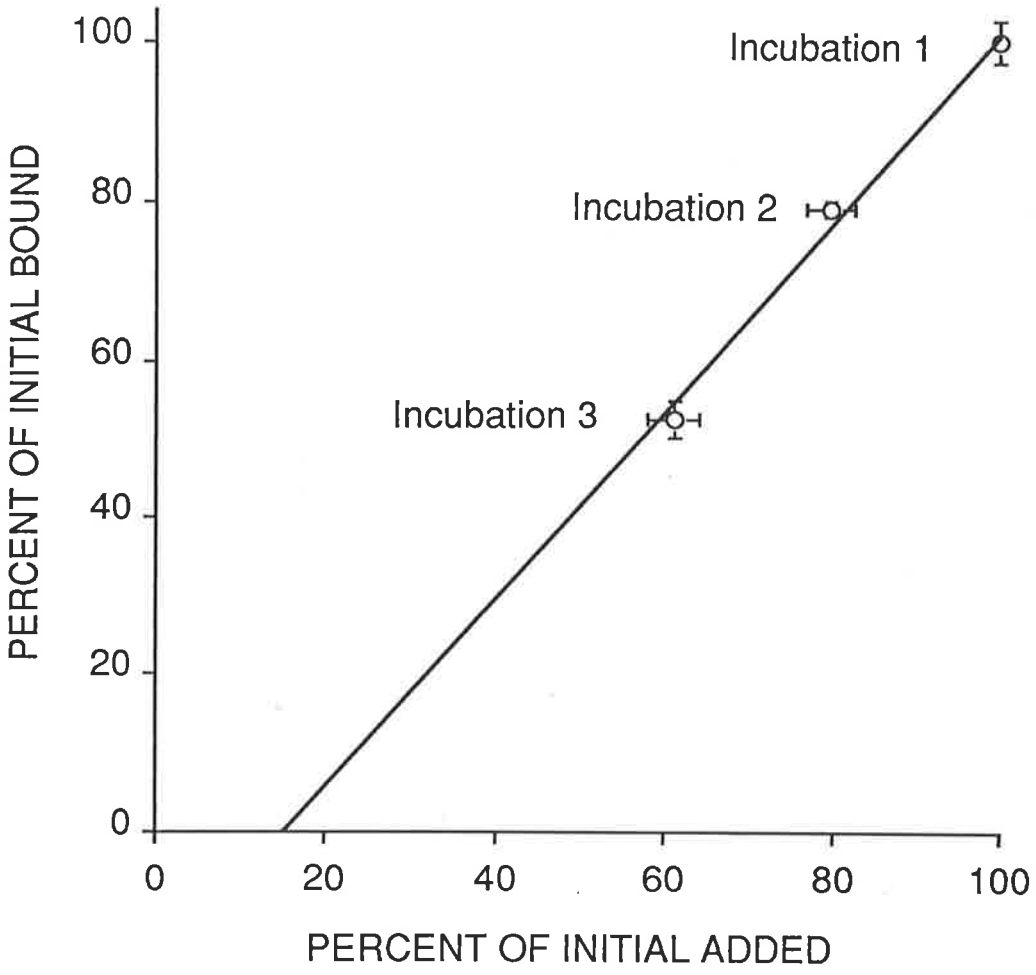
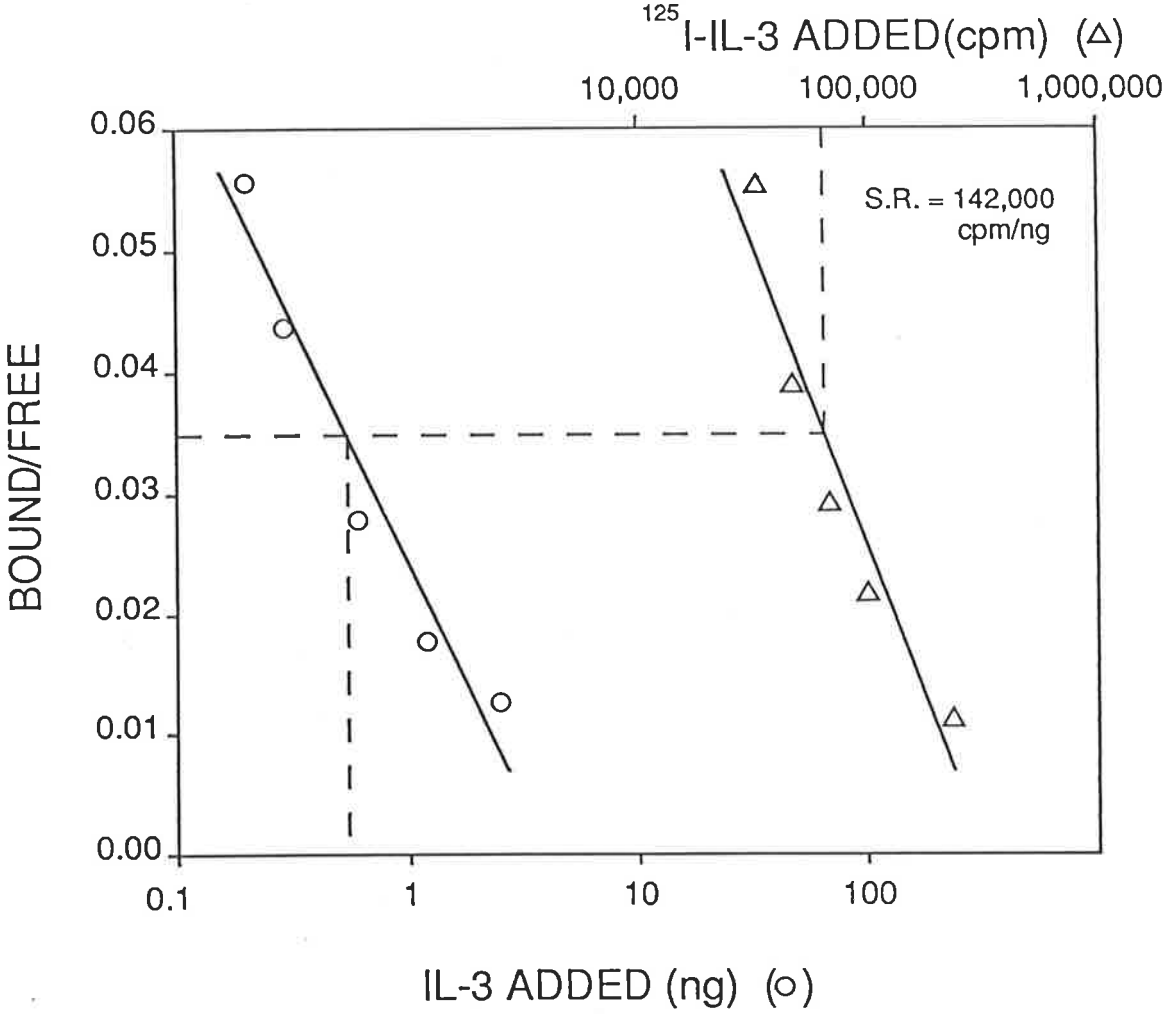

100
80
60
40
20
0
PERCENT OF INITIAL BOUND
Incubation 1
Incubation 2
Incubation 3
0
20
40
60
80
100
PERCENT OF INITIAL ADDED

Figure 2.4 Determination of specific radioactivity for a preparation of ^{125}I-IL-3 by self-displacement analysis. A constant number of monocytes (4.5×10^6 per point) was incubated with a single concentration of ^{125}I-IL-3 (approximately 0.1ng in 0.15ml final volume) together with increasing concentrations of either ^{125}I-IL-3 (Δ) or unlabeled IL-3 (o). Specific binding was determined at each point after overnight incubation at 4°C. Free radioactivity was corrected for maximal binding capacity. SR was derived by comparing amounts of unlabeled (0.5ng) and labeled (71,000cpm) IL-3 at a given bound/free ratio (0.035). The SR was therefore 142,000cpm/ng. Each point is the mean of 2 replicates.


^{125}I-IL-3 ADDED(cpm) (△)
10,000
100,000
1,000,000
0.06
0.05
0.04
0.03
0.02
0.01
0.00
BOUND/FREE
S.R. = 142,000
cpm/ng
0.1
1
10
100
IL-3 ADDED (ng) (○)

given bound/free ratio, the mass of ligand bound to the receptor must be the same for each plot, the specific radioactivity of the ^{125}I-IL-3 can be directly read from the graph as 142,000cpm/ng or 2.8x10^{6}cpm/pmol.

SR of the radioligand preparations used in these experiments varied from 0.6x10^{6}cpm/pmol (unmodified GM-CSF) to 2.8x10^{6}cpm/pmol (modified IL-3).

2.21 Radioligand binding assay

The experiments reported in Chapter 6 were performed under equilibrium binding conditions; that is, sufficient time was allowed for radioligand and receptor to reach equilibrium, and binding was measured at a single time point. The experiments in Chapter 7, however, recorded patterns of change in binding with time, and were performed under different conditions.

2.21.1 *Equilibrium binding assay*

Freshly purified monocytes were suspended in binding medium consisting of RPMI 1640 supplemented with 20mM HEPES and 0.5% bovine serum albumin. Typically, 4x10^6 monocytes in 0.15ml medium were incubated with iodinated ligand in siliconised glass tubes overnight at 4^{o}C on a rotating table. Cell suspensions were overlayed on 0.2ml FCS at 4^{o}C and centrifuged for 30 seconds at maximum speed in a Beckman Microfuge 12. The tip of each tube was cut off above the visible cell pellet and counted in a Packard Auto-Gamma 5650. Non-specific binding was determined in the presence of a 100-fold excess of unlabeled cytokine. In the case of ^{125}I-IL-3, preliminary experiments demonstrated complete inhibition of radioligand binding by either modified IL-3 (containing the N-terminal octapeptide) or by

unmodified IL-3. Subsequent experiments were performed using unmodified IL-3 as competitor. Specific binding was calculated by subtracting nonspecific from total binding, and free radioligand was defined as the difference between total radioactivity added and that specifically bound.

For determination of receptor **number** and **affinity** by equilibrium methods, at least 10 different concentrations of radioligand were used per experiment, each concentration in duplicate. The data were analysed by the method of Scatchard (1949), where the ratio of specifically bound:free radioactivity is plotted against bound cpm. For this analysis, free radioactivity was corrected for MBC (Section 2.20.1) and the SR was determined by SDA (Section 2.20.2). Scatchard data were analysed by the EBDA and LIGAND computer programs (Elsevier-BIOSOFT, Cambridge, England) (McPherson, 1983, 1985). Comparisons of variance about the regression lines for one site and two site binding models were made by LIGAND using objective statistical measures with weighting for the number of replicates per point (F test, sum of the squares).

For determination of receptor **specificity**, radioligand was incubated with cells and a 100-fold excess concentration of purified competitor cytokine, and processed as described above. These competition experiments were all performed overnight at 4°C, either in the presence or absence of 0.1% sodium azide. To determine whether receptor internalisation could occur under these conditions, acid-resistant radioactivity was measured according to the protocol of Fernandez-Botran et al (1989). In brief, aliquots of cells were taken from the binding mixture after overnight incubation, and were resuspended in two volumes of 0.5M acetic acid/0.5M NaCl, pH 2.0 for two minutes at 4°C followed by centrifugation on FCS as described. Total cell associated radioactivity was measured on duplicate samples

with binding medium, pH 7.2, substituted for the low pH buffer. Dissociable radioactivity was defined as the difference between total specific counts bound and acid-resistant specific counts bound.

2.21.2 *Kinetic binding assays*

Association kinetics were determined at both 4°C and at 37°C, with measurement of specific binding, as outlined in Section 2.21.1, at a number of time points. 0.1% NaN_3 was included in these experiments to prevent receptor-ligand complex internalisation. Dissociation kinetics were determined after establishment of equilibrium binding by overnight incubation of cells and ligand at 4°C. Cells were then centrifuged through an FCS cushion in 1.5ml polypropylene tubes (Eppendorf, Hamburg, West Germany) and like samples pooled. Radiolabeled monocytes were resuspended in binding medium at 4°C, aliquotted into binding tubes, and a temperature shift to 37°C performed. Specific cpm bound were determined at a number of time points after separation of bound and dissociated radioactivity by a second centrifugation step. These experiments were also done in the presence of NaN_3.

Internalisation experiments were performed after equilibrium binding overnight at 4°C, as outlined above, but without NaN_3. Duplicate aliquots of cells were taken at each time point, and total and acid-resistant radioactivity determined as outlined in Section 2.21.1. Internalised radioactivity was defined as acid-resistant radioactivity, while surface-bound radioactivity was total minus acid-resistant cpm.

Kinetic binding data are presented graphically in terms of the number of molecules of ligand bound versus time. Molecules bound were calculated by the formula:

$$\text{molecules bound per cell} = \frac{\text{cpm bound} \times 6.023 \times 10^{23}}{\text{SR of ligand (cpm/mol)} \times \text{cell number.}}$$

The data from association and dissociation experiments was analysed by the computer program KINETIC (Elsevier Biosoft) yielding rate-constants for association and dissociation. The theoretical background for these calculations is presented in Chapter 7. Internalisation rate constants were derived graphically, as outlined in Chapter 7, by a previously published method (Wiley and Cunningham, 1981).

2.22 Statistical methods

Biological data were expressed as the mean ± SD or ± SEM, and significance was determined using the Student's unpaired t test. For comparisons involving a number of different experiments with inter-experiment variability in the magnitude of the response, Wilcoxon's matched pairs test was used. For Scatchard transformation of equilibrium binding data, and for determination of rate constants of radioligand association and dissociation, the computer programes EBDA, LIGAND and KINETIC were used (Elsevier Biosoft). These programs rely on a weighted non-linear curve-fitting technique, and provide several statistical measures of 'goodness of fit'. These include the runs test, the F test, and the sum of squares. Significant results in this thesis were defined as those with $p<0.05$.

CHAPTER 3

REGULATION OF MONOCYTE GROWTH AND ANTIGEN EXPRESSION BY CSF

INTRODUCTION

The life span for cells of the monocyte/macrophage lineage is relatively long, with a turnover time in rodents of 20-60 days at steady state (Nathan et al, 1985). Studies on human subjects undergoing bone marrow transplantation have suggested a life span for tissue macrophages in man of at least 3 months (Thomas et al, 1976). Prolonged survival can also be achieved with the *in vitro* culture of monocytes, where cells with the general features of tissue macrophages develop (Schlesinger et al, 1984). However, such culture techniques generally rely on the presence of either human or fetal calf serum to maintain cell viability and promote differentiation and maturation. While several of the components of serum, including insulin and transferrin, are known to be important in supporting monocyte cultures (Adams, 1979), addition of these proteins alone to serum-free medium is insufficient to promote growth and survival (Becker et al, 1987). Identification of other factors which promote monocyte survival is necessary to explain the prolongation of life of these cells *in vivo*.

The role of the colony-stimulating factors in monocytopoiesis raises the possibility that these factors may also regulate monocyte numbers later in the differentiation pathway, and several *in vitro* studies with mouse cells support this. The survival and proliferation of more than 90% of murine bone-marrow-derived macrophages (BMM) is enhanced by M-CSF (Tushinski et al, 1982, 1985) while proliferation of blood monocytes and peritoneal exudate macrophages (PEM) is stimulated by GM-CSF (Chen et al, 1988A). GM-CSF also acts as a survival factor for a proportion (20%) of murine pulmonary alveolar macrophages (PAM), and at higher concentrations causes proliferation of the same subset of cells (Lin et al, 1989). The activity of IL-3 appears to be more restricted, leading to proliferation in PAM (Chen et al, 1988B), but not in PEM or blood monocytes as a sole stimulus (Chen et al,

1986). Synergism between different CSF in stimulating murine mononuclear phagocyte proliferation has recently been described (Chen et al, 1986, 1988A,B).

The relevance of these findings to monocyte biology *in vivo* is more difficult to determine. However, intraperitoneal administration of IL-3 and GM-CSF in mice leads to increases in mononuclear phagocyte numbers in several organs, and in particular in the peritoneal cavity (Metcalf et al, 1986A, 1987). A number of other changes are seen in these animals, including an increase in splenic haemopoiesis and increases in blood leucocyte counts. Of most importance to this discussion, however, is the observation of enhanced mitotic activity in peritoneal macrophages from mice treated with either cytokine. These findings suggest that at least a proportion of the increase in peritoneal macrophage numbers is due to local proliferation, although other mechanisms are undoubtedly also operative (Metcalf et al, 1986A, 1987).

Information concerning the regulation of human monocyte survival and proliferation is limited to *in vitro* studies with M-CSF, which leads to enhanced survival under serum-free conditions (Becker et al, 1987) and, in contrast to the findings in the mouse, induces proliferation in only 1-5% of cells (Gendelman et al, 1988). The effects of IL-3 and GM-CSF on human monocyte survival and proliferation are unknown.

In addition to regulating mononuclear phagocyte numbers, emerging evidence also suggests a role for CSF in the maturation and differentiation of these cells. M-CSF enhances lysosomal enzyme content in human monocytes *in vitro*, and maintains respiratory burst activity in long term culture (Becker et al, 1987). In addition, studies on the U-937 monocytic cell line have shown the following effects of GM-CSF: altered cell morphology, enhanced reduction of nitroblue tetrazolium (a measure of respiratory burst activity) and increased expression of the c-fms proto-oncogene (the M-CSF receptor) (Geissler et al,

1989). Changes in the surface expression of many antigens have been noted with monocyte/macrophage maturation and differentiation (Goyert et al , 1986). Prominent amongst these is the complement receptor 3 (CR3, also known as MAC-1), which expression is regulated by M-CSF in murine monocytes (Warren et al, 1985) and by M-CSF and GM-CSF in human monocytes (Becker et al, 1987; Geissler et al, 1989). Other M-CSF-regulated antigens described include the leukocyte functional antigen (LFA) adhesion complex, p150/95, the high affinity Fc receptor, and HLA-DR (Becker et al, 1987). Less is known of the influence of IL-3 on antigen expression in mononuclear phagocytes, although one report describes stimulation of M-CSF-receptor expression in murine PEM treated with IL-3 (Chen et al, 1986).

In this chapter, the roles of IL-3 and GM-CSF in the regulation of human monocyte survival, proliferation and antigen expression are described. Data are presented showing that both cytokines are active at picomolar concentrations in supporting monocyte survival, and that proliferation is only stimulated at significantly higher concentrations of CSF. In addition, and in contrast to the findings in the mouse, only a small proportion of cells is shown to be capable of IL-3- or GM-CSF-induced proliferation. The regulation of monocyte size, protein content and antigen expression by CSF is also described.

RESULTS

3.1 Effect of CSF on monocyte numbers *in vitro*

Methods of monocyte purification and culture were first established to allow these experiments to proceed. Cells purified by adherence were used for the survival and proliferation assays, and showed a purity of 88.8±1.2% (mean ± SEM for 5 experiments). The cells were cultured *in situ* in petri dishes at an initial density of 0.5-2x10^6 per dish in

medium consisting of RPMI with 10% pooled human AB serum, plus antibiotics and $NaHCO_3$. Several batches of serum were screened in survival assays, and considerable variability was noted in their capacity to support long term monocyte culture in the absence of added growth factors. Sera with a low inherent capacity to support the cultures were used in subsequent experiments.

The percent survival over time of monocytes cultured in medium alone, and in the presence of 20pM IL-3 or GM-CSF is shown in Fig. 3.1. While control cultures showed a gradual decline in monocyte numbers with time, CSF-treated cultures maintained cell numbers at approximately 70-80% of initial values. CSF-treated cultures showed significantly higher numbers of cells by the unpaired t test at days 6, 8 and 11 of culture ($p<0.01$). Several experiments were performed with cell harvest at a single time point between 7 and 14 days of culture. The results of three such experiments with IL-3 and five experiments with GM-CSF are summarized in Table 3.1 (left hand panel). The data confirm that both cytokines act to maintain monocyte/macrophage numbers *in vitro*.

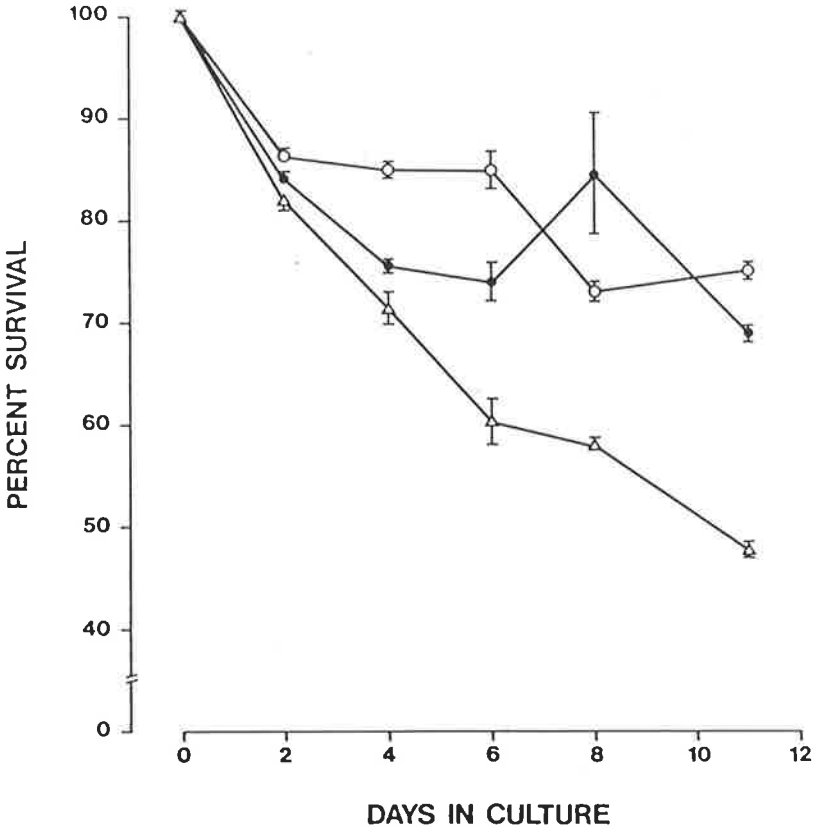
The specificity of the CSF-monocyte interaction is demonstrated in a respresentative experiment (Fig. 3.2). As previously reported (Becker et al, 1987), M-CSF treated cultures showed higher monocyte numbers at day 7 than did controls. IL-3 and GM-CSF showed similar results to M-CSF, but G-CSF did not alter monocyte numbers compared to control cultures.

3.2 Effect of CSF on monocyte ^{3}H-thymidine uptake

The maintenance of monocyte numbers seen in cultures treated with IL-3 or GM-CSF suggests either an effect on cell survival, or the stimulation of low grade monocyte proliferation balanced by cell death. To investigate these possibilities, ^{3}H-thymidine

Figure 3.1. Percent survival over time in control (△) versus IL-3-treated (●) and GM-CSF-treated (○) groups. Monocytes were purified by adherence to plastic and cultured for up to 11 days in the absence or presence of growth factors at a concentration of 20pM. Culture medium and growth factors were replaced at 4 and 8 days of culture, with re-addition of any cells in suspension. Petri dishes were harvested at the indicated times and monocyte counts performed. Each point represents the mean ± SEM of at least three replicates. IL-3 and GM-CSF-treated cultures showed significantly greater monocyte numbers compared with controls at days 6, 8 and 11 of culture ($p<0.01$ by unpaired t test).

Figure 3.2. Monocyte number per culture after a 7 day incubation with 600pM G-CSF, IL-3, GM-CSF, M-CSF or no stimulus. Methods used are detailed in the legend for Fig. 3.1. Data are expressed as the mean ± SEM of three replicates per stimulus.


PERCENT SURVIVAL
100
90
80
70
60
50
40
0
0
2
4
6
8
10
12
DAYS IN CULTURE

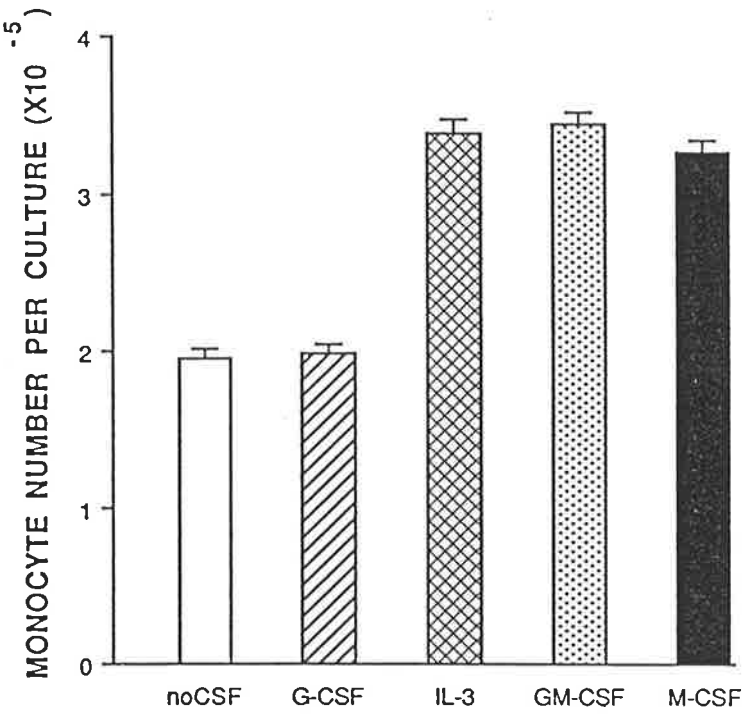

MONOCYTE NUMBER PER CULTURE (X10 -5)
4
3
2
1
0
noCSF
G-CSF
IL-3
GM-CSF
M-CSF

TABLE 3.1 Regulation of Monocyte Growth by CSF

	Monocyte Number* (Percent of control)	Forward Light Scatter** (no. of channels)	Total Protein*** (ug per 10^5 monocytes)
control	-	105.9±0.2	70.6±7.4
IL-3 (600pM)	162.9±4.2	137.0±9.1	106.0±4.9
GM-CSF (600pM)	164.9±5.9	141.3±1.5	140.2±5.5

* Monocytes were purified and grown in tissue culture as described in Materials and Methods. Cells were harvested between days 7 and 14 of culture and counted. Results are expressed as mean ± SEM for three experiments with IL-3 and for five experiments with GM-CSF.

** Forward degree scatter as measured by flow cytometry of 12 day old cultured monocytes. Results are expressed as the mean ± SD for at least two replicates.

*** Total protein per 10^5 cells as measured by coomassie blue binding to 12 day old cultured monocytes. Results are expressed as the mean ± SEM for at least three replicates.

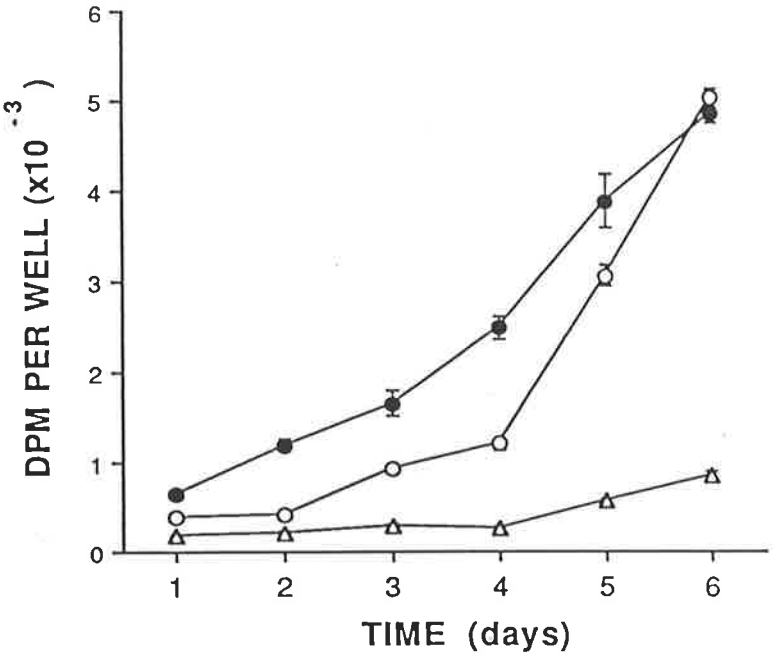
incorporation was used as a measure of monocyte proliferation. Cultures were pulsed with 1uCi/ml ^{3}H-thymidine for 18 hours after varying periods of stimulation with CSF or control. As shown in Fig. 3.3, both IL-3 and GM-CSF stimulated enhanced ^{3}H-thymidine incorporation in monocyte cultures at all time points between days 1 and 6 of culture. The differences between CSF-treated and untreated-monocytes became more pronounced with increasing duration of stimulation. In subsequent experiments comparing different monocyte donors, day 4 was chosen as the time of addition of ^{3}H-thymidine, as the differences in cell numbers between untreated and CSF-treated groups were relatively small at this point (less than 30%, Fig. 3.1).

During the course of these experiments, some variability in the magnitude of the response in different monocyte donors was seen. Fig. 3.4 shows a summary of 5 experiments in which ^{3}H-thymidine uptake was measured at day 4 of monocyte culture. ^{3}H-thymidine uptake was significantly enhanced in 5 of 5 experiments with IL-3, and 3 of 5 with GM-CSF.

In order to determine the sensitivity of monocyte ^{3}H-thymidine uptake as a measure of CSF activity, experiments were performed after 4 days of culture in the presence of varying concentrations of IL-3 and GM-CSF, and the results compared with representative cell number experiments (Fig. 3.5). It can be seen that both cytokines enhanced monocyte numbers at concentrations of <20pM, and showed similar dose-response profiles with plateau at 20-60pM (Fig. 3.5A). In contrast, CSF-induced ^{3}H-thymidine incorporation was only seen with concentrations of cytokine of at least 60pM (Fig. 3.5B), and failed to plateau even at CSF concentrations as high as 600pM.

Figure 3.3. Time course of ^{3}H-thymidine incorporation in cultured human monocytes. 0.5x10^{5} monocytes purified by adherence were incubated in microtitre wells with 600pM IL-3 (●), GM-CSF (○) or control medium (△) for the indicated times, pulsed with 1uCi/ml ^{3}H-thymidine, and harvested 18 hours later onto glass filters. Results are expressed as the mean ± SEM for 6 replicates per point.

Figure 3.4. Per cent increase in ^{3}H-thymidine incorporation in 4 day old CSF-treated monocyte cultures. The effects of IL-3 and GM-CSF at concentrations of 600pM are compared with no stimulus in five separate experiments on different donors. 0.5 x 10^{5} monocytes purified by adherence were incubated for 4 days and pulsed with ^{3}H-thymidine and harvested as described above. Results are expressed as the mean ± SEM for 6 replicates. CSF-treated groups showed significantly higher values by the unpaired t test for 5 of 5 experiments with IL-3 ($p<0.05$) and for 3 of 5 experiments with GM-CSF ($p<0.05$).


DPM PER WELL (x10 -3)
0
1
2
3
4
5
6
1
2
3
4
5
6
TIME (days)

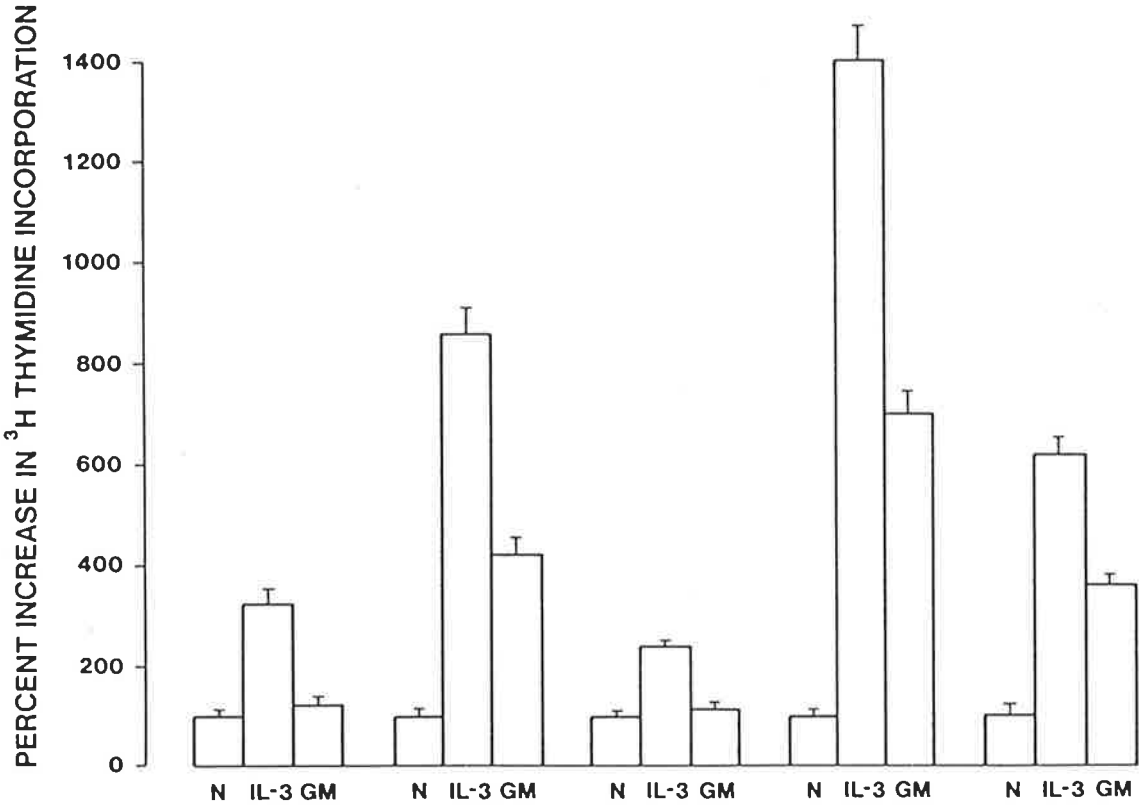
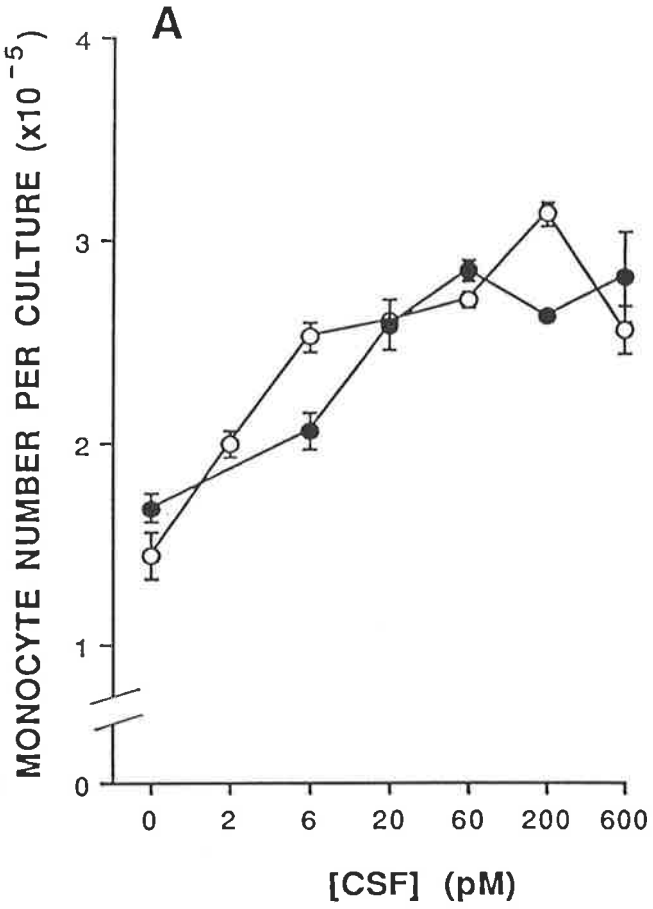
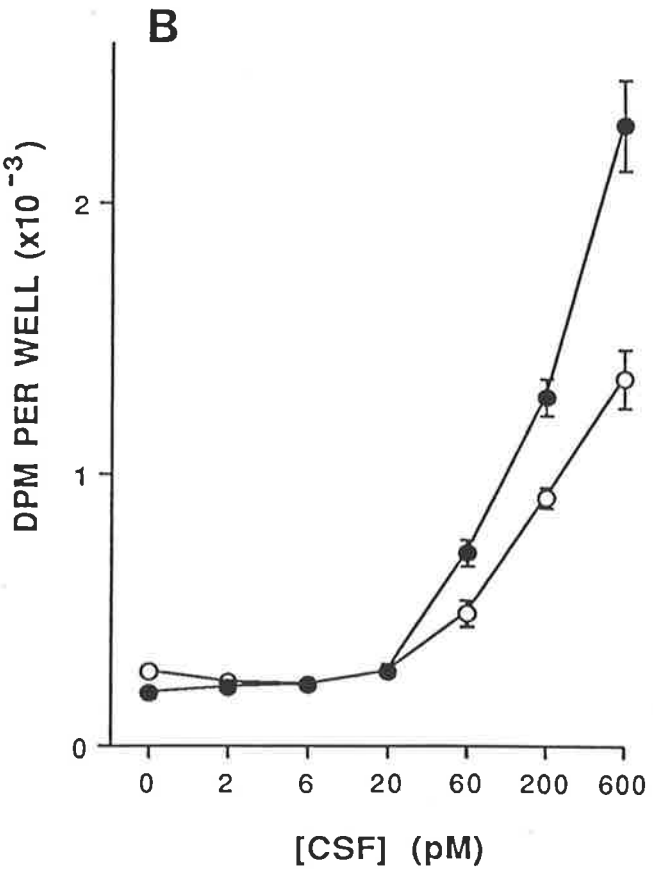

PERCENT INCREASE IN 3H THYMIDINE INCORPORATION
0
200
400
600
800
1000
1200
1400
N IL-3 GM
N IL-3 GM
N IL-3 GM
N IL-3 GM
N IL-3 GM

Figure 3.5. Stimulation of purified monocytes by different concentrations of IL-3 (●) or GM-CSF (o). (A) Number of cells harvested at day 7 of culture. (B) In a separate experiment, ^{3}H-thymidine incorporation over an 18 hour period from day 4 of culture. Results are expressed as the mean ± SEM of 3 replicates (A) and 6 replicates (B) per point.


A
MONOCYTE NUMBER PER CULTURE (x10^{-5})
4
3
2
1
0
0
2
6
20
60
200
600
[CSF] (pM)


B
DPM PER WELL (x10^{-3})
2
1
0
0
2
6
20
60
200
600
[CSF] (pM)

3.3 Labelling index of cultured monocytes

To establish the proportion of cells incorporating ^{3}H-thymidine, and to ensure that the thymidine uptake reflected incorporation into DNA, autoradiographic studies were performed. These demonstrated a small increase in the proporation of cells with >15 nuclear grains in CSF treated cultures compared with medium control (Table 3.2). 2.2±0.4% of monocytes treated with IL-3 (mean ± SEM of 4 experiments) and 1.7±0.1% of monocytes treated with GM-CSF (mean ± SD of 2 experiments) showed >15 grains per nucleus. In contrast, 0.5±0.1% of control monocytes (mean ± SEM of 4 experiments) showed >15 nuclear grains.

While these experiments were performed using highly purified adherent monocytes, a small proportion of contaminating cells was present. To determine whether peripheral blood-derived colony forming units (CFU) might be present in the monocyte preparations, and thereby account for the observed ^{3}H-thymidine uptake, semisolid agar culture of adherent and non-adherent fractions of the mononuclear cell population were performed. GM-CSF at different concentrations was used as a stimulus. Measurements of both day 7 clusters and day 14 colonies showed that while some CFU-GM were present in the non-adherent fraction, there was no significant clone formation in the adherent fraction (Fig. 3.6).

3.4 Trophic effects of CSF on monocytes

Examination of monocytes under phase microscopy after several days in culture revealed differences in apparent cell size between CSF-treated and untreated groups. To confirm this finding, 12 day old monocytes were detached from their culture dishes using EDTA at 4^{o}C and mechanical scraping, and subjected to flow cytometry. Cell size, as determined by forward light scatter, is shown for the different treatment groups in Table 3.1 (middle panel).

TABLE 3.2 Labelling index of day 4 monocytes*

Experiment	Stimulus		
	IL-3	GM-CSF	Medium Control
1	3.0	1.8	0.75
2	1.7	1.6	0.00
3	1.0	ND	0.6
4	2.9	ND	0.5
Mean ± SD	2.2±0.4	1.7±0.01	0.5±0.1

* Monocytes were cultured for four days in the presence or absence of CSF at a concentration of 600pM and pulsed for 18 hours with 1 uCi/ml ^{3}H-thymidine. Cells were harvested and autoradiography performed as described in Materials and Methods. The results of four experiments with IL-3 and medium control and two experiments with GM-CSF are shown. Each value is the mean of two determinations, in which a minimum of 400 cells was counted. The labeling index was defined as the percentage of cells with greater than 15 grains per nucleus. ND = not done.

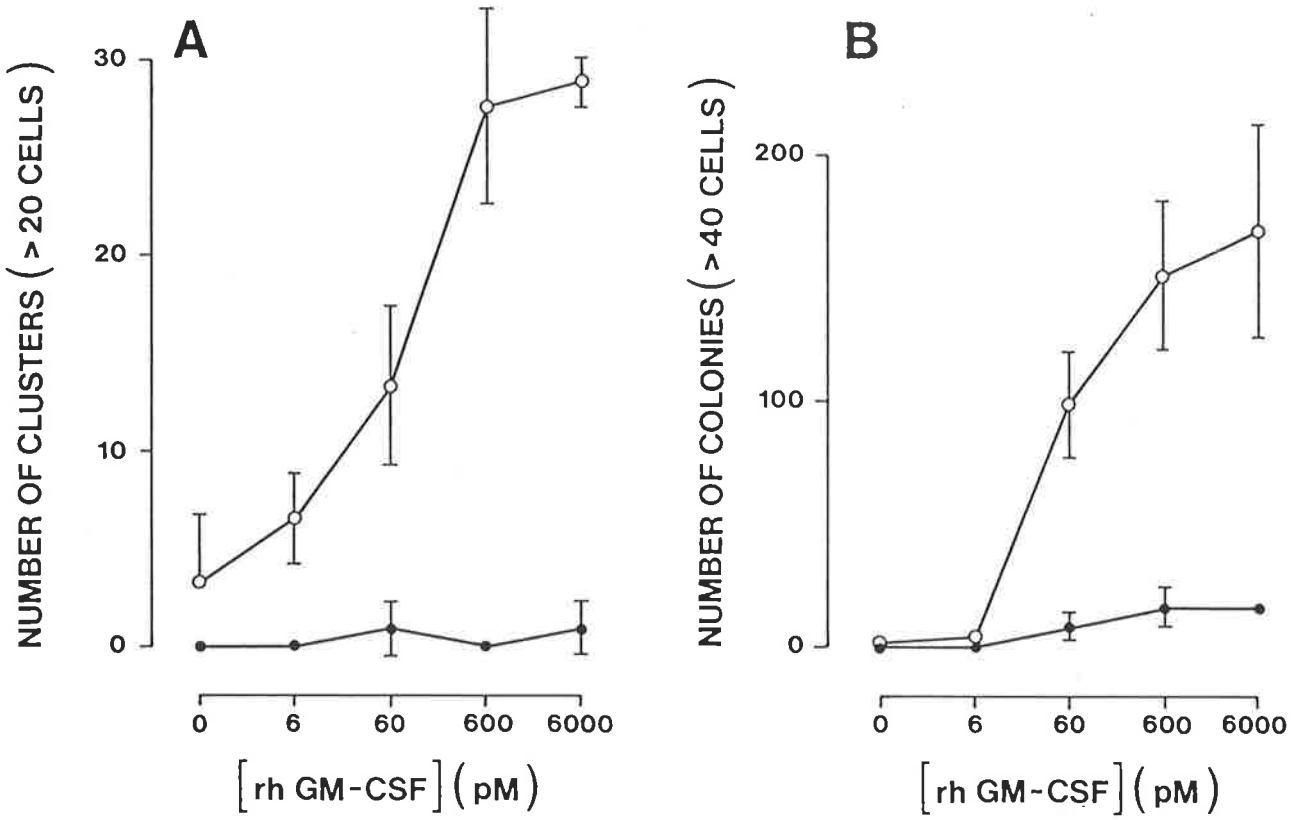


Figure 3.6. Colony forming assay with adherent (●) and non-adherent (○) fractions of peripheral blood mononuclear cells. Cells were incubated with varying concentrations of GM-CSF in soft agar, as described in Section 2.15, with counting of clones at day 7 (A) and day 14 (B) of culture. Results are expressed as mean ± SEM of at least 3 replicates per point.

As the mean channel number for forward light scatter is measured on a linear scale, these results show an enhancement of cell size over controls of approximately 30% for both GM-CSF-treated and IL-3-treated monocytes at day 12.

The trophic influence of IL-3 and GM-CSF on human monocytes was also apparent when total protein measurements were made on 12 day old stimulated and unstimulated cultures (Table 3.1, right hand panel). GM-CSF stimulated a doubling of protein content per 10^5 cultured cells compared with controls, while IL-3 stimulated an increase of approximately 50%.

3.5 Modulation of monocyte antigenic expression by CSF

For immunofluorescence experiments, it was desirable to obtain and culture monocytes in suspension, in order to facilitate handling and to reduce cell damage induced by detachment from plastic dishes. Monocytes were therefore prepared by elutriation, yielding a purity in excess of 90% (see Section 2.8.3). The cells were subsequently cultured in a suspension system (Section 2.9.2). Monocytes cultured for 5 days in this manner were analysed by flow cytometry for expression of a number of cell surface antigens. These include the LFA-family members LFA-1, MAC-1 and p150/95, and the ligand for LFA-1 (ICAM-1) (Fig. 3.7 and Table 3.3). Expression of MAC-1, as detected by two different MAb (60.1, MO-1), increased by greater than 4 fold in CSF-treated monocytes compared with controls, while expression of LFA-1 (MAb TS-1/22) showed approximately 2 fold increase in CSF-treated cells. MAb directed specifically to the common β chain of the LFA family (60.3, TS-1/18) revealed upregulation of this antigen by the CSF (2-4 fold increase), consistent with the findings for individual LFA-family members.

Figure 3.7. Monocyte antigen expression induced by CSF. Monocytes were purified by elutriation, and incubated for 5 days in suspension cultures with 600pM CSF or control. Cells were recovered from suspension by centrifugation at 100g, resuspended to equal concentrations, and stained with a variety of MAb. The specificities of the MAb are: IH-4: ICAM-1; MO-1, 60.1:α chain MAC-1 (CD11b); 150/95:α chain p150/95 (CD11c); TS 1/22:α chain LFA-1 (CD11a); 60.3, TS 1/18:β chain, LFA family (CD18). Forward light scatter for CSF-treated monocytes showed less than 10% increase over that for control cells at this time point. The coefficient of variability of the flow cytometer was 2-3%.

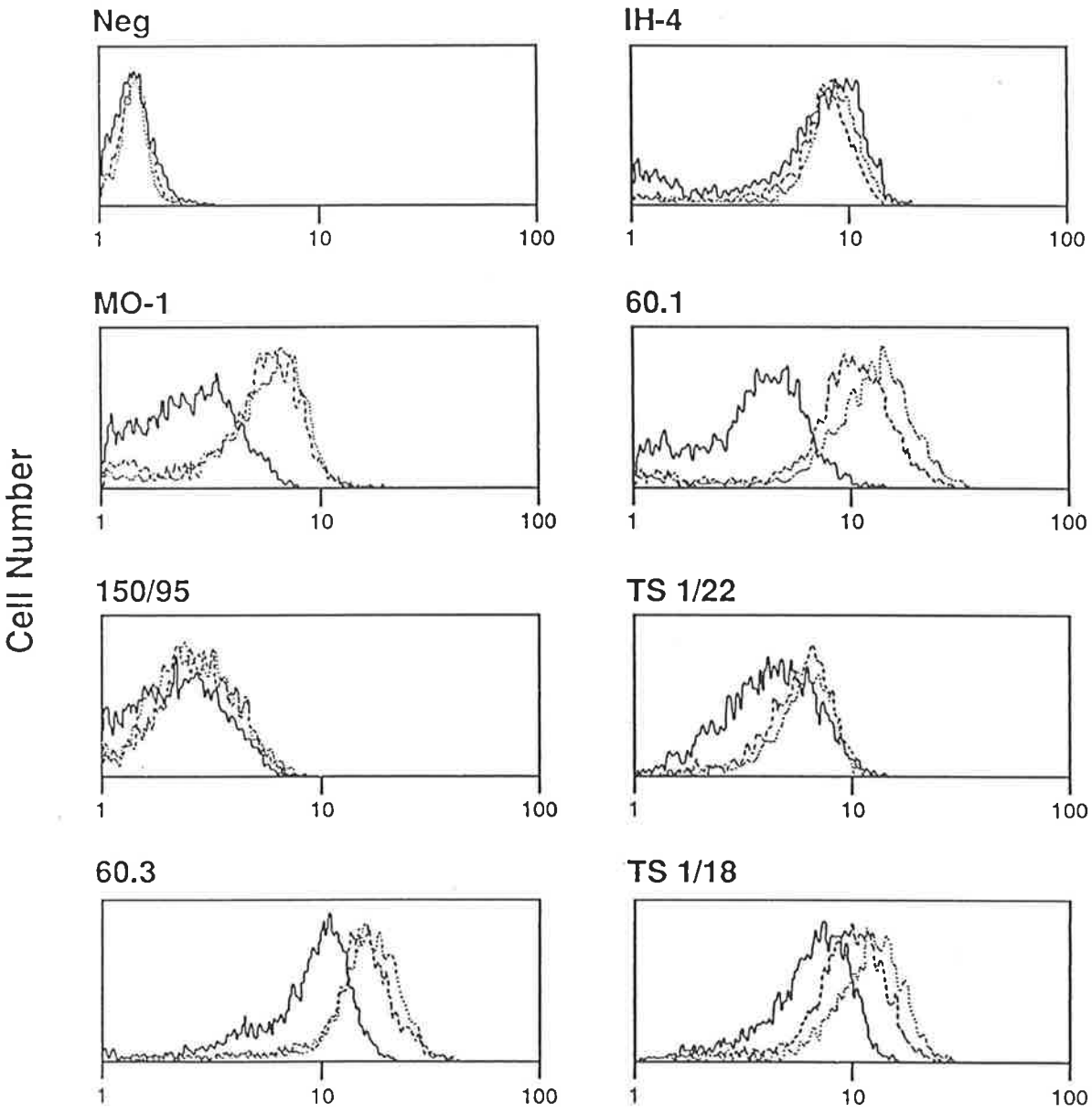

Neg
IH-4
MO-1
60.1
150/95
TS 1/22
60.3
TS 1/18
1
10
100
Cell Number
Relative Fluorescence Intensity
Control
IL-3
GM-CSF

TABLE 3.3 Modulation of Monocyte Antigenic Expression by CSF

MAb	Antigen	Stimulus		
		no CSF	IL-3	GM-CSF
IH-4	ICAM-1	92*	100 (+8)**	110 (+18)
MO-1	MAC-1	35	78 (+43)	85 (+50)
60-1	MAC-1	55	116 (+61)	131 (+76)
150/95	p150/95	30	41 (+11)	41 (+11)
TS 1/22	LFA-1	68	87 (+19)	91 (+23)
60.3	β chain	112	148 (+36)	153 (+41)
TS 1/18	β chain	95	120 (+25)	134 (+39)

* mean fluorescence, after subtraction of background fluorescence, measured on a logarithmic scale.

** shift in mean fluorescence relative to no CSF group.

Methods and MAb specificities are as outlined for Fig. 3.7.

DISCUSSION

Evidence is presented in this chapter that IL-3 and GM-CSF show activity in the regulation of human monocyte survival, growth and antigen expression *in vitro*. Fig. 3.1 shows that IL-3 and GM-CSF maintain monocyte numbers in culture over an 11 day period, while unstimulated cultures show a progressive decline. The specificity of the effect is demonstrated in Fig. 3.2, where IL-3 and GM-CSF but not G-CSF result in increased monocyte numbers relative to control cultures. The sensitivity of the interaction is demonstrated in Fig. 3.5A, where enhanced survival is seen at concentrations of CSF as low as 6pM. Although previously reported to be a human monocyte survival factor in serum-free cultures (Becker et al, 1987), M-CSF was also active in the experiments shown here (Fig. 3.2) which were performed in the presence of 10% human AB serum. Different batches of serum showed varying capacity to support monocyte cultures in the absence of exogenous stimuli. The elements responsible for interbatch variability are not known, but may include platelet-derived or other growth factors.

LPS may also enhance human monocyte survival (Becker et al, 1987), possibly via the mechanism of induction of M-CSF secretion. The experiments reported here employed media containing <40pg/ml LPS (by Limulus assay) at the highest concentration of serum used. In addition, highly purified recombinant cytokines were used, which contained undetectable levels of LPS (<0.03 ng/ml by Limulus assay). These data make it unlikely that the results were an LPS-induced artifact.

The data presented in Fig. 3.3 show that in addition to a survival effect at low dose, IL-3 and GM-CSF can also enhance ^{3}H-thymidine incorporation in human monocyte cultures in a time-dependent fashion. While some variability in the response was noted in different monocyte donors (Fig. 3.4), enhanced ^{3}H-thymidine uptake was seen in 3 of 5 experiments

with GM-CSF, and 5 of 5 with IL-3. The effect was only seen, however, at concentrations of CSF of 60pM and above, and cannot therefore be responsible for the observed influence of IL-3 and GM-CSF on cell numbers at lower cytokine concentrations. The dose-response curves shown here for IL-3 and GM-CSF-stimulated ^{3}H-thymidine uptake are similar to those recently published by Koyanagi et al (1988), where significant uptake was only seen with concentrations of CSF of 100pM or greater. In addition, those authors referred to a CSF-induced increase in cell numbers of 'at most twofold' over the 14 day course of their experiments, but no details were provided. In the experiments reported here, increases in cell numbers over and above initial values were not seen, despite the use of CSF concentrations as high as 600pM. The reasons for the difference in observations is not clear, but it is noteworthy that Koyanagi et al used a different purification protocol, and did not provide data on either fresh or cultured monocyte purity.

The autoradiographic data (Table 3.2), demonstrate two further points. Firstly, only a small subpopulation of cells (1-3%) were seen to have nuclear grains. Secondly, the localisation of grains over the nucleus rather than randomly distributed over the cells shows that the enhanced ^{3}H-thymidine uptake seen in these cultures was a consequence of uptake into DNA, and not due to binding of degraded ^{3}H-methyl groups to cytoplasmic macromolecules (Maurer, 1981).

The most likely explanation for these findings is that a subpopulation of cells is capable of proliferating in response to high concentrations of IL-3 or GM-CSF. This conclusion is supported by the finding of occasional mitotic figures in CSF-treated, but not in untreated cultures (data not shown). An alternative hypothesis, namely that CSF might have simply enhanced DNA repair mechanisms in a subpopulation of cells, seems less likely but has not been totally excluded by these experiments. The question of the identity of the dividing cells is partially addressed by the colony-forming assays (Fig. 3.5). These show that using

adherence as a purification step effectively removes CFU-GM from the monocyte population. The identification of occasional myeloblasts and promyelocytes in monocytes prepared by elutriation (data not shown), led to the avoidance of this purification method for survival and ^{3}H-thymidine experiments. Thus the enhanced ^{3}H-thymidine incorporation seen in monocyte cultures treated with high concentrations of IL-3 and GM-CSF is most likely due to proliferation in a small subset of monocytes, although uptake by a contaminating cell type other than CFU cannot be entirely excluded. The small numbers of dividing cells seen after stimulation with IL-3 or GM-CSF are in keeping with another recent report, in which hM-CSF induced division in a subpopulation of human monocytes only (up to 5%, Gendelman et al, 1988). The results in man might therefore appear to differ from those in the mouse, where much higher proportions of various mononuclear phagocyte populations can be stimulated to proliferate with CSF treatment. However, the majority of murine studies have employed peritoneal, pulmonary alveolar, or bone-marrow-derived macrophages rather than blood monocytes, and may therefore have involved more responsive target cell populations. Support for this hypothesis is provided by work with mIL-3, which stimulates proliferation in PAM but not in PEM or blood monocytes (Chen et al, 1988B). In contrast, work with mGM-CSF by the same group (Chen et al, 1988A) found similar proliferative potential in PEM, blood monocytes and BMM. Whether other mononuclear phagocyte populations in man show greater CSF-induced proliferation than do monocytes, remains to be established.

In addition to their effects on cell numbers, IL-3 and GM-CSF clearly exhibit trophic effects on human monocytes, with CSF-induced enhancement of cell size and protein content compared with controls (Table 3.1). In Table 3.3 and Fig. 3.7 the effects of IL-3 and GM-CSF on monocyte antigen expression can be seen. All of the antigens studied are important in leukocyte adhesive functions (see chapter 4), and three of them (MAC-1, p150/95, LFA-1) can be grouped into a single family on the basis of a common β chain: the

leukocyte-functional antigen (LFA) family. The fourth antigen, ICAM-1, forms the ligand for LFA-1. In addition to its role in adhesion, MAC-1 also functions as a receptor for fibrinogen and clotting factor X (Altieri et al, 1988A,B), and as the complement receptor 3. IL-3 and GM-CSF produced a strong increase in the expression of MAC-1 after 5 days in culture (Table 3.3, Fig. 3.7). Lesser, but still significant, increases were seen in the expression of LFA-1, while the upregulation of p150/95 and of ICAM-1 was less marked. The regulation of MAC-1 by GM-CSF in human monocytes has also been recently reported by Geissler et al (1989) who found similarly large increases in expression after 3 days of suspension culture. Modulation of other LFA family antigens was not described, however, and no previous reports of IL-3-regulated LFA expression in human monocytes could be found.

These data illustrate the capacity of IL-3 and GM-CSF to influence human monocyte phenotype as well as growth, and raise the possibility of CSF-induced functional change as a consequence of altered antigen expression. The issue of regulation of monocyte function by CSF will be discussed further in chapter 4.

The question of synergy between different CSF in the stimulation of monocytes has not been directly addressed in this chapter. Such interactions have, however, been previously reported in both murine monocyte/macrophages and in a human monocytic cell line (Chen et al, 1986, 1988A,B; Geissler et al, 1989). The presence of M-CSF in normal serum (Tushinski et al, 1982, and Bartocci et al, 1987) raises the possibility that the phenomena reported here result from synergy between M-CSF and the exogenous growth factors. Even in the absence of serum-derived M-CSF, induction of M-CSF secretion by monocytes in response to IL-3 or GM-CSF (Vellenga et al, 1988) might allow subsequent synergy to develop. Monocytes *in vivo* are likely to be exposed to a wide range of different regulators, and future studies with different combinations of these will be of great interest.

SUMMARY

In this chapter, the regulation of monocyte number, size and antigen expression by IL-3 and GM-CSF have been described. Both IL-3 and GM-CSF enhanced monocyte survival between days 4 and 14 of culture at concentrations as low as 6pM, while G-CSF was inactive. In contrast, stimulated ^{3}H-thymidine uptake was only seen at CSF concentrations of 60pM and above. Autoradiographic studies demonstrated that a subpopulation of cells (1-3%) showed nuclear grains. The question of the identity of the dividing cells at high CSF concentrations was partially addressed by performing colony assays with adherent and non-adherent mononuclear cells. These showed no CFU-GM present in the adherent fraction used in the ^{3}H-thymidine studies. The similarities between IL-3 and GM-CSF extended to their effects on monocyte maturation. Both cytokines stimulated increased cell size, increased protein content, and enhanced surface expression of CR3 and other LFA family antigens, raising the possibility of CSF regulation of monocyte adhesion-dependent functions.

CHAPTER 4

REGULATION OF MONOCYTE ADHESION BY CSF

INTRODUCTION

Several recent reports have presented data on the stimulation of human monocyte function by both IL-3 and GM-CSF. Amongst these are the stimulation of non-antibody dependent tumour cell cytotoxicity (Cannistra et al, 1988A), of gene expression (Vellenga et al, 1988, Oster et al, 1989, Ernst et al, 1989), and of anticandidal function (Wang et al, 1989). In addition, both growth factors have been shown to induce large increases in the recovery of human immunodeficiency virus (HIV) from 14 day old HIV-infected monocyte cultures (Koyanagi et al, 1988). These publications are consistent in showing little difference between IL-3 and GM-CSF in their effects on monocyte biology. Each study employed relatively long period of stimulation (greater than 2 hours), and where time courses were shown, the patterns for IL-3 and GM-CSF were similar. Dose-response profiles, where reported, were identical. Other studies have reported findings for GM-CSF alone, and additional effects ascribed to this cytokine include the stimulation of intracellular killing of *Leishmania Donovani* by monocytes (Weiser et al, 1987) and induction of morphological changes (Hart et al, 1988). While these data firmly establish IL-3 and GM-CSF as 'macrophage activating factors', the scope of their activities, their relative efficacy in the rapid stimulation of monocyte function, and their importance relative to other macrophage activating factors, remains to be determined.

One area which has received scant attention is the potential role of CSF in the regulation of monocyte adhesion. Several aspects of normal monocyte function are dependent upon adhesion events, as outlined in the general introduction to this thesis. In particular, monocyte-endothelial adhesion is a necessary step in the transmigration of monocytes into the extravascular space. The large increase in transendothelial migration of monocytes seen during inflammation and the accumulation of such monocytes at the inflammatory site (Issekutz et al, 1981) show that monocyte adhesion is a highly regulated phenomenon.

Several studies in this area have focused either on monocyte or on endothelial cell stimulation and have highlighted the importance of each cell type in contributing to adhesion events. Thus, the complement product C5a and the chemotactic peptide formyl-methionyl-leucyl-phenylalanine (f-Met-Leu-Phe) both stimulate monocytes to adhere to endothelium (Doherty et al, 1987) as do the phorbol ester phorbol myristate acetate (PMA) and autologous plasma (Wallis et al, 1985). Endothelial monolayers can be stimulated to increase monocyte adhesion by LPS (Carlos et al, 1989) and by the inflammatory cytokine Interleukin-1 (Bevilacqua et al, 1985).

IFNγ, the classic 'macrophage activating factor', may also help control monocyte adhesion. This cytokine induced homotypic adhesion in suspension cultures of human monocytes, while the inclusion of MAb to LFA-family antigens showed that this process was LFA-1 dependent (Mentzer et al, 1986). Homotypic monocyte adhesion may form a model for monocyte-tumour cell conjugation (Mentzer et al, 1986) and may therefore be of relevance to tumour cell lysis. More recently, Shaw et al (1989) have shown stimulation of murine PEM adhesion to the basement membrane glycoproteins laminin, fibronectin and collagen IV by IFNγ. Such interactions are likely to be of importance in the penetration by monocytes of the endothelial basement membrane during their emigration from the blood.

Whether CSF are involved in the control of any aspect of human monocyte adhesion is unknown. However, the modulation of surface expression of monocyte adhesion complexes by CSF (Chapter 3), and the development of rapid but transient margination of leucocytes in patients receiving intravenous GM-CSF (Devereux et al, 1989) provide some support for the concept.

In this chapter, data are presented showing that both IL-3 and GM-CSF are potent stimulators of monocyte adhesion. The response seen is biphasic, and adhesion is seen to a

variety of surfaces including human umbilical vein endothelial cells (HUVEC), extracellular matrix proteins (EMP) and to plastic. While the two growth factors show identical temporal and dose-response profiles during the late phase of stimulated adhesion, GM-CSF induces a more rapid response than IL-3 during early phase stimulation, raising for the first time a question of differences between the two growth factors in their mode of cell-signalling.

Data are also presented showing that while late phase stimulated adhesion is dependent upon *de novo* protein synthesis, early phase stimulated adhesion is not. Further, it is shown that stimulated adhesion during both phases is inhibitied by an antibody to CD18, establishing the role of the LFA family of adhesion molecules in this phenomenon.

RESULTS

4.1 IL-3 and GM-CSF stimulate two phases of monocyte adhesion

The adhesion experiments in this chapter and in Chapter 5 were performed using freshly elutriated monocytes of high purity (see Section 2.8.3). Adhesion was measured by an isotopic method (2.16) after one of two different culture methods (2.9).

The pattern of adhesion recorded during a 21 hour culture of monocytes on plastic is shown in Fig. 4.1. Adhesion at 60 minutes was significantly greater in the presence of IL-3 ($p<0.0004$ by unpaired t test) or GM-CSF ($p<0.00001$) than in their absence, and will be referred to as the 'early phase' of stimulated adhesion. By 2 hrs of culture this effect was no longer evident. Between 2 and 9 hours of culture, adhesion of control- and CSF-treated cells gradually declined, and control cells showed a continuing decline in adhesion to 21 hours. In contrast, IL-3 and GM-CSF-treated monocytes showed increasing levels of adhesion from 9 hours onwards, and reached maximal levels at 15 hours of culture. This

pattern of adhesion, including the decrease between 2 and 9 hours of culture, was seen in each of 3 experiments performed, and demonstrates that IL-3 and GM-CSF do not simply maintain the high levels of adhesion seen at 2 hours, but stimulate enhanced adhesion during this 'late phase' over and above 9 hr levels.

4.2 Early phase stimulation of monocyte adhesion

Subsequent experiments aimed to examine the two phases of stimulated adhesion in detail, both on endothelium and on plastic. As shown in Fig. 4.2A, adhesion of freshly prepared monocytes to endothelium increased in a time-dependent fashion over the first 75 minutes of culture. Addition of 600pM GM-CSF to the monocytes at the onset of culture led to a significant enhancement of adhesion at t=10,20,30 and 45 minutes ($p<0.001$). At 75 minutes of culture, GM-CSF-stimulated adhesion was not significantly greater than for controls, reflecting the continuing rise in adhesion in the latter group. A similar pattern was seen for IL-3-treated monocytes ($p<0.007$ at t=10,20,30,45 minutes, $p<0.02$ at t=75 minutes), although the rise in stimulated adhesion was less steep than seen with GM-CSF ($p<0.004$ at t=10,20 minutes). Parallel experiments performed on plastic (Fig. 4.2B) showed similar results to endothelium. The slower rate of stimulation by IL-3 was a consistent feature of these experiments, seen in 4 of 4 time courses performed. Dose response curves for the two cytokines measured at 20 minutes of culture (insets, Fig. 4.2) showed that the magnitude of the IL-3 effect could not be increased at higher concentrations of CSF.

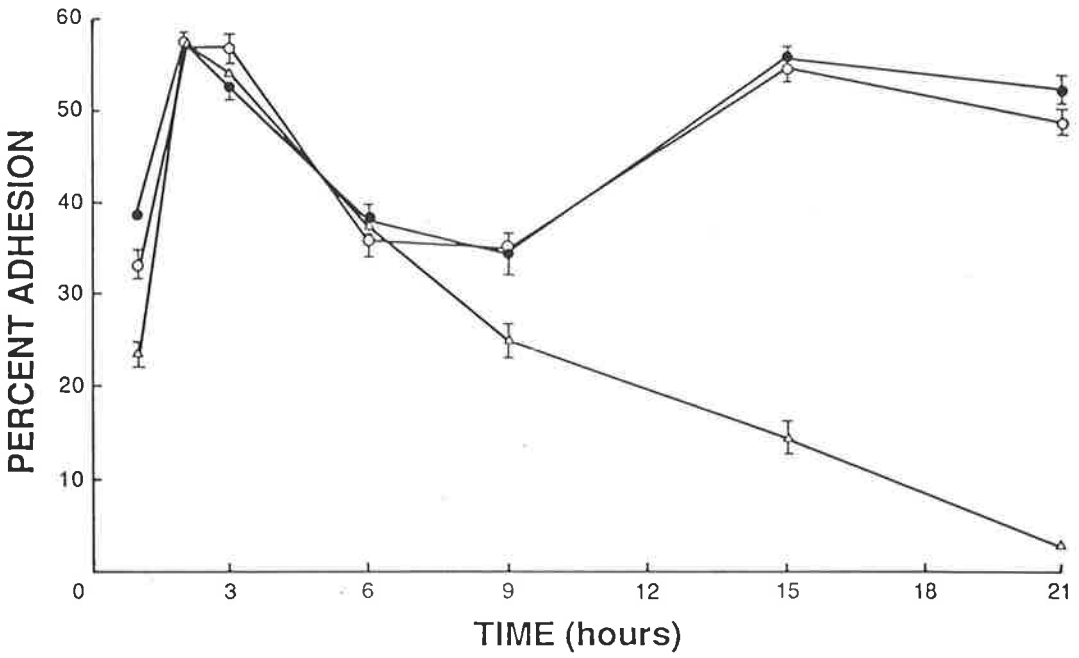
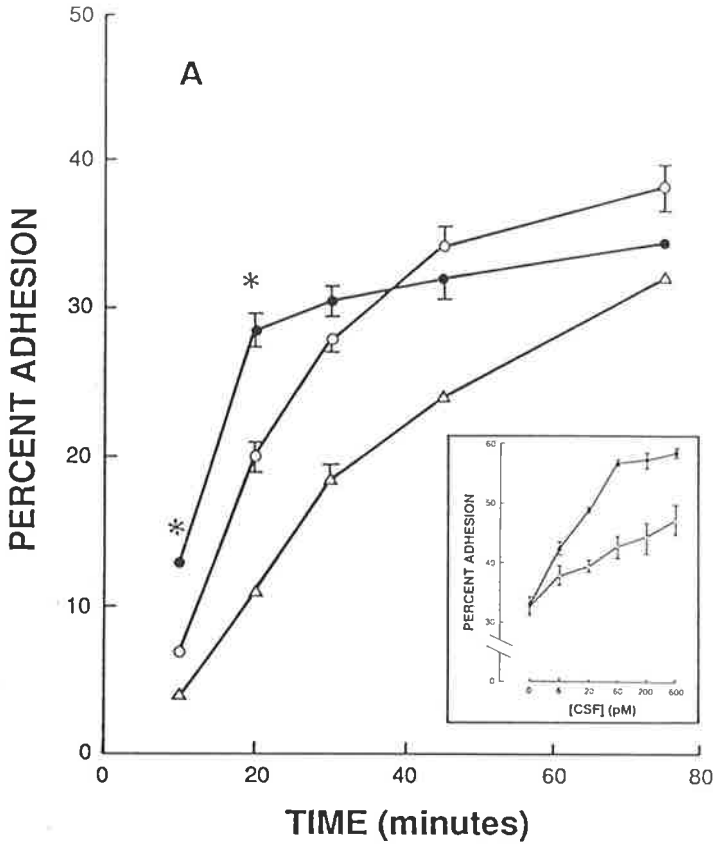
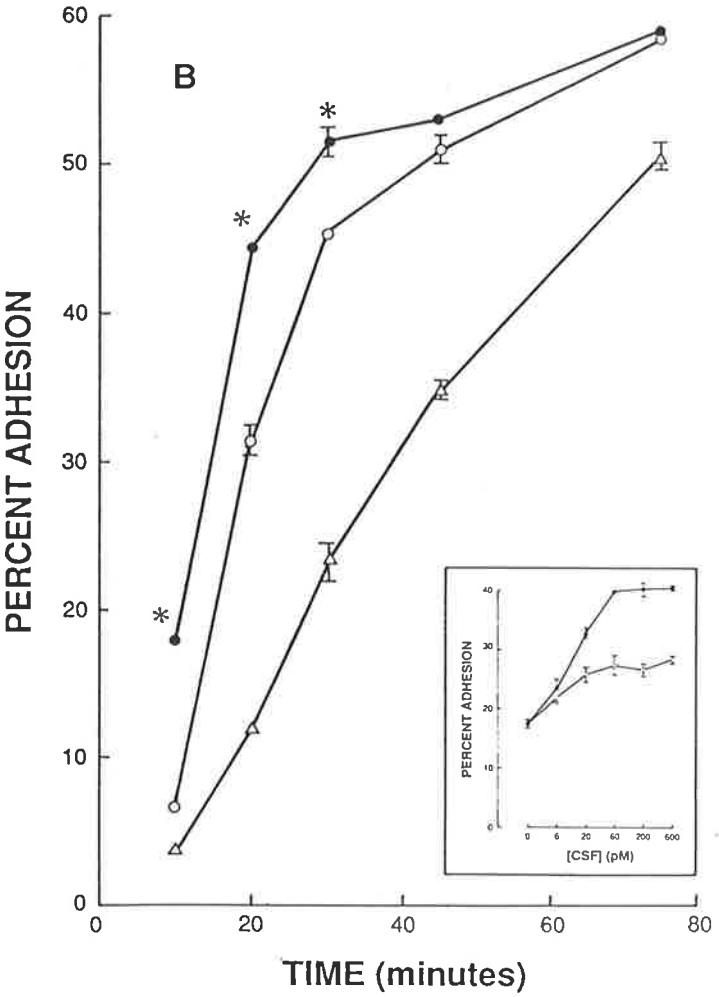


Figure 4.1 Effect of IL-3 and GM-CSF on monocyte adhesion to plastic over 21 hours of culture. Monocytes were prepared by elutriation and ^{51}Cr-labeled as described in Materials and Methods. The cells were resuspended in RPMI 1640 with 10% FCS and aliquotted into microtitre wells containing medium (△), IL-3 (o) or GM-CSF (●) (final concentration 600pM). Adhesion was measured at the times indicated as described in the text. Each point represents the mean ± SEM of 6 replicates in a representative experiment of 3 similar experiments. Errors are omitted where these are smaller than the size of the symbol.

Figure 4.2 Effect of IL-3 and GM-CSF on monocyte adhesion over 75 minutes. ^{51}Cr-labeled monocytes were resuspended in warm RPMI 1640 with 10% FCS and aliquotted into microtitre wells containing pre-warmed (37°C) medium (△), IL-3 (o) or GM-CSF (●) (final concentration 600pM). Adhesion to confluent monolayers of HUVEC (A) and to plastic (B) was measured at the indicated times. Each point represents the mean ± SEM of 4 replicates in a representative experiment of 4 performed. Errors are omitted where these are smaller than the size of the symbol. IL-3-stimulated adhesion was significantly higher than control at t=10,20,30 and 45 minutes on both HUVEC and on plastic ($p<0.007$ by unpaired t test) and at t=75 minutes on both surfaces ($p<0.02$). GM-CSF-stimulated adhesion was significantly higher than control at t=10,20,30 and 45 minutes on HUVEC and at t=75 minutes in addition on plastic ($p<0.001$). In separate experiments monocytes were incubated on HUVEC (Inset, A) and on plastic (Inset, B) with different concentrations of IL-3 (o) and GM-CSF (●), and were harvested at 20 minutes of culture. Each point represents the mean ± SEM of 4 replicates.

* $p<0.004$ compared with IL-3 treated monocytes.


A
PERCENT ADHESION
TIME (minutes)
0
10
20
30
40
50
0
20
40
60
80
*
*
*
PERCENT ADHESION
[CSF] (pM)


B
PERCENT ADHESION
TIME (minutes)
0
10
20
30
40
50
60
0
20
40
60
80
*
*
*
PERCENT ADHESION
[CSF] (pM)

4.3 Late phase stimulation of monocyte adhesion

To study the late phase of stimulated adhesion to cultured endothelium, the same co-incubation techniques used for the early phase were attempted. Using these methods monocytes adhered very strongly to HUVEC at 21 hours either in the presence or absence of cytokines (data not shown). The reason for enhanced adhesion between monocytes and HUVEC following prolonged co-incubation is unclear but may involve the development of immunological or other reactions between HLA-incompatible monocyte and HUVEC donors, with subsequent stimulation of the adhesive capacity of either or both cell types. To avoid this problem, ^{51}Cr-labeled monocytes were preincubated with cytokines or control medium for 21 hours in supension cultures, washed once, and resuspended in culture medium without the addition of further stimuli. The cells were then allowed to adhere to endothelial monolayers or to plastic for 30 minutes prior to harvest. A representative experiment of 4 performed using these methods is shown in Fig 4.3. Both growth factors enhanced monocyte adhesion to endothelial monolayers (Fig 4.3A) and to plastic (Fig 4.3B). Experiments comparing monocyte adhesion to plastic at 21 hours using the two protocols showed that background or unstimulated adhesion to this surface was higher using the suspension method than the plastic-monocyte co-incubation method (data not shown).

The late phase of stimulated adhesion induced by IL-3 and GM-CSF was not only temporally identical for the two cytokines (Fig 4.1) but seen at equivalent molarities. Fig. 4.4 shows pooled data from 4 experiments in which IL-3 (Fig 4.4A, circles) and GM-CSF (Fig 4.4B, circles) demonstrate similar dose-response profiles. Inclusion of antisera to IL-3 (Fig 4.4A, triangles) or to GM-CSF (Fig 4.4B, squares) as specificity controls abolished the stimulation at low concentrations of added homologous cytokine. In contrast, anti-GM-CSF was ineffective at inhibiting the effect of IL-3 (Fig. 4.4A, squares) and anti-IL-3 caused only partial inhibition of the pro-adhesive effects of GM-CSF (Fig. 4.4B, triangles). Similar

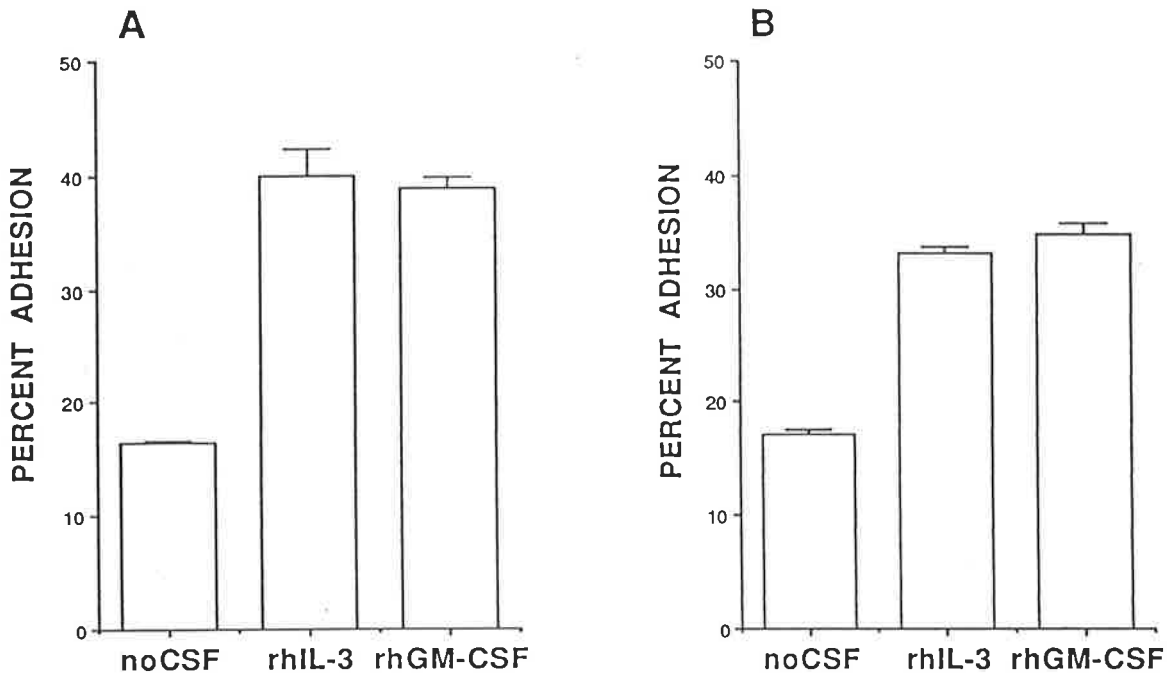
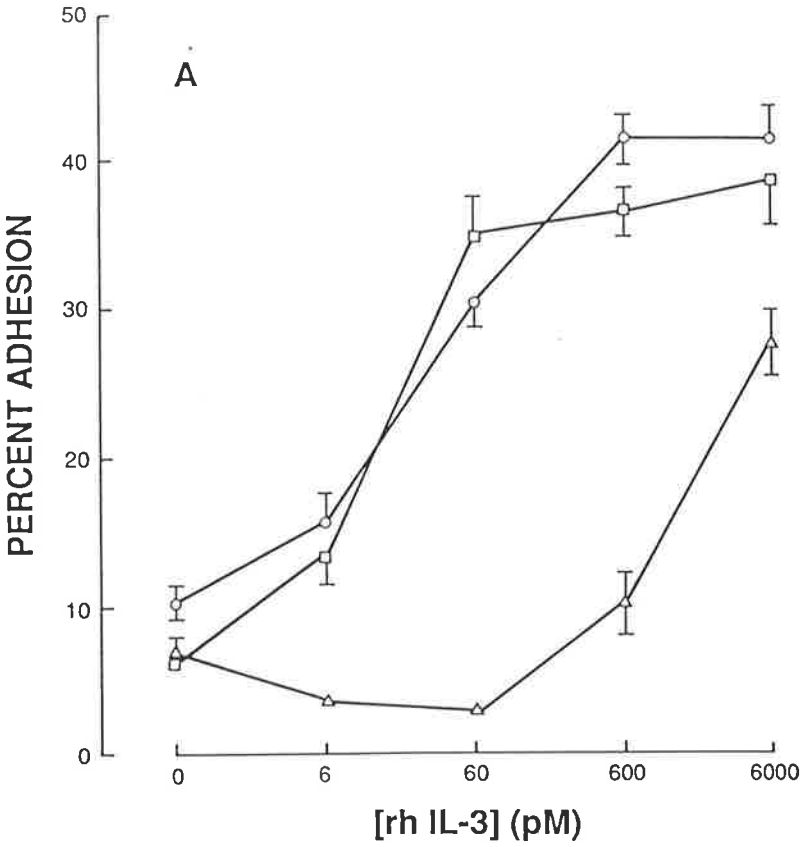
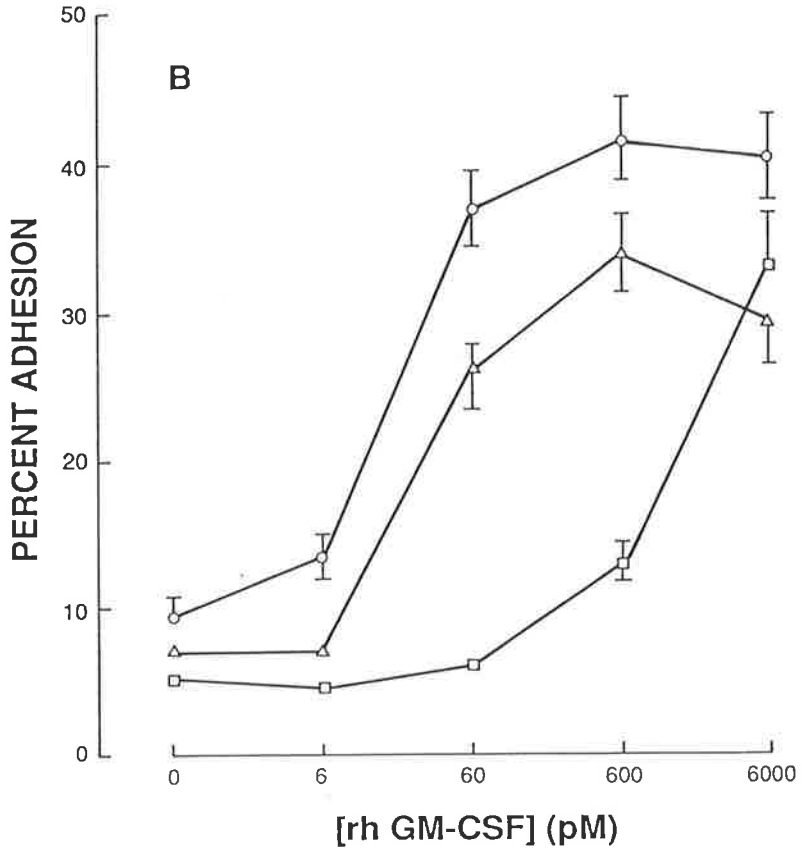


Figure 4.3 Effect of IL-3 and GM-CSF on monocyte adhesion after suspension culture. ^{51}Cr-labeled monocytes were suspended in RPMI 1640 with 10% FCS and incubated at 37°C in 10ml polypropylene tubes on a roller apparatus. 600pM IL-3, GM-CSF or medium control were included from the beginning of culture. At 21 hours, monocytes were gently centrifuged from suspension, resuspended in warm medium and aliquotted into microtitre wells containing either confluent monolayers of HUVEC (A) or plastic (B). Adhesion was measured after a further 30 minutes. Monocyte recovery following suspension culture was >90% with no significant differences between different treatment groups. Each point represents the mean ± SEM of four replicates in a representative experiment of 5 performed.

Figure 4.4 Inhibition of CSF-stimulated monocyte adhesion to plastic by specific anti-CSF antisera. IL-3 (A) or GM-CSF (B) at different concentrations were pre-mixed in microtitre wells with antisera or medium for 30 minutes at 24°C. Rabbit antiserum to IL-3 (△) and sheep antiserum to GM-CSF (□) were used at a final dilution of 1/1000, and compared with medium (o). 1.5×10^5 ^{51}Cr-labeled monocytes were then aliquotted to each well and adhesion was measured at 21 hours. Each point is the mean ± SEM of 24 replicates pooled from 4 experiments. Errors are omitted where these are smaller than the size of the symbol.


A
PERCENT ADHESION
50
40
30
20
10
0
0
6
60
600
6000
[rh IL-3] (pM)


B
PERCENT ADHESION
50
40
30
20
10
0
0
6
60
600
6000
[rh GM-CSF] (pM)

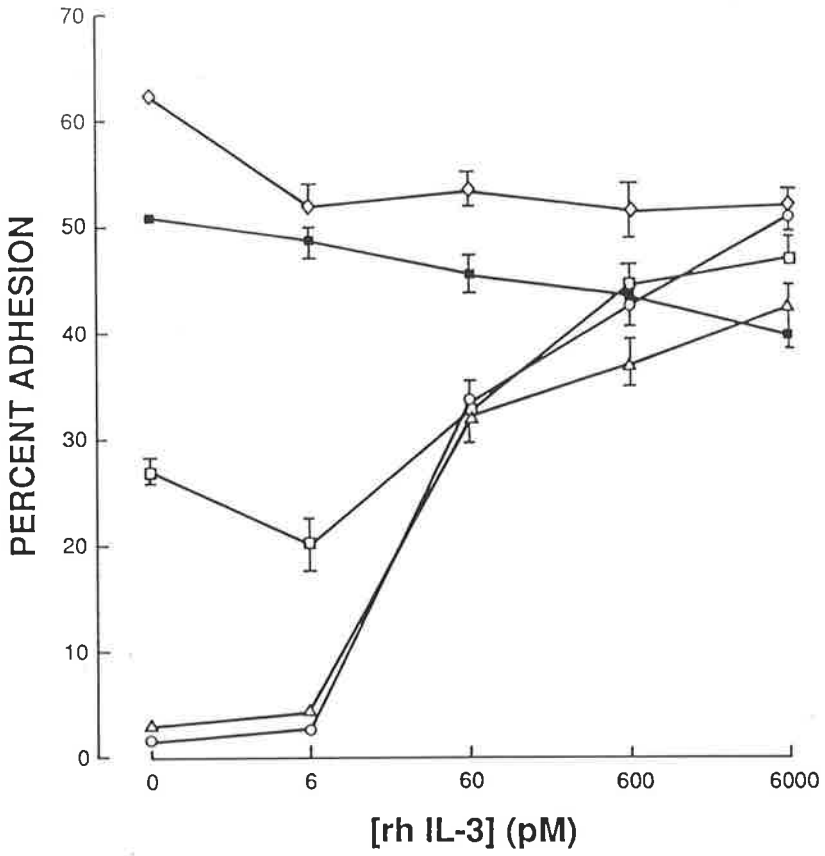


Figure 4.5 Co-stimulation of monocytes with IL-3 and GM-CSF. ^{51}Cr-labeled monocytes were added to microtitre wells containing different concentrations of IL-3 in combination with GM-CSF at final concentrations of 0 (o), 6 (△), 60 (□), 600 (■), and 6000 (◇) pM. Adhesion was measured at 21 hours of culture, with each point representing the mean ± SEM of 6 replicates in a representative experiment of 3 performed. Errors are omitted where these are smaller than the size of the symbol.

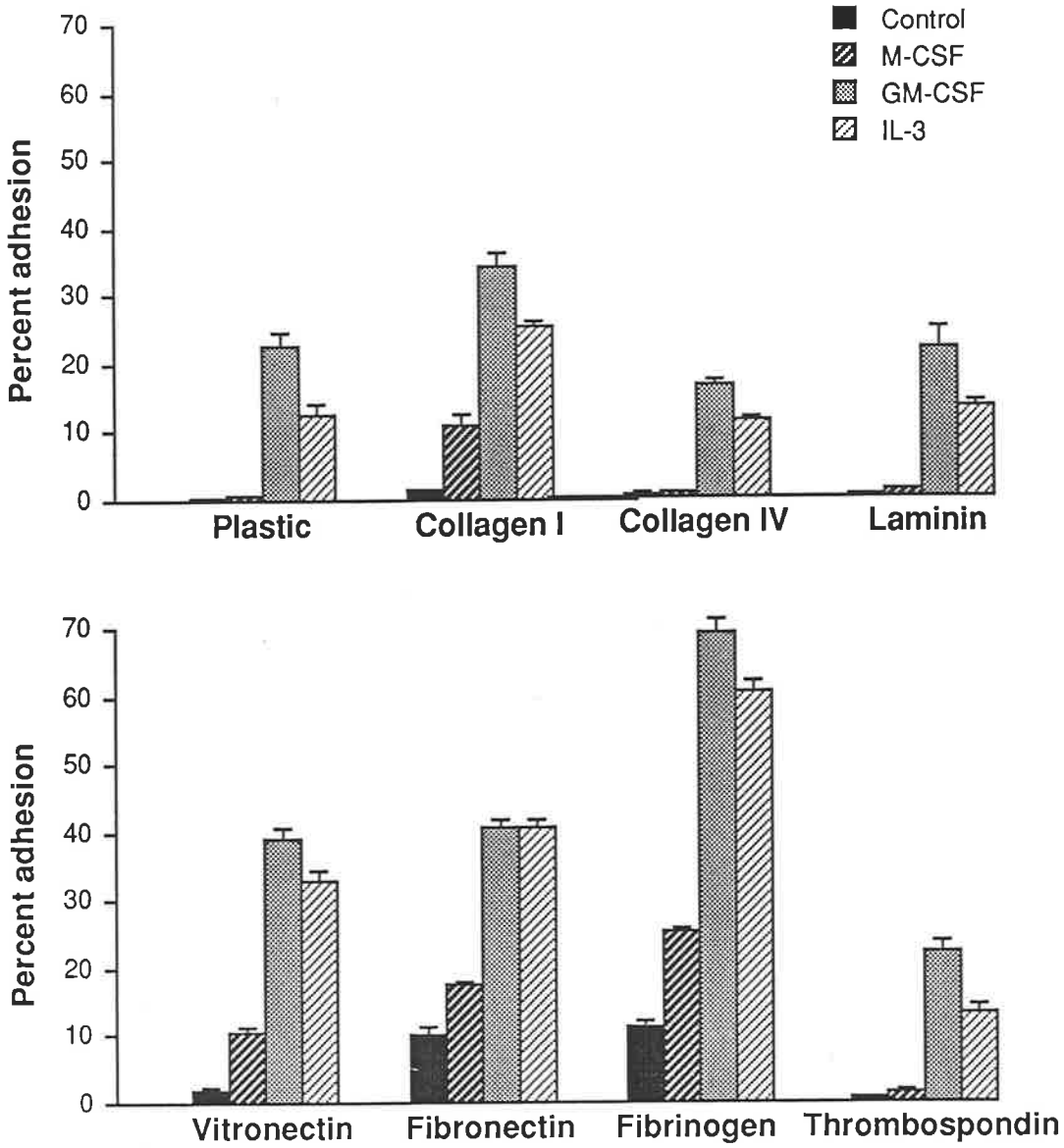
partial inhibition of stimulated monocyte adhesion by other rabbit antisera (data not shown) suggest that the latter effect may be a non-specific or species-dependent phenomenon.

Experiments to determine if the two cytokines might act in an additive or synergistic manner were carried out using IL-3 and GM-CSF at different concentrations. No evidence was found for synergy between the two cytokines and their effects were not additive at optimal concentrations (Fig 4.5).

4.4 Stimulation of monocyte adhesion to EMP

Following adhesion to, and migration through, the endothelium, monocytes *in vivo* must traverse the endothelial basement membrane in order to reach the extravascular space. Experiments were therefore performed to assess the capacity for CSF-stimulated monocyte adhesion to purified components of basement membrane. These include collagen IV, laminin and fibronectin. Adhesion was also measured to serum proteins (fibrinogen, vitronectin), to the platelet product, thrombospondin, and to collagen I, an important structural component of connective tissues. Mitrotitre wells were precoated with the various proteins, and late phase adhesion measured using the co-incubation protocol. Basal monocyte adhesion to many of the EMP was higher than to plastic. An experiment showing relatively low basal adhesion is shown in Fig. 4.6. IL-3 and GM-CSF stimulated increased adhesion to all substrates, although the levels of both basal and stimulated adhesion varied from one substrate to the next. In general, a higher level of basal adhesion was associated with a greater increase in CSF-stimulated adhesion. Of the basement membrane proteins, collagen IV and laminin bound monocytes relatively weakly, while fibronectin showed intermediate capacity to act as on adhesion substrate. The strongest adhesion was seen to fibrinogen. M-CSF was included in these experiments as a control, and showed some

Figure 4.6 Stimulation of monocyte adhesion to EMP. Microtitre wells were precoated with EMP as described in Materials and Methods and adhesion of ^{51}Cr-labeled monocytes measured after 21 hours of co-culture. CSF were used at a final concentration of 600pM. Results are mean ± SEM for 6 replicates per point.


Control
M-CSF
GM-CSF
IL-3
Percent adhesion
70
60
50
40
30
20
10
0
Plastic
Collagen I
Collagen IV
Laminin
Percent adhesion
70
60
50
40
30
20
10
0
Vitronectin
Fibronectin
Fibrinogen
Thrombospondin

activity on collagen I, vitronectin, fibronectin and fibrinogen. However, M-CSF-stimulated adhesion was much lower than that for IL-3 and GM-CSF.

4.5 Inhibition of CSF-stimulated monocyte adhesion by cycloheximide

The rapidity of the early phase of stimulated adhesion suggested a mechanism independent of new protein synthesis. To test this hypothesis, cycloheximide (5 ug/ml) was included in some experiments from the onset of culture (Fig. 4.7A). Cycloheximide is a reversible inhibitor of protein synthesis, which acts by inhibiting ribosomal function and hence chain initiation and elongation, and has previously been shown to have a rapid onset of action (Oleinick et al, 1977). The results show that at 30 minutes of culture, cycloheximide did not inhibit either unstimulated or CSF-stimulated monocyte adhesion. Instead, an increase in adhesion was seen in all groups with cycloheximide, although this was only significant in control-treated monocytes (Fig. 4.7A, and legend) ($p<0.02$). When data from 4 experiments performed at 30 minutes of culture were pooled, a similar pattern of significance was seen, with only control-treated monocytes increasing adhesion significantly in the presence of cycloheximide ($p<0.03$, data not shown). A control experiment designed to determine if cycloheximide was active at inhibiting protein synthesis during this time is shown in Table 4.1. In this experiment, monocytes were suspended in methionine-free medium containing either IL-3, GM-CSF or medium control, and warmed to 37°C. Medium or cycloheximide was then added, and ^{35}S-methionine added approximately 30 seconds later. Incorporation of ^{35}S-methionine into protein was measured after 30 minutes in culture. No stimulation of protein synthesis by either cytokine was observed during this time (Table 4.1), and protein synthesis in all treatment groups was reduced by 85% in the presence of cycloheximide, confirming the activity of the drug.

In contrast to the findings at 30 minutes, inclusion of cycloheximide for 21 hours of culture completely abolished both basal and stimulated monocyte adhesion to plastic (Fig. 4.7B). As this period with cycloheximide was associated with some loss of cell viability, shorter periods of inhibition were examined, with addition of cycloheximide at 5 hours or 30 minutes prior to harvest at 21 hours (Fig. 4.7C, 4.7D). These results show that both unstimulated and stimulated adhesion was virtually abolished when cycloheximide was present for the last 5 hours of culture only. Monocyte viability by Trypan-blue exclusion was >94% for all groups at 21 hours using this protocol. The addition of cycloheximide 30 minutes prior to harvest resulted in stimulation of adhesion in control-treated and in GM-CSF-treated monocytes (Fig. 4.7D) ($p<0.04$, <0.03 respectively). The stimulation of adhesion by cycloheximide in IL-3-treated monocytes at this point (Fig. 4.7D) did not reach significance in the experiment shown ($p<0.06$) but when data were pooled from 4 separate experiments, the increase observed with cycloheximide was significant ($p<0.04$).

4.6 Time course of inhibition of adhesion by cycloheximide

In order to determine the kinetics of inhibition of adhesion by cycloheximide, experiments were performed with addition of the inhibitor at various times during the last 5 hours of 21 hour cultures. Significant inhibition of adhesion in IL-3 and GM-CSF-stimulated cultures was seen with the addition of cycloheximide at t=19 hrs (Fig. 4.8B,C). Maximal inhibition was seen with addition of the inhibitor at t=16 hours, representing a period of co-incubation of 5 hours. Unstimulated monocytes also showed inhibition of adhesion with cycloheximide (Fig. 4.8A), but this was only significant after 4 hours of incubation with the drug.

Figure 4.7 Cycloheximide inhibits late but not early-phase monocyte adhesion. ^{51}Cr-labeled monocytes were aliquotted into wells and pulsed with control medium (open bars) or cycloheximide (final concentration 5ug/ml, hatched bars) at t=0 hours (A,B) t=16 hours (C) and t=20.5 hours (D). 600pM CSF or medium control were present throughout the incubation, and harvest was at 30 minutes (A) or 21 hours (B,C,D) of culture. Cell viability as measured by Trypan-blue exclusion was >98% for all groups at 30 minutes and >94% for groups C & D at 21 hours. Cell viability after 21 hours of co-incubation with cycloheximide (B) was reduced to 62%. Each point represents the mean ± SEM of 6 replicates in a representative experiment of 4 performed. Cycloheximide stimulated significant increases in adhesion at 30 minutes (A) in control-groups ($p<0.02$) but not in IL-3-($p<0.15$) or GM-CSF-groups ($p<0.45$). At 21 hours, in the presence of cycloheximide for 30 minutes only (D), increased adhesion was seen with cycloheximide in control- ($p<0.04$) and GM-CSF-groups ($p<0.03$) but did not reach significance in IL-3 groups ($p<0.06$).

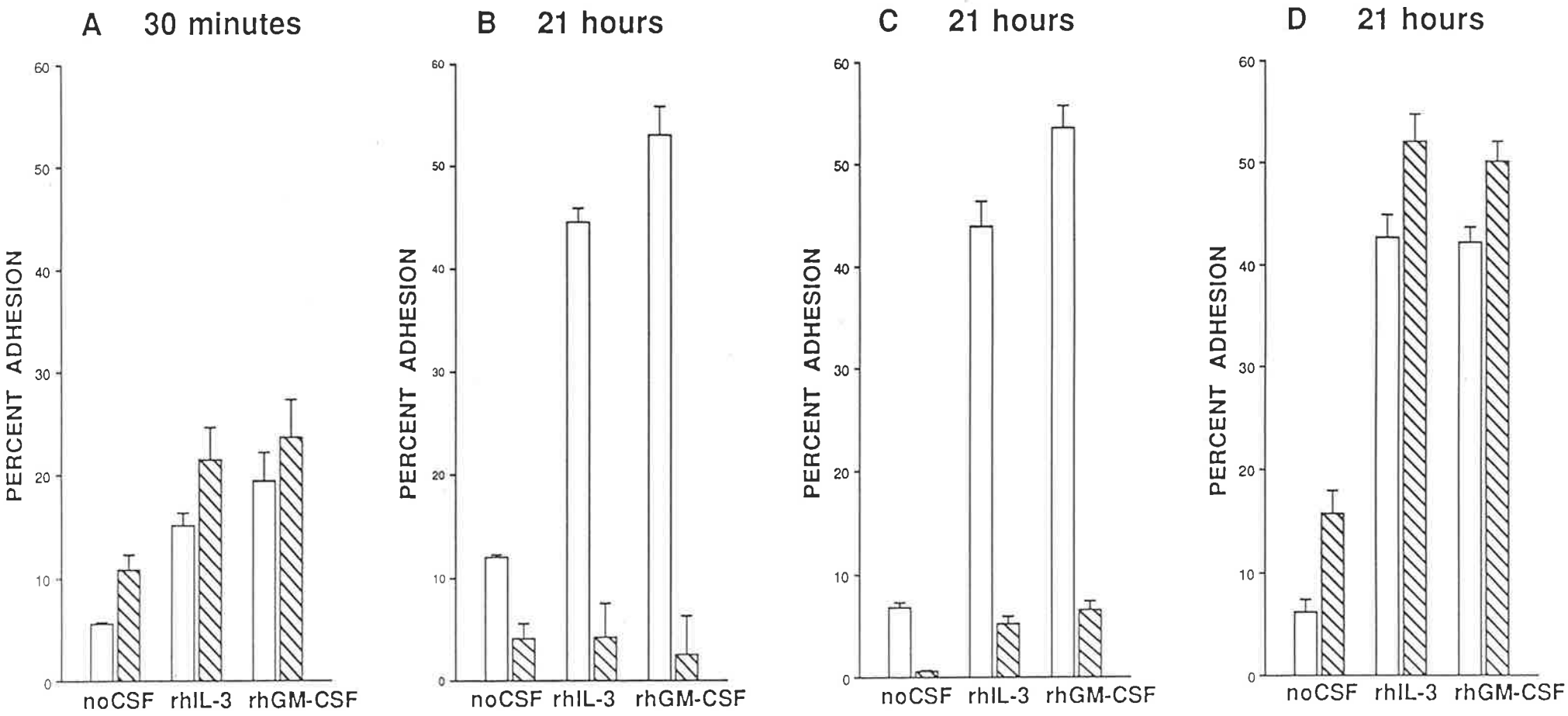
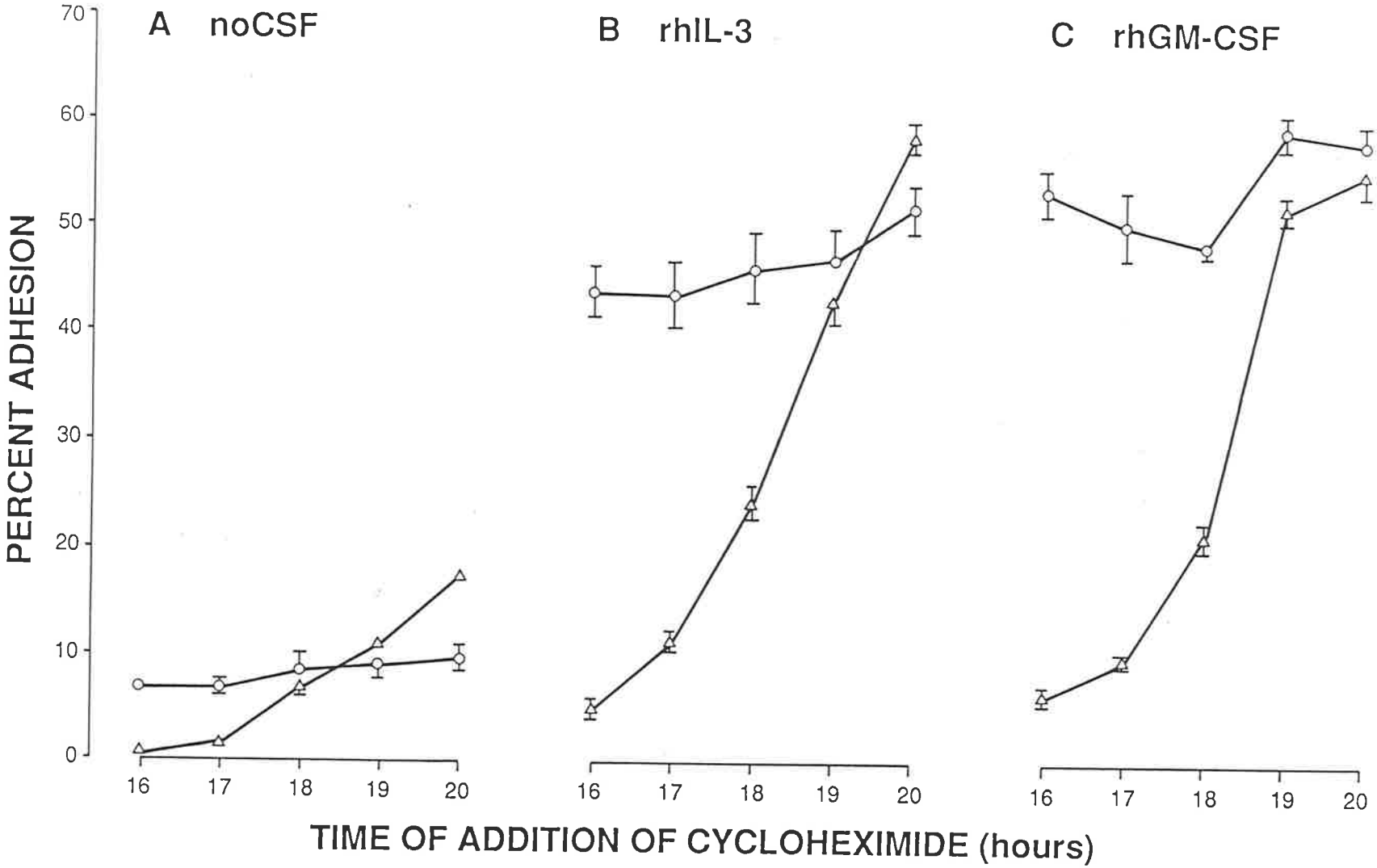

A 30 minutes
B 21 hours
C 21 hours
D 21 hours
PERCENT ADHESION
0
10
20
30
40
50
60
noCSF
rhIL-3
rhGM-CSF

Table 4.1 ^{35}S-methionine incorporation in short-term monocyte culture*

	^{35}S-methionine incorporation (cpm)	
Stimulus	Control	+ Cycloheximide
Control	22,621±477	3,40±71
IL-3	23,543±614	3,223±77
GM-CSF	22,624±363	3,163±157

*Monocytes were prepared by elutriation and suspended at a concentration of 4x10^6/ml in methionine-free RPMI 1640 with 1% FCS. The cells were aliquotted into 2ml polypropylene tubes, warmed to 37°C and control medium or CSF were added to give a final concentration of 600pM. Control medium or cycloheximide was then added (final concentration 5ug/ml) and 30 seconds later each tube was pulsed with 10uCi ^{35}S-methionine. The monocytes were incubated for 30 minutes at 37°C with gentle agitation, followed by trichloroacetic acid-precipitation of incorporated ^{35}S-methionine onto paper filters, as described in Materials and Methods. Each value represents the mean ± SEM of three replicates.

Figure 4.8 Time course of inhibition of adhesion by cycloheximide. ^{51}Cr-labeled monocytes were incubated with or without CSF (600pM) for 21 hours, with addition of 5ug/ml cycloheximide (△) or control medium (o) at t=16,17,18,19 or 20 hours, as shown. Harvest was at 21 hours for all groups. Results are mean ± SEM for 4 replicates per point.


A noCSF
B rhIL-3
C rhGM-CSF
PERCENT ADHESION
0
10
20
30
40
50
60
70
16
17
18
19
20
TIME OF ADDITION OF CYCLOHEXIMIDE (hours)

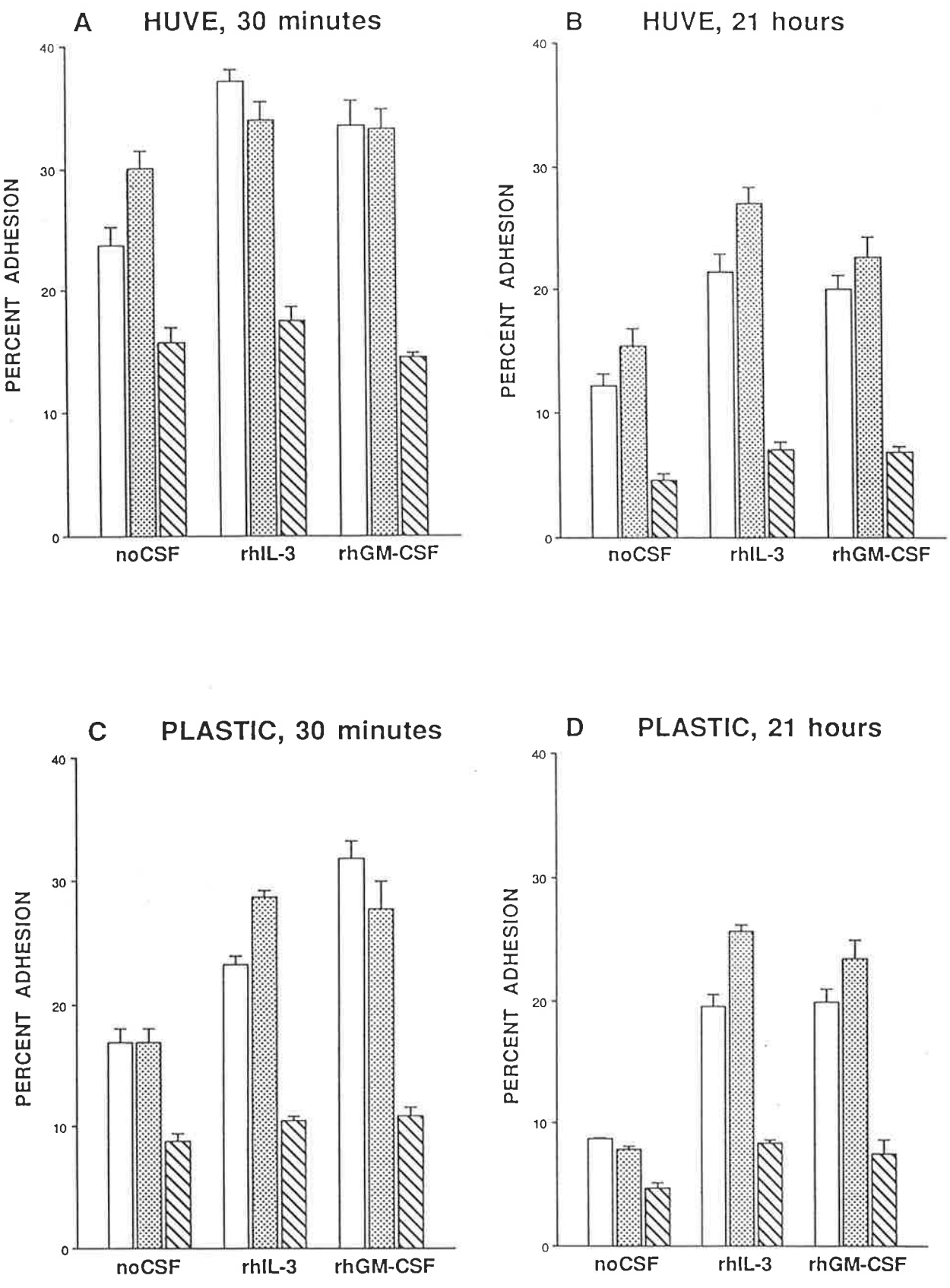
4.7 CSF-stimulated monocyte adhesion is dependent upon CD18

The adhesion of unstimulated monocytes to HUVEC and to plastic has been shown previously to be at least partially dependent on the LFA family of adhesion molecules (Carlos et al, 1989; Keizer et al, 1987; Arnaout et al, 1988). To determine the role of this family in the two phases of IL-3 and GM-CSF-stimulated monocyte adhesion, MAb 60.3 (anti-CD18) and MAb 60.1 (anti-CD11b) were included in adhesion assays both at 30 minutes of culture (Fig. 4.9A,C) and after 21 hours in suspension culture (Fig. 4.9B,D). Both antibodies were used at 1/300 final dilution, a concentration which has been shown to be saturating in immunofluorescence experiments with monocytes as measured by flow cytometry. IL-3 and GM-CSF enhanced monocyte adhesion to both substrates and at both times compared with controls ($p<0.007$, $p<0.02$ respectively). MAb 60.1 did not significantly inhibit the levels of adhesion seen (Fig. 4.9), but in control experiments it inhibited the adhesion of neutrophils to tumour necrosis factor-stimulated HUVEC by approximately 80%, confirming the activity of the antibody. In contrast to the findings with 60.1, MAb 60.3 inhibited background monocyte adhesion to a variable but incomplete degree in all combinations of time and substrate, and reduced the CSF-stimulated adhesion to a similar level. Non-LFA-directed antibodies of IgG 2a (Hy 1.2) and IgG 1 (Hy 5.19) isotypes used at similar concentrations did not inhibit monocyte adhesion (data not shown). These results suggest that while basal monocyte adhesion is only partially dependent on CD18, IL-3 and GM-CSF-stimulated adhesion is dependent almost entirely on this molecule.

4.8 Dependence of stimulated monocyte adhesion on ICAM-1

The dependence of stimulated monocyte adhesion on CD18, but not on CD11b (the α chain of MAC-1) suggested a role for the adhesion molecules LFA-1 or p150/95. Lack of

Figure 4.9 Effect of monoclonal antibodies to CD11b (60.1, stippled bars) and to CD 18 (60.3, hatched bars) versus medium control (open bars) on monocyte adhesion to HUVEC (A,B) and to plastic (C,D). For experiments harvested at 30 minutes (A,C), ^{51}Cr-labeled monocytes were suspended in warm medium and aliquotted into microtitre wells containing prewarmed medium, IL-3 or GM-CSF (final concentration 600 pM) plus antibody or control at a final dilution of 1/300. Adhesion was measured as described in the text. For experiments harvested at 21 hours (B,D), ^{51}Cr-labeled monocytes were cultured in suspension with CSF (600pM) or control medium for 21 hours, washed, resuspended in warm medium and aliquotted into microtitre wells containing antisera or medium control. Adhesion was measured after a further 30 minutes. Isotype-specific control antisera for 60.1 (IgG1, subclass) and 60.3 (IgG2a, subclass) did not inhibit monocyte adhesion. Each point represents the mean ± SEM of at least 4 replicates in a representative experiment of 3 performed. Significantly higher adhesion was seen in all experiments with IL-3 ($p<0.007$) and with GM-CSF ($p<0.02$) in the absence of antisera. Levels of adhesion in the presence of MAb 60.3 were similar for the three treatment groups within each substrate/time combination. The only significant differences seen in 60.3-inhibited adhesion levels were between IL-3-treated and untreated monocytes at 21 hours (B,D; $p<0.05$).


A HUVE, 30 minutes
B HUVE, 21 hours
C PLASTIC, 30 minutes
D PLASTIC, 21 hours
PERCENT ADHESION
0
10
20
30
40
noCSF
rhIL-3
rhGM-CSF

sufficient quantities of MAb to these molecules, however, prevented the performance of direct inhibition experiments. In order to at least partially circumvent this problem, experiments were performed using a MAb prepared against ICAM-1, a major ligand for LFA-1. Fig. 4.10 shows inhibition by this MAb (IH-4) of GM-CSF-stimulated monocyte adhesion to plastic at 21 hours. Both whole antibody and Fab fragments induced a maximal inhibition of adhesion of approximately 40%, but with differing dose-response profiles.

The role of ICAM-1 in monocyte adhesion was further investigated using an ICAM-1-transfected murine L-cell line. This cell line has been previously shown to stably express high levels of ICAM-1 (Wawryk et al, in press). Early phase adhesion to the transfected L cells and to L cell controls grown to confluence in microtitre wells is shown in Fig. 4.11. Unstimulated monocyte adhesion to the transfectants was significantly higher than to L cells (Fig. 4.11A) and was reduced down to L cell-levels by IH-4 Fab fragments at 30ug/ml. Neither GM-CSF nor IL-3 stimulated adhesion to L cells or to the transfectants using these methods (Fig. 4.11B,C), perhaps reflecting the very strong adhesion seen even in the absence of CSF (Fig. 4.11A).

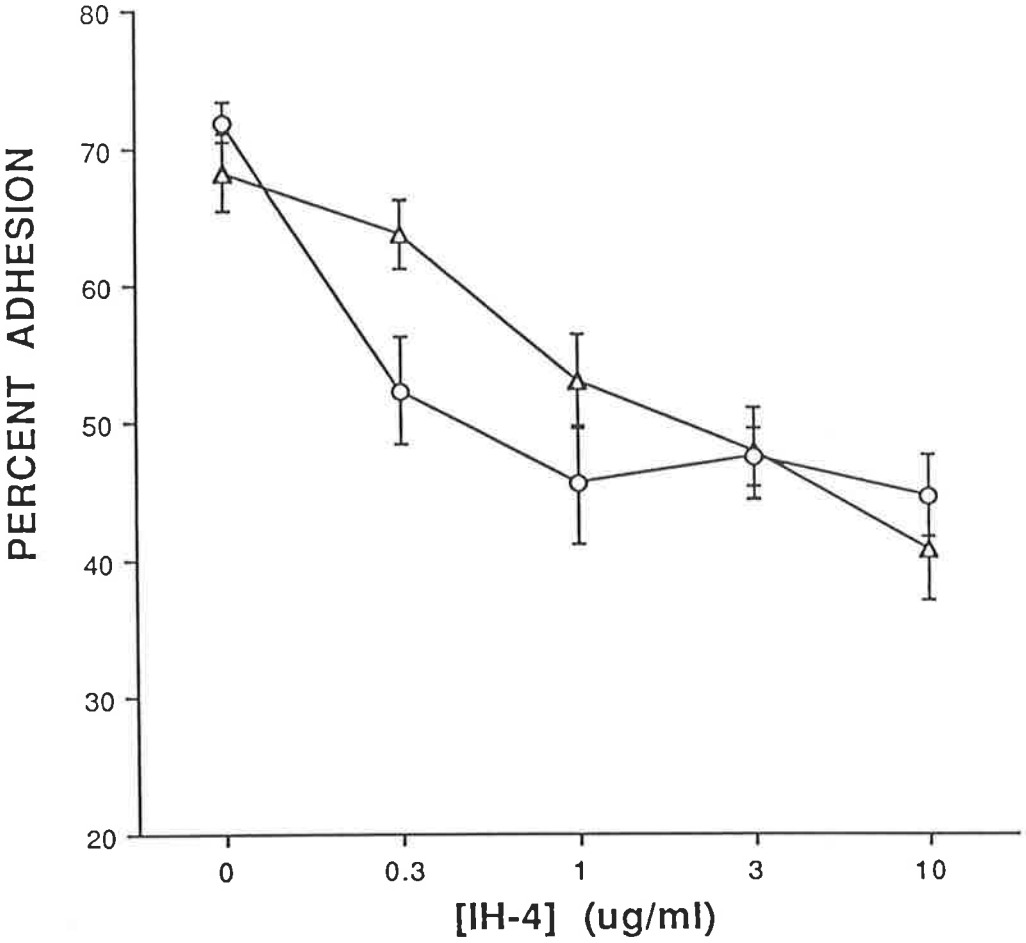
4.9 Expression of LFA-family surface markers

The preceding sections support the contention that LFA members LFA-1 or p150/95 are involved in the adhesion of monocytes stimulated with IL-3 or GM-CSF. Previous studies have shown that GM-CSF stimulates MAC-1 upregulation on human neutrophils *in vivo*, while having no effect on other LFA complexes (Socinski et al, 1988). Geissler et al (1989) recently reported a similar change in human monocyte MAC-1 expression with GM-CSF, and the immunofluorescence data in Chapter 3 of this thesis show regulation of all LFA complexes by both IL-3 and GM-CSF at 5 days of culture. In order to determine if shorter periods of stimulation might also induce upregulation of these antigens,

immunofluorescence studies using a fluorescence-activated cell sorter (FACS) were performed on monocytes stimulated for 30 minutes (early phase) or for 21 hours (late phase) in a suspension culture system. Centrifugations and other cell manipulations were reduced to a minimum, as described in Materials and Methods, in order to reduce the effects of physical handling on surface marker expression (Miller et al, 1987). Adhesion experiments were performed in parallel with the FACS studies, using cells which had been through identical stages of preparation and handling. No significant changes were seen in surface expression of LFA-family markers in three experiments performed at 30 minutes of stimulation (Table 4.2). While the stimulation of adhesion seen in these experiments was variable (Table 4.2, legend), upregulation of CD18 or other markers was not seen, even in experiments with the highest CSF-induced adhesion increases (Exp. 1, 3, GM-CSF). In contrast, the findings at 21 hours were of significant increases in surface marker expression with both IL-3 and GM-CSF, compared with controls (Table 4.3). Expression of MAC-1, as detected by MAb 60.1 and Mo-1, was increased approximately 2-fold by both CSF, and expression of CD-18, as detected by MAb 60.3, increased to a similar degree. CD18, as detected by MAb TS1/18, and LFA-1 (TS1/22) and p150/95 (150/95) showed lesser, but still significant increases, with similar results for the two cytokines. Large increases in adhesion was seen in parallel for both experiments (Table 4.3, legend).

Figure 4.10 Inhibition of monocyte adhesion to plastic by MAb IH-4. Monocytes were incubated for 21 hours in microtitre wells with 600pM GM-CSF, together with different concentrations of MAb IH-4 (o) or of IH-4 Fab fragments (△). Adhesion at 21 hours in the absence of GM-CSF was 2.5±0.5%. IL-3-stimulated monocytes showed a similar pattern to GM-CSF-stimulated cells. Results are mean ± SEM of 4 replicates per point.

Figure 4.11 Inhibition of monocyte adhesion to ICAM-1 transfectants by MAb IH-4. Monolayers of L cells or ICAM-1 transfected L cells (ICAMs) were formed in microtitre wells, and washed twice before use. ^{51}Cr-labeled monocytes were then incubated in the wells with no CSF (A), or 600pM GM-CSF (B) or IL-3 (C), and adhesion measured at 30 minutes of culture. MAb IH-4 Fab fragments at a final concentration of 30ug/ml were included in some wells in two of the three experiments. Results are mean ± SEM of 4 replicates per point.


PERCENT ADHESION
80
70
60
50
40
30
20
0
0.3
1
3
10
[IH-4] (ug/ml)

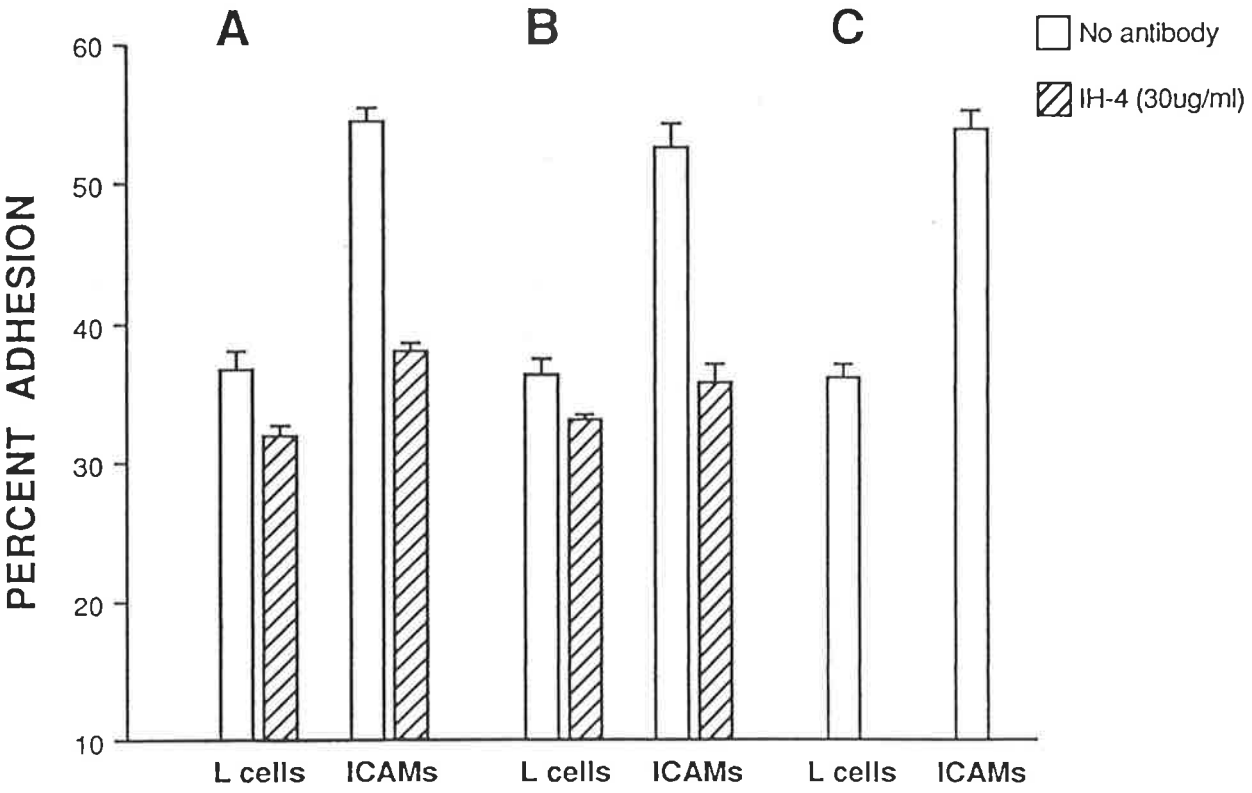

A
B
C
No antibody
IH-4 (30ug/ml)
PERCENT ADHESION
60
50
40
30
20
10
L cells
ICAMs
L cells
ICAMs
L cells
ICAMs

Table 4.2 LFA surface expression on unstimulated and 30 minute-stimulated monocytes. Monocytes were purified by elutriation, resuspended in RPMI with 10% FCS and incubated at 37° for 30 minutes with CSF (600pM) or control. Cell suspensions were then cooled to 4°C, aliquotted into FACS tubes, and stained according to the protocol in Materials and Methods. The specificity of the MAb was: 60.1, MO-1:α chain MAC-1 (CD11b); TS1/22:α chain, LFA-1 (CD11b); 150/95:α chain, p150/95 (CD11c); 60.3, TS1/18:β chain, LFA family (CD18). Parallel adhesion experiments on the same donor cells yielded the following percent adhesion at 30 minutes: Exp. 1: no CSF, 10.7±0.7; IL-3, 13.3±0.7; GM-CSF, 20.1±1.6. Exp. 2: 9.2±0.5; 11.6±0.5; 10.2±0.5. Exp. 3: 5.4±0.4; 7.1±0.6; 11.6±0.2.

Table 4.2 LFA surface expression on unstimulated and 30 minute-stimulated monocytes

MAb (Antigen)	Stimulus	Experiment 1	2	3	Fluorescence Shift** (Mean±SEM)
60.1 (MAC-1)	no CSF	155*	141	124	
	IL-3	148(-7)**	148 (+7)	123 (-1)	0±3
	GM-CSF	151(-4)	146 (+5)	125 (+1)	+1±2
MO-1 (MAC-1)	no CSF	97	112	90	
	IL-3	90 (-7)	114 (+2)	88 (-2)	-2±2
	GM-CSF	104 (+7)	106 (-6)	94 (+4)	+2±3
TS1/22 (LFA-1)	no CSF	162	134	114	
	IL-3	161 (-1)	133 (-1)	112 (-2)	-1±0
	GM-CSF	159 (-3)	128 (-6)	116 (+2)	-2±2
150/95 (p150/95)	no CSF	37	49	33	
	IL-3	36 (-1)	49 (0)	26 (-7)	-3±2
	GM-CSF	36 (-1)	46 (-3)	28 (-5)	-3±1
60.3 (β chain)	no CSF	135	171	155	
	IL-3	131 (-4)	181 (+10)	158 (+3)	+3±3
	GM-CSF	129 (-6)	183 (+12)	155 (0)	+2±4
TS1/18 (β chain)	no CSF	126	164	143	
	IL-3	125 (-1)	161 (-3)	139 (-4)	-3±1
	GM-CSF	124 (-2)	159 (-5)	145 (+2)	-2±2

* mean fluorescence, after subtraction of background fluorescence, measured on a logarithmic scale.

** shift in mean fluorescence relative to no CSF group.

Table 4.3 LFA surface expression on unstimulated and 21 hour-stimulated monocytes. Monocytes were purified by elutriation, resuspended in RPMI with 10% FCS, and incubated at 37°C in a suspension culture system for 21 hours with CSF (600pM) or control. Cells were then centrifuged at 100g, resuspended in medium at 4°C, and staining with MAb performed as outlined in Materials and Methods. Specificities for the MAb are outlined in Table 2.1 and Table 4.2. Parallel adhesion experiments on the same donor cells after overnight suspension culture yielded the following percent adhesion at 30 minutes. Exp. 1: no CSF, 19.6±0.8; IL-3, 49.1±2.7; GM-CSF, 46.6±3.6. Exp. 2.: 21.2±0.8; 36.2±1.5; 34.1±3.8.

Table 4.3 LFA surface expression on unstimulated and 21 hour-stimulated monocytes

MAb (Antigen)	Stimulus	Experiment 1	Experiment 2	Fluorescence Shift ** (Mean ± SEM)
60.1 (MAC-1)	no CSF	83*	74	
	IL-3	103 (+20)**	89 (+15)	+18±2
	GM-CSF	106 (+23)	99 (+25)	+24±1
MO-1 (MAC-1)	no CSF	53	39	
	IL-3	77 (+24)	54 (+15)	+20±3
	GM-CSF	76 (+23)	69 (+30)	+27±2
TS1/22 (LFA-1)	no CSF	112	79	
	IL-3	129 (+17)	84 (+5)	+11±4
	GM-CSF	129 (+17)	84 (+5)	+11±4
150/95 (p150/95)	no CSF	39	10	
	IL-3	56 (+17)	15 (+5)	+11±4
	GM-CSF	56 (+17)	23 (+13)	+15±1
60.3 (β chain)	no CSF	127	117	
	IL-3	148 (+21)	127 (+10)	+16±4
	GM-CSF	146 (+19)	139 (+22)	+21±1
TS1/18 (β chain)	no CSF	102	99	
	IL-3	116 (+14)	111 (+12)	+13±1
	GM-CSF	117 (+15)	112 (+13)	+14±1

* mean fluorescence, after subtraction of background fluorescence, measured on a logarithmic scale.

** shift in mean fluorescence relative to no CSF group.

DISCUSSION

The central finding in this chapter is that IL-3 and GM-CSF regulate human monocyte adhesion. Stimulated adhesion was seen to both endothelium and to purified basement membrane and other proteins, suggesting a role for CSF-regulated adhesion *in vivo*. The kinetics of this regulation were complex (Fig. 4.1, 4.2) with an early phase of stimulated adhesion seen between 10 minutes and 1 hour of culture, and a late phase after 9 hours of stimulation. While levels of background or unstimulated adhesion varied with different monocyte donors, the stimulation seen was reproducible and highly significant, and was observed at concentrations of cytokine as low as 6pM (Fig. 4.2, 4.4). Further, the experiments performed with antisera to IL-3 and GM-CSF (Fig. 4.4) confirm that the stimulated adhesion seen was due to these cytokines and not to an unknown contaminant.

The rapid onset of the early phase of stimulated adhesion suggested a mechanism independent of new protein synthesis, and the failure of cycloheximide to inhibit adhesion at 30 minutes supported this hypothesis (Fig. 4.7A). Cycloheximide has previously been shown to inhibit protein synthesis in Chinese hamster ovary cells after 30 seconds (Oleinick et al, 1977), and a similarly rapid action in human monocytes is shown here (Table 4.1). These findings show that early phase adhesion, both background and stimulated, does not result from the synthesis of new adhesion molecules, and suggests instead that a change in existing structures may be responsible.

The late phase of stimulated adhesion (Fig. 4.1, 4.3, 4.6) was seen on a background of declining adhesion in unstimulated wells (Fig. 4.1) and after a decrease in overall adhesion between 2-9 hours. Although both growth factors support monocyte survival in longer term culture (greater than 4 days, Fig. 3.1), there were no differences in monocyte numbers or viability at 21 hours between CSF-treated and untreated groups. In addition, unstimulated

cells showing low adhesion at 21 hours could be stimulated with IL-3 or GM-CSF to increase their adhesiveness within 30 minutes (data not shown). These findings demonstrate that IL-3 and GM-CSF stimulate genuine increases in adhesion during the late phase and are not simply maintaining cell viability or the high levels of adhesion seen in all groups at two hours.

The ability of cycloheximide to inhibit adhesion at 21 hours, even when present for the last 5 hours of stimulation only (Fig. 4.7C), further distinguishes the two phases, and suggests that synthesis of adhesion molecules or related structures is necessary to maintain a pro-adhesive state. The failure of cycloheximide to inhibit late phase adhesion when present for the last 30 minutes of culture only (Fig. 4.7D), is not surprising, and may simply reflect a low rate of turnover of adhesion structures which had been synthesized during the previous 20 hours of culture with CSF. An estimate of the rate of turnover of these structures is given by the time course of inhibition of adhesion (Fig. 4.8), where major inhibition is seen between 2 and 4 hours of co-incubation with cycloheximide.

The enhancement of both unstimulated and CSF-stimulated adhesion after the addition of cycloheximide at t=20.5 hours (Fig. 4.7D) was also seen in the presence of cycloheximide during early phase adhesion, although it only reached significance in control-groups (Fig. 4.7A and legend). While the reasons for this enhancement remain unclear, the phenomenon may represent inhibition of synthesis by cycloheximide of an endogenous adhesion inhibitor of short half-life.

Previous reports regarding the structures involved in unstimulated monocyte adhesion to endothelium and plastic have implicated, at least in part, the LFA family of molecules. These studies demonstrated only minor inhibition of monocyte-endothelial adhesion by antibodies to CD11b (alpha chain, MAC-1) (Keizer et al, 1987, Mentzer et al, 1987; Arnaout

et al, 1988), but found significant albeit incomplete inhibition of adhesion by antibodies to CD18 (beta chain, LFA family). This ranged from 35 per cent (Keizer et al, 1987) to 56, 57 and 62 per cent inhibition (Carlos et al, 1989; Arnaout et al, 1988; Mentzer et al, 1987, respectively). Our findings with unstimulated fresh monocytes (Fig. 4.9A,C) are consistent with these reports, showing partial inhibition with MAb 60.3 (anti-CD18), but no inhibition with MAb 60.1 (anti-CD11b). In addition, however, we demonstrate two novel findings. Firstly, unstimulated adhesion to HUVEC and to plastic at 21 hours is also reduced by about 50 per cent by 60.3 but not by 60.1 (Fig. 4.9B,D). Secondly, IL-3 and GM-CSF-stimulated adhesion at both time points and on both endothelium and on plastic is mainly dependent on CD18 (Fig. 4.9). These findings are consistent with a previous report of autologous plasma-and PMA-stimulated monocyte adhesion to endothelium, in which 60.3 abolished the response (Wallis et al, 1985), and suggest that CD18 is of fundamental importance in regulated monocyte adhesion.

The failure of MAb 60.1 to inhibit either unstimulated or CSF-stimulated monocyte adhesion, despite its activity in inhibiting neutrophil adhesion, suggested that monocyte adhesion was not primarily dependent on MAC-1. Experiments were therefore conducted to investigate the role of a second LFA-family member, LFA-1, in this phenomenon. MAb directed to ICAM-1 (IH-4), the major ligand for LFA-1 (Staunton et al, 1989), inhibited CSF-stimulated monocyte adhesion to plastic by approximately 40% (Fig. 4.10), and similar degrees of inhibition were seen with whole MAb and with Fab fragments. In addition, the capacity for monocytes to adhere in an ICAM-1-dependent fashion was demonstrated by significantly higher adhesion to an ICAM-1 transfected cell line compared with controls (Fig. 4.11). However, the adhesion of unstimulated monocytes to these transfectants was very strong (Fig. 4.11), and no further adhesion was seen in the presence of CSF. While incomplete, these experiments point to a possible role for ICAM-1, and hence LFA-1, in CSF-stimulated monocyte adhesion.

The dependence of CSF-stimulated monocyte adhesion on CD18 may arise from one or more of a number of mechanisms. Increased surface expression of LFA members LFA-1 or p150/95 could explain the findings reported here, and could result either from the synthesis of new molecules during late phase adhesion, or from the redistribution of molecules to the cell surface at either time. Such rapid redistribution events have been previously demonstrated for MAC-1 and p150/95 in human monocytes (Miller et al, 1987). The results of the immunofluorescence experiments performed with late-phase monocytes (Table 4.3) are consistent with these postulates. CD18 expression was increased two-fold with CSF stimulation, and both LFA-1 and p150/95 also showed significant upregulation. The increased expression of MAC-1 seen in the same experiments is of uncertain significance, in the light of the functional inhibition data (Fig. 4.9), which show no role for MAC-1 in either basal or stimulated adhesion. The CSF-induced upregulation of MAC-1 on monocytes may therefore have a functional correlate in increased binding of C3bi, or increased phagocytosis. These hypotheses have not yet been tested.

In contrast to the findings at 21 hours, no change in expression of surface markers was seen in monocytes stimulated for only 30 minutes with CSF (Table 4.2), suggesting that upregulation of adhesion molecules is not responsible for stimulated adhesion at this time.

The significance of increases in the surface expression of adhesion molecules in general has recently been questioned. Inhibition of stimulated MAC-1 upregulation in neutrophils by an anion-blocking agent did not prevent stimulated neutrophil adhesion to endothelium, despite such adhesion being MAC-1 dependent (Vedder et al, 1988). A second study by Buyon et al (1988) showed similar findings, in that MAC-1-dependent homotypic adhesion in neutrophils stimulated with F-met-leu-phe was not dependent on the coincident increase in MAC-1 expression seen. In addition, recent reports demonstrate that the monocyte MAC-1 complex may be stimulated to undergo conformational change leading to the novel binding

of fibrinogen and factor X (Altieri et al, 1988A,B). These studies provide a model for functional change in monocyte adhesion structures, and may be of particular relevance to the rapid early phase of CSF-stimulated adhesion.

A conceptual problem arises in the interpretation of experiments performed on plastic. In particular, inhibition of monocyte adhesion to plastic by MAb to CD18 implies the presence of a ligand for LFA-1 or p150/95 on the plastic surface. While unproven, two potential sources for such ligands are present within the assay system. Firstly, the experiments were performed in the presence of 10% FCS, which contains a large number of proteins including fibrinogen, fibronectin, chondronectin (Yamada, 1983) and vitronectin (Parker et al, 1989), and possibly others not as yet described. The coating of the plastic surface by such proteins might therefore provide attachment points for the relevant adhesion receptor. Secondly, monocytes synthesize and secrete a huge range of products including fibronectin (Nathan, 1987) and thrombospondin (Jaffe et al, 1985), and contain intracytoplasmic ICAM-1, which is capable of redistribution to the membrane upon adhesion to fibronectin-coated surfaces (Dougherty et al, 1988). The deposition of such synthesized proteins by monocytes on plastic surfaces remains a possible, if unproven, mechanism for subsequent receptor-ligand-mediated adhesion.

The experiments involving adhesion to EMP also require comment. Adhesion of unstimulated monocytes to the basement membrane proteins laminin and collagen IV was negligible at 21 hours, but significant adhesion was seen to fibronectin (Fig. 4.6). The pattern of stronger adhesion to fibronectin was also seen with CSF-stimulated cells, and has been previously noted with murine PEM adhesion at 24 hours of culture (Shaw et al, 1989). These findings are of interest in view of the demonstration of increased levels of endothelial-derived fibronectin in the vessel walls at the site of tissue injury (Clark et al, 1982), providing a mechanism for the selective accumulation of CSF-stimulated monocytes

at the wound site. IL-3 and GM-CSF also stimulated adhesion to the serum and connective tissue proteins tested (Fig. 4.6), and the biological significance of these findings is not clear. However, local production of CSF at the site of inflammation (Williamson et al, 1988; Alvaro-Gracia et al, 1989) may stimulate monocyte adhesion to neighbouring connective tissue proteins, leading to an accumulation of cells by preventing onward migration. A similar 'migration inhibition' activity has been previously described for GM-CSF and human neutrophils *in vitro* (Weisbart et al, 1985; Atkinson et al, 1988).

In most respects, IL-3 and GM-CSF performed in these experiments in a similar fashion, with an identical temporal profile and dose-response relationship during the late phase, and a similar dependence on CD18 for manifestation of the response. In addition, the failure of the two cytokines to synergise, or to act in an additive fashion at optimal concentrations (Fig. 4.5) suggests that IL-3 and GM-CSF stimulate the same subpopulation of monocytes, and that they may act through a common mechanism of cell activation. Interestingly, however, in 4 of 4 time-courses performed during the early phase, IL-3 was significantly slower at stimulating enhanced adhesion than was GM-CSF (Fig. 4.2), although subsequently reaching comparable levels of efficacy. While several reports have demonstrated that these 2 growth factors have similar or identical effects on human monocytes (Cannistra et al, 1988A; Vellenga et al, 1988; Oster et al, 1989; Koyanagi et al, 1988; Ernst et al, 1989; and Wang et al, 1989), each of these reports was based on relatively prolonged periods of stimulation (greater than 2 hours). This report is therefore the first to describe a rapid effect of either cytokine on monocyte function and the temporal profiles revealed suggest that there may be differences between the two factors in the kinetics of their interaction with their receptors. This question will be addressed further in Chapter 7.

The findings reported here may be of significance *in vivo*. The occurrence of marked tissue accumulations of monocytes and macrophages in mice transgenic for GM-CSF (Lang et al,

1987), despite normal bone marrow cellularity and histology, raises the possibility that the pathology results from abnormal CSF-induced monocyte-endothelial adhesion and subsequent diapedesis. In inflammatory disorders in man, such as rheumatoid arthritis (RA), monocytes can be observed in close apposition to the vessel wall (Kurosaka et al, 1983), and large increases in transendothelial migration and intrasynovial accumulation of monocytes are observed. Local production of haemopoietic growth factors (Williamson et al, 1988; Alvaro-Gracia et al, 1989) may play a role in the regulation of such disordered monocyte trafficking. As already discussed, the demonstration of CSF-induced monocyte adhesion to EMP may be of relevance to the accumulation of monocytes at the inflammatory site and at sites of wound healing. CSF-induced monocyte adhesion to collagen I, a major structural component of bone, tendons and ligaments, may contribute to the joint destruction seen in severe erosive arthropathies such as RA. Abnormal adhesion of monocytes to endothelium in the early phases of development of atheroma (Endemann et al, 1987 and Joris et al, 1983), and the subsequent migration of fat-laden monocytes into the intima to become foam cells (Endemann et al, 1987 and Joris et al, 1983) may also be manifestations of IL-3 or GM-CSF-induced hyperadhesiveness. Finally, the transient and very rapid monocytopenia seen after intravenous injection of GM-CSF (Devereux et al, 1989) and the occurrence of local phlebitis in such patients (Groopman et al, 1987) are likely to be in vivo manifestations of the early phase of adhesion reported here.

SUMMARY

IL-3 and GM-CSF have been revealed as potent stimulators of human monocyte adhesion. Stimulated adhesion was seen to HUVEC, basement membrane and other extracellular matrix proteins, and to plastic. The pattern of stimulated adhesion was complex, with a rapid 'early phase' seen within 10 minutes, followed by a 'late phase' after 9 hours of culture. Early phase adhesion was independent of new protein synthesis, inhibited by MAb

to the LFA antigen CD18, and unassociated with changes in the number of LFA molecules expressed on the cell surface. These findings suggest that a redistribution or functional change in existing LFA molecules may be responsible for stimulated adhesion at this time point. Late phase adhesion was dependent on continuing protein synthesis, was also LFA dependent, and was associated with increased surface expression of all LFA members. The temporal and dose-response profiles for IL-3- and GM-CSF-stimulated monocyte adhesion were identical during late phase adhesion, but GM-CSF stimulated more rapid adhesion during the early phase. The finding raises the question of differences between the two factors in their mode of cell signalling.

CHAPTER 5

INHIBITION OF MONOCYTE ADHESION BY IL-4

INTRODUCTION

The data presented in previous chapters argues for an important role for IL-3 and GM-CSF in inflammatory disease, both by controlling monocyte numbers at the inflammatory site, and by stimulating monocyte function. The pro-adhesive effects of IL-3 and GM-CSF on monocytes are of particular importance, because few other physiological agents are known to show such function. If little is known of the stimulation of monocyte adhesion by cytokines, however, even less is understood of factors which inhibit this process. The identification of a factor causing inhibition of monocyte adhesion could be of significance not only in the understanding of monocyte biology, but also as a therapeutic agent in inflammatory or vascular diseases. A number of the activities of interleukin 4 (IL-4) suggested that this cytokine might act as an adhesion inhibitor, and these are outlined below.

IL-4 is a T-cell derived glycoprotein of 129 amino acids with a molecular weight of about 20kD (Yokota et al, 1986). Although human and murine IL-4 show extensive homology, there is no biological cross-reactivity between species, and homology is otherwise limited to small regions of similarity between human IL-4 and murine IL-3 (Yokota et al, 1986). High affinity receptors for IL-4 have been detected on murine spleen cells, and on B cells, T cells, monocyte/macrophages, mast and other myeloid cells in the mouse (Paul et al, 1987). High affinity receptors for IL-4 have also been described on human mononuclear cells and gingival fibroblasts, as well as on a number of human B, T and other cell lines (Park et al, 1987).

IL-4 was first described as a costimulant for proliferation in murine B cells incubated with anti-immunoglobulin antibodies, and was referred to as B-cell stimulatory factor-1 (BSF-1) (Howard et al, 1982). Subsequent reports showed that IL-4 is a multifunctional cytokine, with diverse activities amongst cells of lymphoid and myeloid origin (reviewed by Paul et

al, 1987). These include stimulation of MHC class II molecules on resting B cells, enhancement of IgG_1 and IgE secretion from B cells stimulated with LPS, with a concomitant decrease in secretion of IgM and IgG_2, and stimulation of the growth of normal T cells and T cell tumour lines. There are conflicting reports on the role of IL-4 in the generation of antigen-independent lymphokine activated killer (LAK) cells. Mule et al (1987) have reported enhanced IL-4-induced LAK cell formation in the mouse, while in human cells, no stimulation of LAK cell formation was seen (Widmer et al, 1987). IL-4 exerted both positive and negative regulatory influences on the generation of antigen-specific cytotoxic T lymphocytes (CTL) depending on the time of addition to culture (Widmer et al, 1987), and displays potent anti-tumour activity in the intact animal (Tepper et al, 1989).

IL-4 is also a co-stimulant for the growth of some mast cell lines, and acts as a modifier in haemopoiesis. Although possessing no intrinsic colony-stimulating-activity when used alone, IL-4 synergises with erythropoietin (EPO), G-CSF, M-CSF and IL-3 in the production of murine erythroid, granulocytic, monocyte/macrophage and mast cell colonies respectively (Rennick et al, 1987). In contrast, the same investigators found that murine IL-3-induced granulocytic and monocyte/macrophage colony formation was inhibited with IL-4, illustrating the diverse regulatory capacity of this cytokine (Rennick et al,1987). In similar studies with human bone marrow cells, Bronxmeyer et al (1988) showed synergy between IL-4 and EPO for erythroid colony formation, and with G-CSF for granulocytic colony formation, but not with M-CSF, GM-CSF or IL-3. Jansen et al (1989) showed identical synergy, but in addition showed IL-4-induced inhibition of macrophage colony formation in response to M-CSF and GM-CSF, and McInnes et al (1988) showed that IL-4 was the factor responsible for the formation of monocyte-derived multinucleate cells in liquid bone marrow culture.

The effects of IL-4 on mature myeloid cells have been most studied for monocytes and macrophages. IL-4 was first shown to be a MAF by Crawford et al (1987), who demonstrated enhanced tumouricidal activity in IL-4-stimulated murine PEM. IL-4 was active both alone, and in synergy with LPS and with GM-CSF and IL-3. In contrast, IL-4 decreased the tumouricidal response to IFNγ in the same cells. IL-4 has also been shown to increase the surface expression of Ia antigens on murine PEM (Crawford et al, 1987) and of HLA-DR on human monocytes (Te Velde et al, 1988) although the latter effect may be via IL-4-stimulated IFNγ release from contaminating T cells (Littman et al, 1989). Other stimulatory effects of IL-4 include enhanced uptake and killing of *Trypanosoma cruzi* in murine PM (Wirth et al, 1989), enhanced secretion of complement protein 2, G-CSF and M-CSF, and tissue plasminogen activator from human monocytes (Littman et al, 1989; Wieser et al, 1989; and Hart et al, 1989A), and the induction of macrophage differentiation in the same cells (Te Velde et al, 1988).

As with the actions of IL-4 on haemopoiesis, IL-4 may also act as a suppressor molecule for macrophage function, causing inhibition of IL-1α and β, TNFα and prostaglandin E2 (PGE2) secretion by human monocytes (Hurme et al, 1988; Hart et al, 1989B; Essner et al, 1989). These findings may explain the failure of IL-4 to induce antibody-dependent tumour cell cytotoxicity in PEM (Ralph et al, 1988), as this function has been shown to be mediated by production of TNFα (Cannistra et al, 1988A).

Despite this extensive literature on IL-4/monocyte interactions, nothing is known of the role of this cytokine in the regulation of monocyte adhesion. The anti-inflammatory actions of IL-4 in suppressing IL-1 and TNFα secretion by monocytes, and the observations that, unlike IFNγ, IL-4 appeared not to induce spreading of human monocytes (Hart et al, 1989B) suggested a possible role for IL-4 as a physiological inhibitor of monocyte adhesion. In this chapter, both unstimulated and CSF-stimulated monocyte adhesion is shown to be inhibited

by IL-4 in a dose-dependent manner, while surface expression of monocyte adhesion structures is shown to be increased. Possible mechanisms by which IL-4 exerts its anti-adhesive influence are discussed.

RESULTS

5.1 Inhibition of monocyte adhesion by IL-4

The effect of varying concentrations of IL-4 on IL-3-stimulated monocyte adhesion to plastic is shown in Fig. 5.1. Both cytokines were present from the beginning of culture, and harvest was after 21 hours, representing late-phase adhesion. IL-4 inhibited both IL-3-stimulated and unstimulated adhesion, and showed activity at concentrations as low as 20pM. Maximal inhibition of unstimulated adhesion, or adhesion stimulated by low concentrations of IL-3, was seen with 60pM IL-4. In contrast, when higher concentrations of IL-3 were used to stimulate adhesion (60pM, 600pM), concentrations of IL-4 of at least 600pM were required to achieve maximal inhibition. Maximal inhibition by IL-4 ranged from 79% (6pM IL-3) to 48% (600pM IL-3). Similar results were observed with IL-4 inhibition of GM-CSF-stimulated monocyte adhesion (Fig. 5.2), where inhibition ranged from 80% (6pM GM-CSF) to 37% (600pM GM-CSF). No significant differences in cell survival or viability (trypan blue exclusion) were seen between IL-4-treated and untreated monocytes at 21 hours. In addition, spontaneous ^{51}Cr release was similar for the two groups, indicating that IL-4 was not exerting a toxic effect on monocytes (data not shown).

5.2 Analysis of pooled data on IL-4 inhibition

The results of 10 experiments concerned with the effect of IL-4 on late-phase monocyte adhesion are summarised in Table 5.1. Each experiment included IL-3 and GM-CSF-

Figure 5.1 Inhibition of IL-3-stimulated monocyte adhesion by IL-4. ^{51}Cr-labelled monocytes were incubated for 21 hours in plastic microtitre wells with varying combinations of IL-3 and IL-4. Adhesion was measured as previously described. Each point represents the mean ± SEM of 4 replicates.

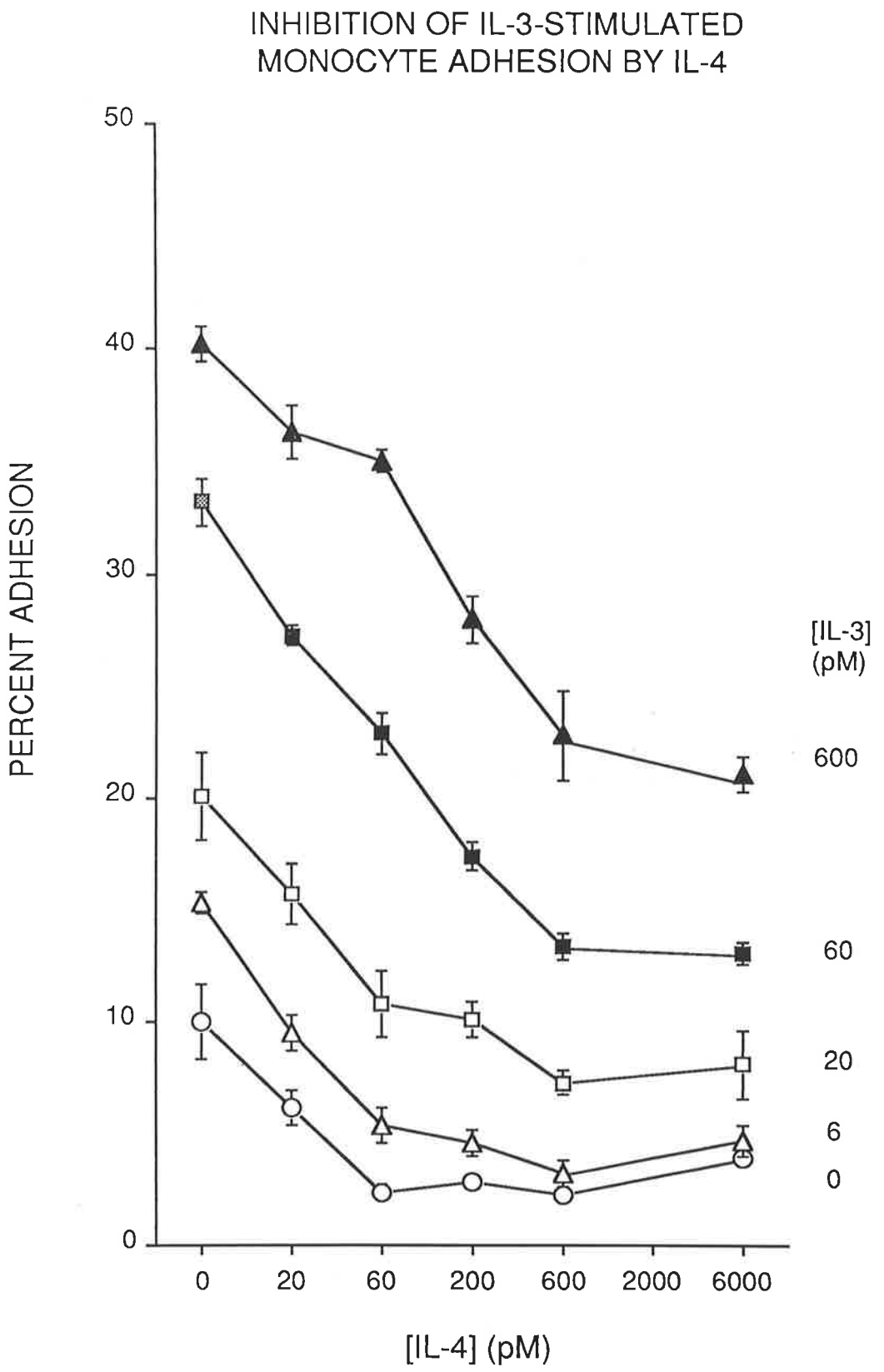
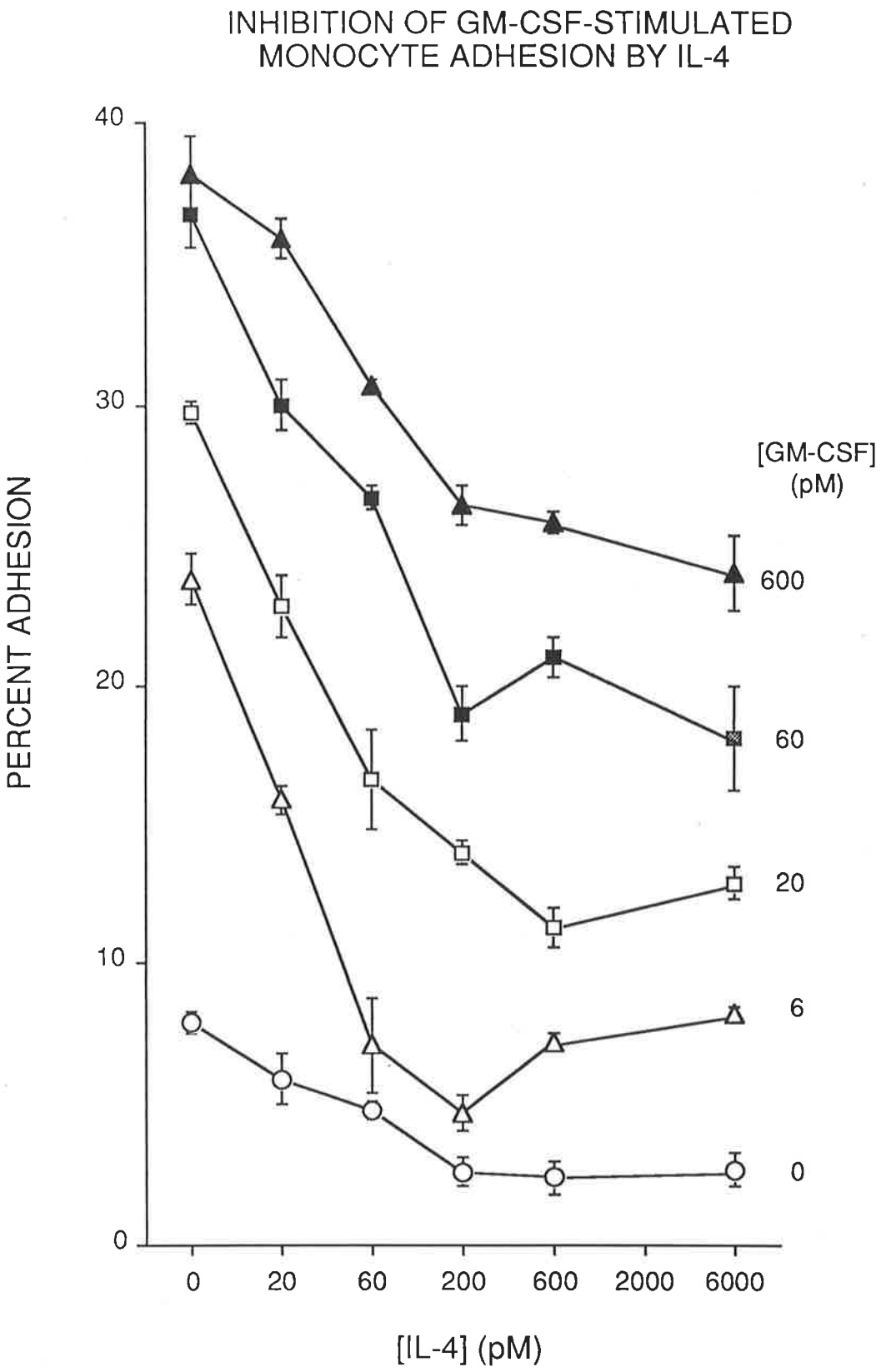

INHIBITION OF IL-3-STIMULATED
MONOCYTE ADHESION BY IL-4
PERCENT ADHESION
50
40
30
20
10
0
0
20
60
200
600
2000
6000
[IL-4] (pM)
[IL-3]
(pM)
600
60
20
6
0

Figure 5.2 Inhibition of GM-CSF-stimulated monocyte adhesion by IL-4. Methods used are described in Figure 5.1.


INHIBITION OF GM-CSF-STIMULATED
MONOCYTE ADHESION BY IL-4
PERCENT ADHESION
40
30
20
10
0
0
20
60
200
600
2000
6000
[IL-4] (pM)
[GM-CSF]
(pM)
600
60
20
6
0

stimulated groups, as well as control groups, with and without IL-4. In most experiments, IL-3 or GM-CSF were used at a final concentration of 60pM, and IL-4 at 600pM, which combination has been shown to yield maximal inhibition (Fig. 5.1 and 5.2). Each value is the mean of at least 4 replicates, and mean values for pooled data for each cytokine combination are also shown in Table 5.1. IL-4 inhibited basal monocyte adhesion at 21 hours by an average of 45%, while IL-3-stimulated and GM-CSF-stimulated adhesion were reduced by an average of 31% and 30% respectively. The percent inhibition by IL-4 varied from one experiment to the next, suggesting differences in responsiveness to this cytokine in different individuals. Statistical analysis of the pooled data was performed using Wilcoxon's matched pairs test. IL-4 significantly inhibited adhesion in both control monocytes ($p<0.025$) and in IL-3- and GM-CSF-stimulated monocytes ($p<0.01$, $p<0.001$ respectively). When individual experiments were analysed using the unpaired t test, significant inhibition of adhesion by IL-4 was seen in 7 of 10 experiments with IL-3-treated monocytes, and in 9 of 10 experiments with GM-CSF-treated cells.

5.3 Inhibition of monocyte adhesion to EMP and to HUVEC

Monocyte adhesion to vitronectin, fibronectin and collagen I in the presence of various cytokine combinations is shown in Fig. 5.3. These EMP were chosen as representing serum-derived, basement membrane and connective tissue proteins respectively, although there is clearly some overlap in their distributions *in vivo*. The EMP were laid down as described previously, and adhesion measured after 21 hours of co-incubation of cells, EMP and cytokines. IL-3 and GM-CSF induced large increases in adhesion on all 3 substrates, although basal adhesion to fibronectin was relatively high. IL-4, used at a concentration of 600pM, inhibited CSF-stimulated adhesion by 17-34%, with the greatest reductions seen with adhesion to collagen. There was no significant inhibition of adhesion to any substrate in control cells.

Table 5.1

Inhibition of monocyte adhesion to plastic by IL-4. ^{51}Cr-labelled monocytes were incubated for 21 hours in microtitre wells in the presence of control medium, IL-3 or GM-CSF. IL-4 was included in some wells from the onset of the experiment, and adhesion measured as described previously. The results of 10 separate experiments are shown, together with mean values for adhesion in the presence of the various cytokines. Levels of significance for IL-4 inhibition of adhesion were $p<0.025$ (control cells), $p<0.01$ (IL-3-stimulated cells) and $p<0.001$ (GM-CSF-stimulated cells) by Wilcoxon's matched pairs test.

Table 5.1 Inhibition of monocyte adhesion to plastic by IL-4

Experiment	Percent Adhesion		
	Stimulus		
	Control (+IL-4)*	IL-3** (+IL-4)	GM-CSF** (+IL-4)
1	30.9 (6.4)	73.6 (21.9)	73.7 (34.5)
2	8.8 (7.4)	39.8 (30.7)	46.3 (29)
3	2.2 (6.7)	25.3 (23.3)	32.9 (31.7)
4	7.9 (2.4)	33.2 (13.3)	36.8 (21.0)
5	16.4 (4.2)	34.9 (17.0)	31.8 (12.1)
6	0.7 (0.4)	36.2 (22.6)	32.4 (24.2)
7	14.7 (5.4)	48.8 (58.1)	56.7 (49.6)
8	2.2 (3.0)	60.4 (44.1)	62.8 (48.1)
9	4.9 (3.0)	18.4 (12.1)	32.5 (23.0)
10	27.6 (25.0)	48.1 (45.8)	58.7 (53.7)
Mean SEM	11.6±3.2 (6.4±2)	41.9±4.9 (28.9±4.6)	46.5±4.6 (32.7±4.1)

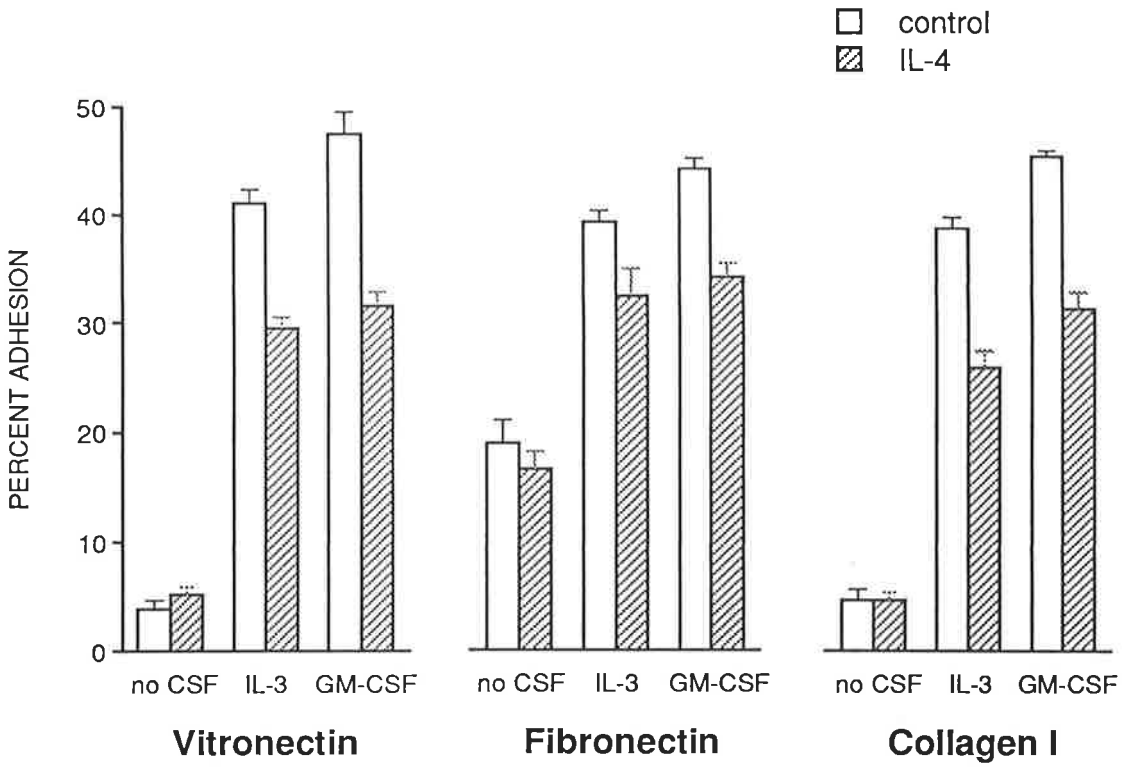
* IL-4 was used at a final concentration of 600pM, except in experiments 9 and 10 (200pM) and experiment 7 (6,000pM).

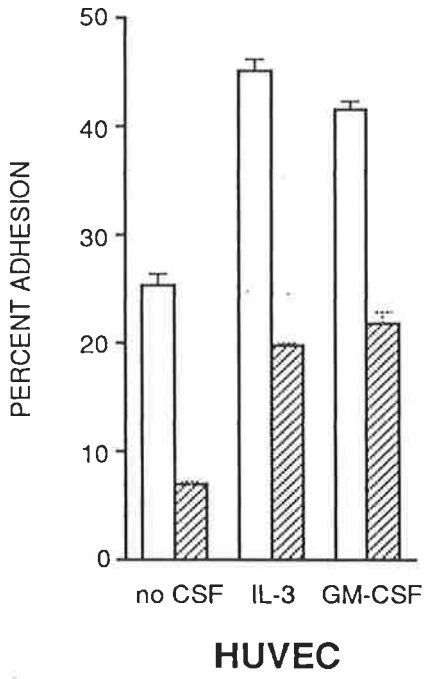
** IL-3 and GM-CSF were used at a final concentration of 60pM, except in experiments 9 and 10 (20pM) and experiments 6, 7 and 8 (600pM).

Figure 5.3 Inhibition of monocyte adhesion to EMP by IL-4. Microtitre wells were precoated with vitronectin, fibronectin, or collagen I as described in Materials and Methods, and adhesion of ^{51}Cr-labelled monocytes measured after 21 hours of co-incubation. All stimuli were present from the beginning of the culture period. IL-3 and GM-CSF were used at a final concentration of 60pM, and IL-4 at a final concentration of 600pM. Results are the mean ± SEM of 4 replicates per point in a representative experiment of 2 performed.

Figure 5.4 Inhibition of monocyte adhesion to HUVEC by IL-4. Monocytes were cultured for 21 hours in a suspension system with IL-3 or GM-CSF (60pM) or control medium, with or without IL-4 (600pM). Adhesion was measured to HUVEC as previously described. Each point is the mean ± SEM of 5 replicates in a representative experiment of 3 performed.

INHIBITION OF MONOCYTE ADHESION BY IL-4





In separate experiments, IL-4 was included in 21 hour suspension cultures of monocytes, prior to adhesion to HUVEC, as previously described (Fig. 5.4). IL-4 inhibited both basal and CSF-stimulated adhesion on HUVEC to a similar degree to that seen previously on plastic.

5.4 Mechanism of action of IL-4

The inhibitory effects of IL-4 on monocyte adhesion were characterised by two major features. Firstly, inhibition of both basal and CSF-stimulated adhesion was seen, suggesting that IL-4 was unlikely to be acting by interfering with CSF-receptor interactions or associated signalling mechanisms. Secondly, the inhibition was seen on a variety of substrates, suggesting that it might result from a general effect of IL-4 on monocytes. Experiments were therefore performed to assess the influence of IL-4 on monocyte protein synthesis. Cells were incubated in suspension for 21 hours with and without IL-4, washed, and resuspended in a low-methionine medium. Incorporation of ^{35}S-methionine into the cells was then measured as described in Section 2.14.2. No inhibition of ^{35}S-methionine uptake was seen in the presence of IL-4, and instead a small increase in uptake was seen in 3 of 3 experiments performed (data not shown).

With no evidence to suggest a general effect of IL-4 on protein synthesis, a discrete effect on the expression of cell surface adhesion molecules was sought. Previous reports have shown IL-4-induced upregulation of monocyte HLA-DR expression at 20 hours of culture (Te Velde et al, 1988), providing a precedent for IL-4-induced surface molecule regulation within the time-scale relevant here. The experiments summarised in Table 5.2 show changes in LFA-family expression after overnight suspension culture with or without IL-4. Instead of a decrease in expression, as might have been predicted, IL-4 induced strong increases in the expression of CD18 and MAC-1, with a lesser but still significant increase

Table 5.2

LFA surface expression on unstimulated and IL-4-stimulated monocytes. Monocytes were incubated in suspension for 21 hours with or without 600pM IL-4, and stained according to the protocol in Table 4.3 and in Section 2.17. The specificities of the MAb are: 60.1, MO-1: α chain MAC-1 (CD11b); TS1/22: α chain LFA-1 (CD11a); 150/95: α chain p150/95 (CD11c); 60.3, TS1/18: β chain LFA family (CD18). Parallel adhesion experiments on the same donor cells after overnight suspension culture yielded the following percent adhesion at 30 minutes. Exp. 1: no CSF, 48.7±3.3; IL-4, 21.4±1.6; Exp. 2: no CSF, 12.5±0.5; Il-4, 7.1±0.5

Table 5.2 LFA surface expression on unstimulated and IL-4-stimulated monocytes.

MAb (Antigen)	Stimulus	Experiment 1	2	Fluorescence Shift (Mean ± SD)
60.1 (MAC-1)	no CSF	92*	101	
	IL-4	122 (+30)**	121 (+20)	+25±5
MO-1 (MAC-1)	no CSF	63	65	
	IL-4	84 (+21)	85 (+20)	+21±1
TS1/22 (LFA-1)	no CSF	112	105	
	IL-4	121 (+9)	107 (+2)	+6±4
150/95 (p150/95)	no CSF	41	41	
	IL-4	50 (+9)	52 (+11)	+10±1
60.3 (β chain)	no CSF	142	135	
	IL-4	157 (+15)	150 (+15)	+15±0
TS1/18 (β chain)	no CSF	128	124	
	IL-4	144 (+16)	135 (+11)	+14±3

* mean fluorescence, after subtraction of background fluorescence, measured on a logarithmic scale.

** shift in mean fluorescence relative to no CSF group.

in expression of p150/95. Good concordance was seen between the two experiments, both in terms of levels of expression in unstimulated cells, and in the IL-4-induced increases seen. Parallel adhesion experiments showed approximately 50% inhibition of monocyte adhesion after pre-treatment with IL-4.

DISCUSSION

The data presented in this chapter show that IL-4 is a negative regulator of monocyte adhesion, and provide further support for a possible anti-inflammatory role for this cytokine *in vivo*. Inhibition of adhesion was seen at concentrations of IL-4 as low as 20pM, while maximal inhibition required concentrations of 60-600pM, depending on the strength of the pro-adhesive stimulus. Both basal and CSF-stimulated adhesion was inhibited, and decreased adhesiveness was seen to endothelium, EMP and to plastic.

The data presented in Table 5.1 show that the magnitude of the response to IL-4 was variable in different donors, but that when data from 10 experiments were pooled, the effect of IL-4 on both unstimulated and CSF-stimulated cells was highly significant. The mean inhibition of adhesion was 45% for control cells, and 31% and 30% for IL-3-stimulated and GM-CSF-stimulated monocytes respectively.

The mechanism of action of IL-4 was investigated by studying protein synthesis in IL-4-treated and untreated cells, and by measuring the expression of cell surface adhesion molecules. IL-4 did not inhibit overall protein synthesis in monocytes after overnight culture, and instead caused a small increase in ^{35}S-methionine uptake. This is in keeping with a previous publication showing IL-4-stimulated protein synthesis in human monocytes after 6 days of culture (Te Velde et al, 1988).

The dependence of CSF-stimulated adhesion on CD18, and the partial dependence of basal monocyte adhesion on the same molecule (see Chapter 4) suggested that IL-4 might exert an antiadhesive effect by down-modulation of this antigen. In fact, IL-4 induced the opposite change, with significant increases in CD18, MAC-1, and to a lesser extent, p150/95 expression at 21 hours. Similar results were obtained for two separate experiments, and parallel adhesion assays showed IL-4-induced inhibition of adhesion of approximately 50% (Table 5.2). These results are in keeping with a previous report, which showed increased expression of MAC-1 and p150/95 but not LFA-1 on human monocytes after 3 days culture with IL-4 (Te Velde et al, 1988). The results raise further questions about the relevance to cell adhesion of changes in expression of cell surface adhesion structures as discussed in Chapter 4. Indeed, the increased expression of MAC-1 and p150/95 upon stimulation of human monocytes by IL-3, GM-CSF and IL-4 (Tables 4.3 and 5.2) may reflect more the influence of each of these cytokines on monocyte differentiation (Chapter 3, and Te Velde et al, 1988) than on their adhesive capacity. Whether upregulation of these molecules correlates with increased binding of C3bi (MAC-1 and p150/95) or of fibrinogen and coagulation factor X (MAC-1) remains to be determined.

These experiments have not resolved the question of the mechanism by which IL-4 inhibits adhesion, and further studies in this area would be of interest. Recent results from this laboratory and elsewhere suggest that stimulation of adhesion in human neutrophils is accompanied by an increase in intracellular levels of platelet activating factor (PAF, M. Denichilo, personal communication; Braquet et al, 1989). Inhibition by IL-4 of intracellular PAF formation, or of other unknown second messengers, might provide a mechanism for the action of IL-4. At the level of the adhesion molecules themselves, IL-4 might cause a 'deactivation' in their functional state, in a converse manner to that proposed in Chapter 4 to explain CSF-mediated adhesion.

The effects of IL-4 on adhesion may provide an explanation for certain other inhibitory effects of IL-4, including those of inhibition of IL-1 and TNFα production (Hurme et al, 1988; Hart et al, 1989B, Essner et al, 1989). Adhesion has been shown to be an important signalling step in itself in the expression of a number of monokines, including IL-1β, TNFα, M-CSF, IL-6 and the oncogene product *c-fos* (Haskill et al, 1988; Navarro et al, 1989). IL-4-induced inhibition of monocyte adhesion to the culture vessel may therefore provide an explanation for the inhibition of production of IL-1 and TNFα. While unproven, this hypothesis has the attraction of providing for a common mechanism for the anti-inflammatory effects of IL-4. The converse hypothesis, namely that IL-1 and TNFα promote monocyte adhesion, and that inhibition of their production might therefore cause cell detachment, might also be proposed. However, experiments in this laboratory with exogenous TNFα have not shown significant pro-adhesive effects of this cytokine on human monocytes (J.R. Gamble, personal communication).

Hart et al (1989B) have highlighted the opposing effects of IL-4 and IFNγ on a number of cell functions. Amongst these are IL-4-induced inhibition of the effects of IFNγ and LPS on monocyte IL-1 and TNFα production, and the inhibitory effect of IFNγ on IL-4-stimulated macrophage cytotoxicity (Crawford et al, 1987). In addition, IFNγ inhibits IL-4-induced CD23 expression on, and IgE production by B cells (Defrance et al, 1987). Others have pointed out that IL-4 and IFNγ can act in a synergistic manner, with enhanced HLA-DR expression, antimicrobial capacity and secretion of the complement protein 2 when both factors are present together (Littman et al, 1989). While not reported here, IFNγ has been shown in this laboratory to have modest pro-adhesive effects on human monocytes (J.R. Gamble, personal communcation) and others have reported enhanced homotypic adhesion in monocytes treated with IFNγ (Mentzer et al, 1986) and stimulation of murine PEM adhesion to basement membrane glycoproteins by IFNγ and LPS (Shaw et al, 1989). Adhesion

therefore provides a further example of a monocyte function controlled in different ways by IL-4 and IFNγ.

Information concerning the detection of IL-4 in different clinical conditions is rudimentary. The presence of multinucleate giant cells at sites of chronic infection or inflammation might imply local production of IL-4, although giant cell formation has also been described with other mediators (Weinberg et al, 1984). A recent report, however, mentions the detection of IL-4 mRNA in synovial lymphocytes in inflammatory arthritis (Ogilvie et al, 1989). Production of IL-4 in such circumstances might lead to reduced monocyte-endothelial adhesion and hence transmigration, leading to reduced numbers of monocytes at the inflammatory site. Reduced monocyte adhesion to structural proteins such as collagen 1 may also prevent monocyte-mediated tissue damage. While enhanced tissue plasminogen activator secretion (Hart et al, 1989A) and reduced TNFα and IL-1 production may also help reduce inflammation, the other functions ascribed to IL-4 in monocytes cannot be characterised as anti-inflammatory.

Several questions remain unanswered about the phenomenon reported here. The role of IL-4 in early phase stimulated adhesion has not been addressed and the mechanism of inhibition of monocyte adhesion has not been resolved. In addition, the possibility that IL-4 may be a more effective inhibitor if preincubated with monocytes prior to the addition of a pro-adhesive stimulus has not been investigated. Similar temporal restrictions apply to the priming of macrophage tumouricidal activity by IFNγ, and merit further study.

SUMMARY

IL-4 was shown to inhibit adhesion of monocytes to endothelium, EMP and plastic after 21 hours of culture. The effect was sensitive, with a threshold for activity at concentrations of

IL-4 of only 20pM, and was saturable at concentrations of 60-600pM and above. While showing some variability in different experiments, IL-4-induced inhibition of both basal and CSF-stimulated adhesion was highly significant when pooled experiments were analysed. Inhibition of adhesion was not associated with reduced overall protein synthesis, and no evidence of IL-4-induced cell toxicity was found. Paradoxically, IL-4-inhibited adhesion was associated with an upregulation of LFA members MAC-1 and p150/95, and of CD18, providing further evidence that regulated monocyte adhesion is not dependent on changes in the number of surface adhesion molecules.

CHAPTER 6

EQUILIBRIUM BINDING STUDIES WITH ^{125}I-IL-3 AND ^{125}I-GM-CSF

INTRODUCTION

Much interest has focussed on the mechanisms by which CSF stimulate their target cells, and in particular on their interaction with cell surface receptors. IL-3 and GM-CSF receptors (IL-3-R, GM-CSF-R) have been extensively characterised in the mouse, and a number of common features emerge. Firstly, receptors are found on both bone marrow cells, and on more mature cells of the granulocytic and monocyte/macrophage lineages, and receptor numbers generally decrease to a few hundred or less per cell with increasing maturity (Nicola, 1989). Secondly, the receptors are of high affinity, with equilibrium dissociation constants (K_D) of 200-300pM, although low affinity sites have also been reported on murine cells (Walker et al, 1985A). In general, biological effects are seen at concentrations of CSF considerably less than the high affinity K_D values, suggesting either that these are an underestimation of the true receptor affinity, or that only a fraction of the available receptors need be occupied for a biological response. Thirdly, binding of CSF to its receptor at 37°C leads to internalisation of the CSF-receptor complex, and ultimately to CSF degradation (Nicola, 1989). Finally, a complex hierarchical pattern of CSF receptor trans-downmodulation is seen in murine bone marrow cells, in which the binding of IL-3 causes internalisation of receptors for GM-CSF, M-CSF, and G-CSF, as well as the IL-3-IL-3-R complex. In turn, GM-CSF binding leads to the internalisation of its own receptor and of those for M-CSF and G-CSF, but not of the IL-3-R. The G-CSF and M-CSF receptors are placed at the bottom of the hierarchy, in that they cannot trans-downmodulate receptors for either GM-CSF or IL-3 except at high concentrations of cytokine (Walker et al, 1985A,B).

Characterisation of CSF-receptor interactions in human cells, and in particular, in human monocyte/macrophages, is less well advanced. The binding of IL-3 to high affinity receptors on human monocytes has recently been described, however (Park et al, 1989A),

and the ability of GM-CSF to partially inhibit IL-3 binding was noted. GM-CSF binding to human monocytes has also been reported, based either on autoradiographic methods (Gasson et al, 1986) or on Scatchard analysis of equilibrium binding data (Park et al, 1986; DiPersio et al, 1988), but no specificity controls were reported.

In this chapter, the patterns of binding of IL-3 and GM-CSF to monocytes are analysed under equilibrium binding conditions, and the data displayed as Scatchard plots. The stimulation of monocyte survival at CSF concentrations as low as 6pM, and of monocyte adhesion at concentrations of only 20pM, suggested the presence of high affinity receptors for both IL-3 and GM-CSF on these cells, and an appropriately high affinity receptor class was indeed identified. In addition, however, low affinity binding sites similar to those previously described in the mouse were seen. Because of their low affinity, and the relatively low numbers of receptors present, the experiments required large numbers of monocytes and radioligands of high specific radioactivity. The high monocyte yields and purity achieved with countercurrent elutraition (Section 2.8.3), and the use of a modified IL-3 molecule for iodination (Section 2.18) were therefore critical to the identification of the low affinity sites.

In further experiments designed to determine the specificity of CSF binding, IL-3 and GM-CSF were shown to exhibit partial but reciprocal inhibition of each other's binding, while a range of other cytokines were shown to lack such cross-competition. These experiments were performed under conditions shown to prevent receptor internalisation, suggesting a direct interaction between IL-3 and GM-CSF, or between their receptors, on the monocyte membrane.

RESULTS

6.1 Theory of Scatchard analysis

The interaction between a radioligand and its receptor at equilibrium is described by the generalised Michaelis-Menten model which was first used to characterise enzyme-substrate kinetics (McPherson, 1983).

The amount of bound radioligand (B) is given by the equation:

$$B = (B\ max.F) / (K_D + F) \qquad \text{(McPherson, 1983)}$$

where Bmax is the maximum density of binding sites, F is the concentration of unbound or free ligand, and K_D is the apparent dissociation constant. This is equal to the concentration of radioligand needed for 50% receptor occupancy (McPherson, 1983).

Scatchard transformation of this equation (Scatchard, 1949) allows the use of linear regression to obtain estimates for K_D and Bmax. The development of computer programs using analytical methods involving weighted least squares regression analysis has allowed application of Scatchard analysis to more complex binding systems, where ligand binding is seen to more than one site. The data in this chapter have been analysed by the program of G. McPherson (1985).

6.2 Determination of receptor number and affinity by Scatchard analysis

Incubation of monocytes with increasing concentrations of radioligand for 18 hours at 4°C, followed by determination of specific cell-associated radioactivity, allowed the construction of binding curves, as demonstrated in Fig. 6.1 and 6.2 (insets). Scatchard transformation of specific binding data (Scatchard, 1949) yielded a curvilinear plot for both ^{125}I-IL-3 and

^{125}I-GM-CSF binding (Fig. 6.1 and 6.2), reflecting the failure to reach a plateau seen in the respective binding curves. Statistical comparisons of different binding models performed by LIGAND yielded a preferred two-site binding model ($p<0.001$ for ^{125}I-IL-3, $p<0.022$ for ^{125}I-GM-CSF). Binding affinities, the number of high and low affinity binding sites per cell and levels of significance are shown in Table 6.1 for three experiments with IL-3 and four with GM-CSF. High affinity binding characteristics were similar for the two cytokines (K_D for ^{125}I-IL-3: 8-38pM; K_D for ^{125}I-GM-CSF: 5-39pM) but with higher receptor numbers per cell for ^{125}I-IL-3 (^{125}I-IL-3: 95-580 receptors per cell; ^{125}I-GM-CSF: 8-67 receptors per cell). K_D values and receptor numbers for the low affinity sites were at least 10-fold higher than for high affinity binding (K_D for ^{125}I-IL-3: 513-939pM; 179-5274 receptors per cell; K_D for ^{125}I-GM-CSF: 576-1120pM; 130-657 receptors per cell). While the majority of binding experiments were performed with monocytes purified by elutriation, blood from two donors was purified by adherence (individuals 4 and 5). Monocytes purified by both methods showed curvilinear Scatchard plots and a dual site binding model. Previous reports had demonstrated a single class of high affinity binding sites for GM-CSF on human neutrophils (Park et al, 1986; DiPersio et al, 1988). A direct comparison between neutrophils and monocytes was therefore undertaken with binding experiments conducted for 1 hour at 24^{o}C, in order to ensure adequate neutrophil survival. The Scatchard plots for ^{125}I-GM-CSF binding to monocytes and neutrophils purified from the same donor and using the same range of concentrations of radioligand are shown in Fig. 6.3. The curvilinear plot and dual binding site model for monocytes (high affinity K_D: 4.7pM; receptor number/cell 35; low affinity K_D: 991pM; receptor number/cell 657) contrasts with the straight plot obtained for neutrophils (K_D: 172pM; receptor number/cell 580). These results illustrate the heterogeneous nature of CSF binding to different cell types, and suggest that the low affinity binding site on monocytes is not an experimental artifact.

Figure 6.1 Binding curve (inset) and Scatchard analysis for the binding of ^{125}I-IL-3 to purified human monocytes. ^{125}I-IL-3 was incubated at different concentrations with 2-10 x 10^6 monocytes at 4°C for 18 hours, and cell-bound radioactivity separated from unbound radioactivity by centrifugation on fetal calf serum at 4°C. Non-specific binding was determined in the presence of a 100 fold excess molar concentration of unlabelled IL-3. Each point is the mean of at least 2 replicates. Scatchard data were analysed by EBDA and LIGAND computer programs.

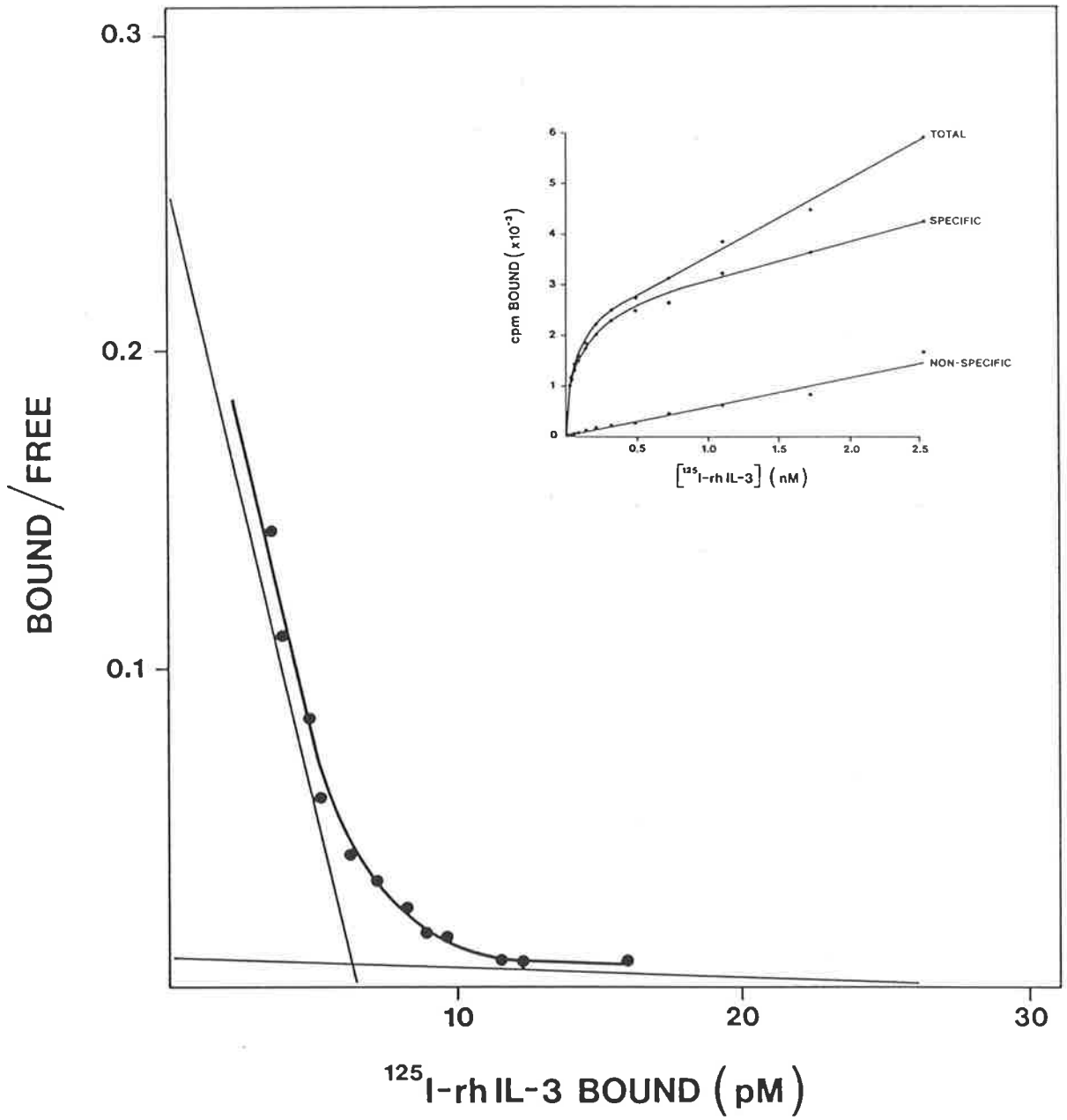

0.3
0.2
0.1
BOUND/FREE
10
20
30
125I-rh IL-3 BOUND (pM)
cpm BOUND (x10-3)
TOTAL
SPECIFIC
NON-SPECIFIC
[125I-rh IL-3] (nM)
0.5
1.0
1.5
2.0
2.5

Figure 6.2 Binding curve (inset) and Scatchard analysis for the binding of ^{125}I-GM-CSF to purified human monocytes. Experimental procedures are outlined in Materials and Methods and in Fig. 6.1.

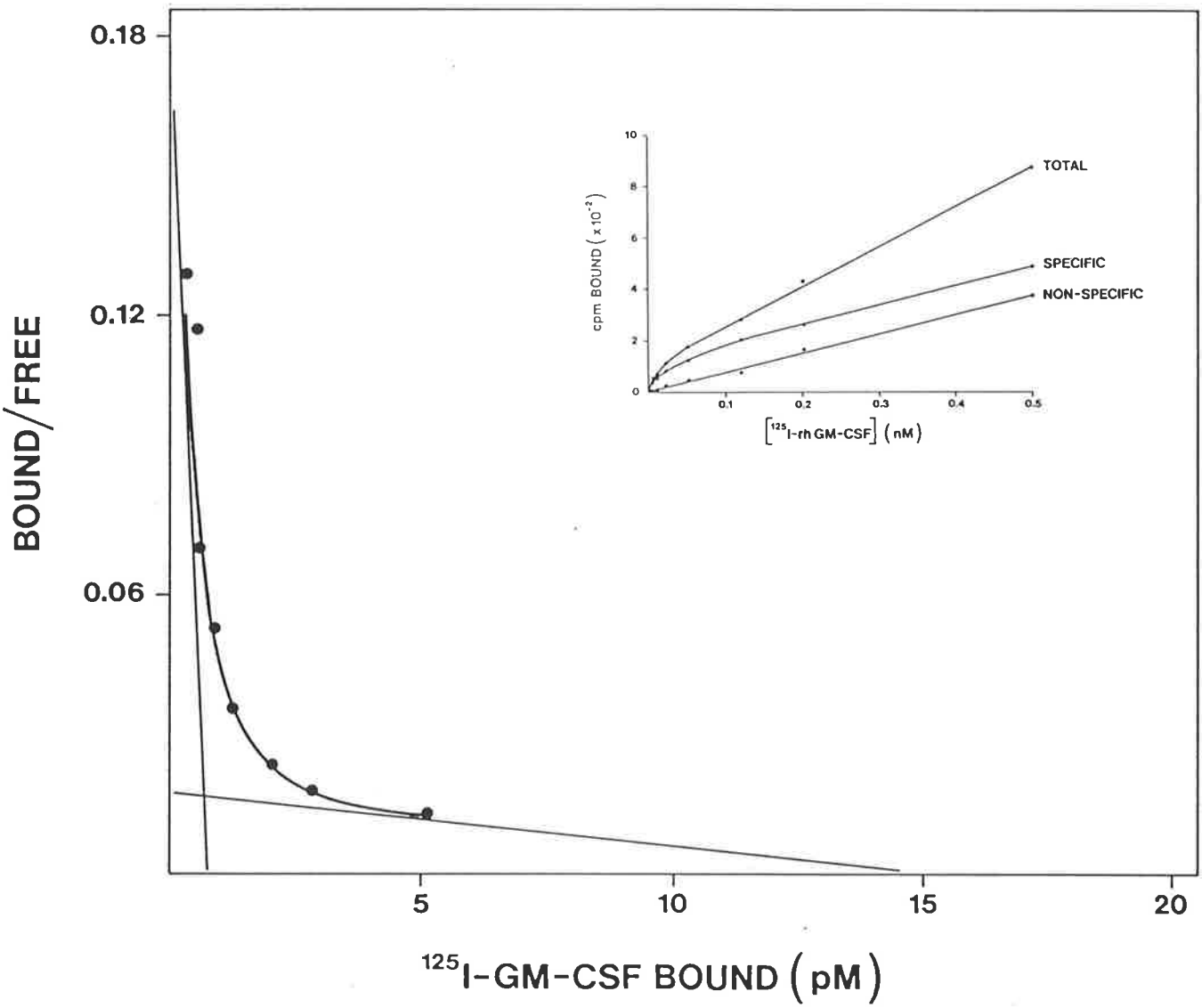

0.18
0.12
0.06
BOUND/FREE
5
10
15
20
^{125}I-GM-CSF BOUND (pM)
cpm BOUND (x10^{-2})
10
8
6
4
2
0
0.1
0.2
0.3
0.4
0.5
[^{125}I-rh GM-CSF] (nM)
TOTAL
SPECIFIC
NON-SPECIFIC

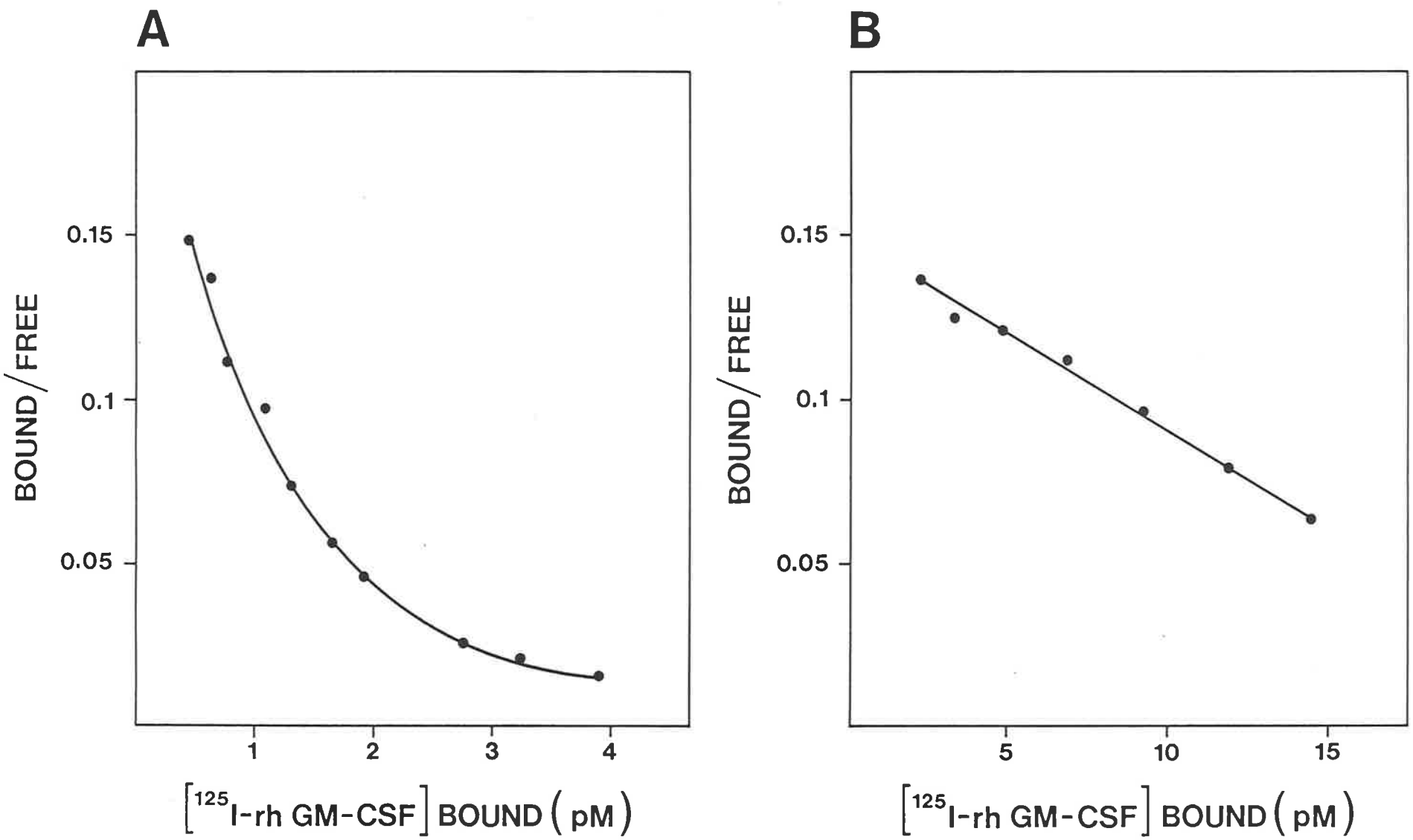
Table 6.1 Equilibrium binding characteristics for IL-3 and GM-CSF on human monocytes

Radiolabelled ligand prepared as described in Sections 2.18 and 2.19 was used at concentrations of up to 3.5nM, with at least ten dilutions per experiment. Radioligand was incubated with either elutriated monocytes (individuals 1,2,3,6,7) or monocytes purified by adherence (individuals 4,5) overnight at 4°C and cell bound radioactivity separated from unbound by centrifugation on fetal calf serum at 4°C. Each point on the binding curve generated was the mean of at least 2 replicates, with between 2 and 10 x 10^6 cells used per replicate. Non-specific binding was determined in the presence of a 100 fold excess of unlabelled ligand. Scatchard data were analysed by EBDA and LIGAND computer programs. A two site binding model was statistically superior to a single site model for all experiments (Exp. 1,2,3,6,7, $p<0.001$, Exp. 4, $p=0.022$, Exp. 5, $p=0.006$).

Table 6.1 Equilibrium binding characteristics for IL-3 and GM-CSF on human monocytes

Cytokine	Individual	High Affinity Site		Low Affinity Site	
		Apparent K_D (pM)	Receptors/ Cell	Apparent K_D (pM)	Receptors/ Cell
IL-3	1	38.2	580	939	5274
	2	26.5	122	513	476
	3	7.7	95	815	179
mean ± SEM		24.1±7.3	266±128	756±103	1976±1348
GM-CSF	4	8.5	8	576	431
	5	38.9	14	1120	130
	6	4.7	35	991	657
	7	10.0	67	588	307
mean ± SEM		15.5±6.8	31±11	819±121	381±96

Figure 6.3 Scatchard analysis for the binding of ^{125}I-GM-CSF to purified human monocytes (A) and neutrophils (B) prepared from the same donor. The experimental protocol, including maximal concentrations of radioligand used, was the same for the two cell types. Cells were incubated for 1 hour at 24°C with different concentrations of ^{125}I-GM-CSF, and cell-bound radioactivity separated from unbound by centrifugation on FCS at 4°C. Non-specific binding was determined at each point in the presence of a 100-fold excess molar concentration of unlabelled GM-CSF. Each point is the mean of at least 2 replicates. Scatchard data were analysed by EBDA and LIGAND computer programs.

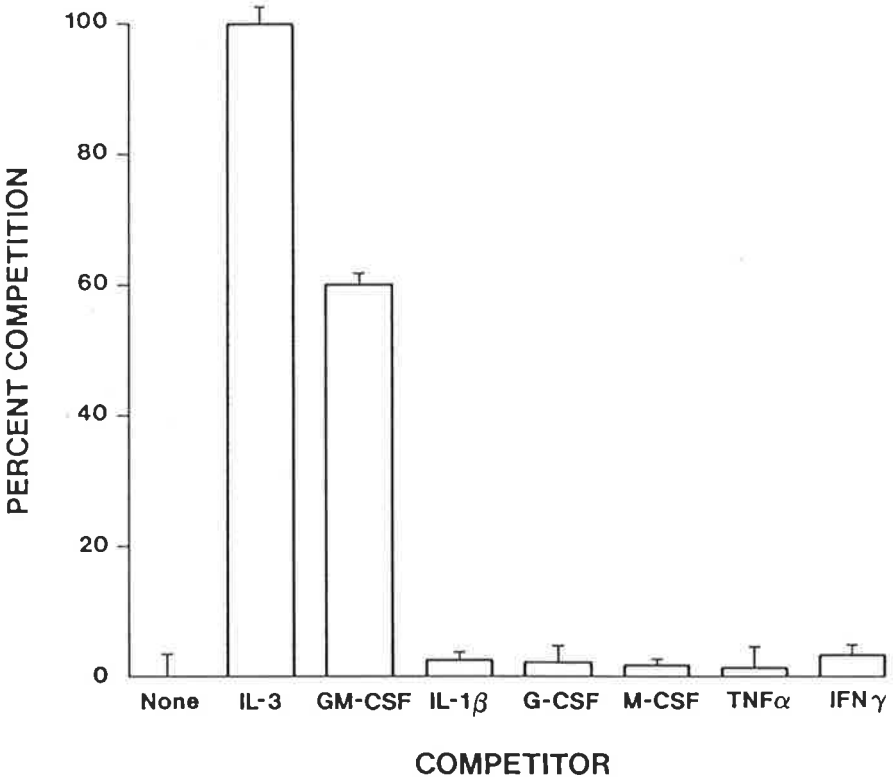

A
B
0.15
0.1
0.05
BOUND/FREE
1
2
3
4
5
10
15
[125I-rh GM-CSF] BOUND (pM)

6.3 Specificity of ^{125}I-IL-3 and ^{125}I-GM-CSF binding to monocytes

To establish the specificity of IL-3 and GM-CSF binding to monocytes, competition experiments were performed in which a variety of purified recombinant cytokines were used to compete for the binding of the radioligand (Fig. 6.4). These experiments were conducted overnight at 4°C. None of the monocyte-active cytokines M-CSF, IFN-γ, and TNF-α, nor IL-1 or G-CSF tested at 100-fold excess molar concentration inhibited ^{125}I-IL-3 or ^{125}I-GM-CSF binding. In contrast, 60% of the binding of ^{125}I-IL-3 to monocytes was inhibited by excess non-iodinated GM-CSF (Fig. 6.4, top panel) and almost 80% of ^{125}I-GM-CSF binding was inhibited by IL-3 (Fig. 6.4, bottom panel). Such inhibition of ^{125}I-CSF binding by the heterologous cytokine will be referred to as 'competition' or as 'competable' binding. As with receptor number and affinity, the degree of competition for ^{125}I-CSF binding varied between different individuals, ranging from 25% to 80%. Parallel experiments using the same conditions showed that IL-3 did not inhibit the binding of ^{125}I-GM-CSF to human neutrophils or HL-60 cells (data not shown), illustrating once again the differences in CSF binding in different cell types.

To determine whether higher concentrations of unlabelled heterologous competitor could completely inhibit ^{125}I-CSF binding to monocytes, quantitative inhibition experiments were performed. Fig. 6.5 shows a representative experiment in which a fixed concentration of each radioligand (10^{-10}M) was incubated with increasing concentrations of homologous and heterologous competitor. Each competitor was seen to completely inhibit the binding of its cognate ligand, but only 40% of the binding of the non-cognate ligand, even at 1,000 fold excess molar concentration. In addition, IL-3 showed greater efficiency at competing for ^{125}I-IL-3 and ^{125}I-GM-CSF binding at low competitor concentrations than did GM-CSF (Fig. 6.5).

Figure 6.4 Competition for the binding of ^{125}I-IL-3 (top panel) and ^{125}I-GM-CSF (bottom panel) to purified human monocytes from different donors. 10^{-10}M radioligand was incubated with 4-6 x 10^6 monocytes in the presence or absence of 10^{-8}M competitor cytokine. Specific cpm bound per 10^7 cells in the absence of competitor were 6,950 for ^{125}I-IL-3 binding and 580 for ^{125}I-GM-CSF binding. Each point represents the mean ± SD of at least 2 replicates.


PERCENT COMPETITION
100
80
60
40
20
0
None
IL-3
GM-CSF
IL-1β
G-CSF
M-CSF
TNFα
IFNγ
COMPETITOR

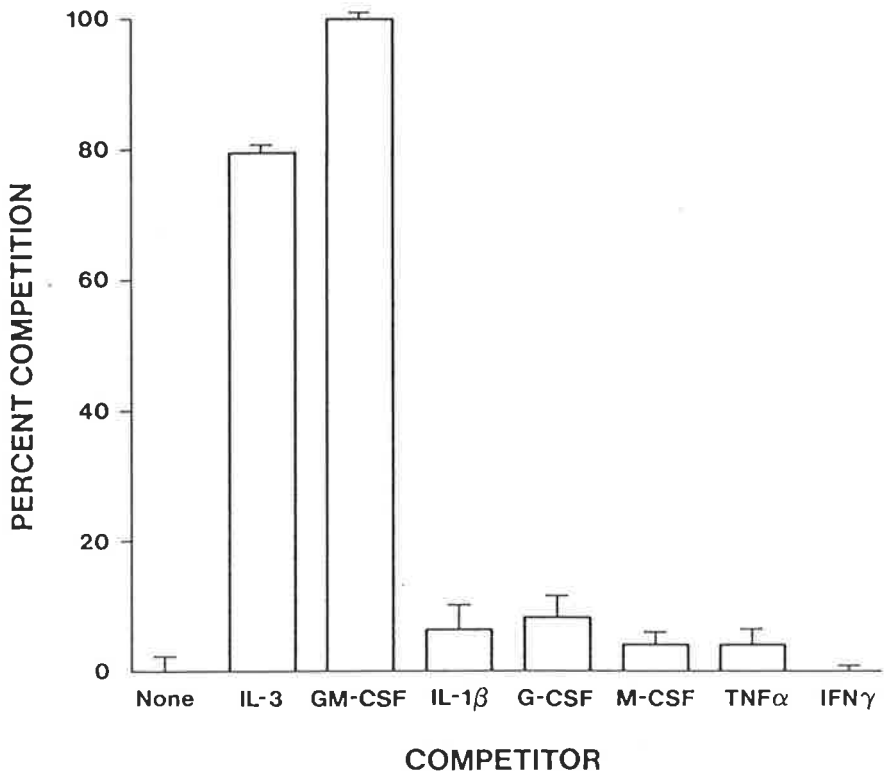
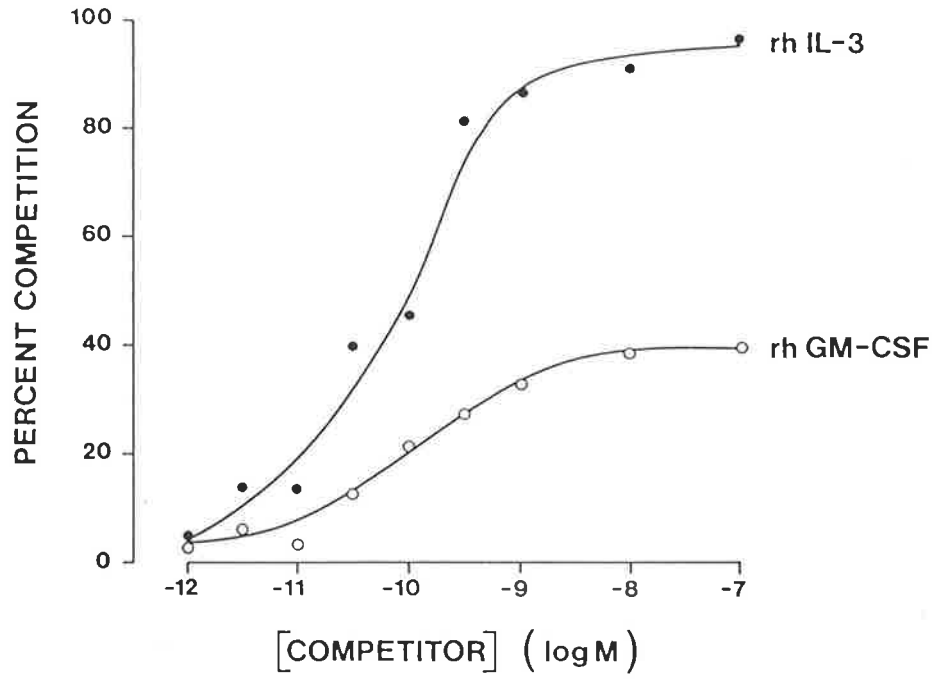
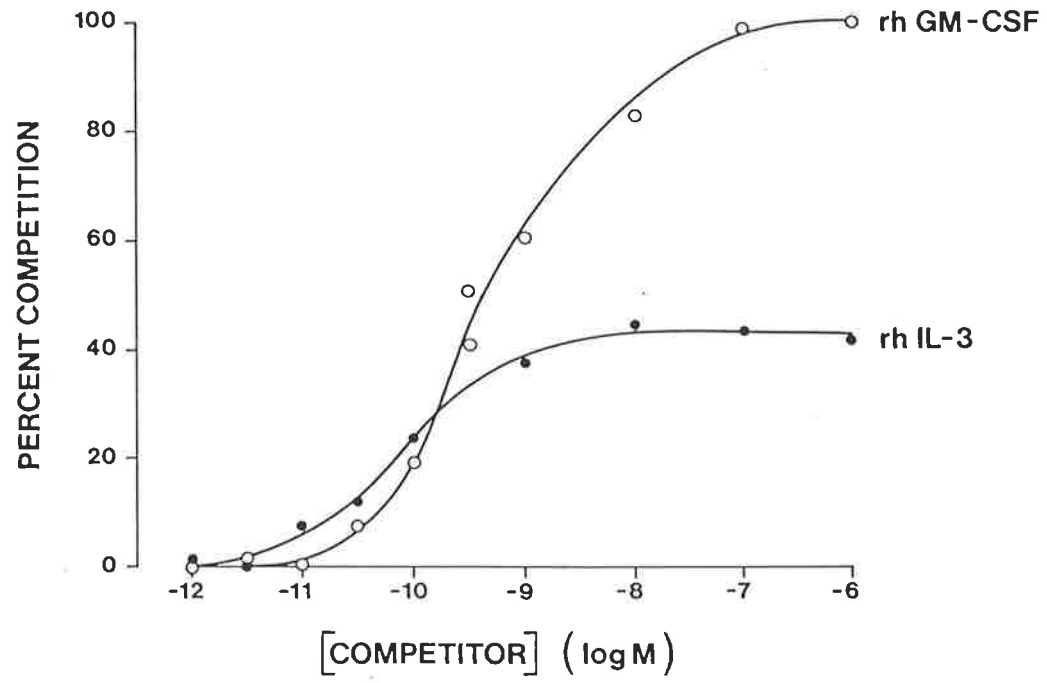

PERCENT COMPETITION
100
80
60
40
20
0
None
IL-3
GM-CSF
IL-1β
G-CSF
M-CSF
TNFα
IFNγ
COMPETITOR

Figure 6.5 Competition for ^{125}I-IL-3 binding (A) and ^{125}I-GM-CSF binding (B) to human monocytes from different donors. 10^{-10}M radioligand was incubated with 4-6 x 10^6 cells and increasing concentrations of homologous and heterologous competitor cytokine. Specific cpm bound per 10^7 cells in the absence of competitor were 7,130 for ^{125}I-IL-3 binding and 1,060 for ^{125}I-GM-CSF binding. Each point represents the mean of at least 2 replicates.

A


PERCENT COMPETITION
100
80
60
40
20
0
-12
-11
-10
-9
-8
-7
[COMPETITOR] (log M)
rh IL-3
rh GM-CSF

B


PERCENT COMPETITION
100
80
60
40
20
0
-12
-11
-10
-9
-8
-7
-6
[COMPETITOR] (log M)
rh GM-CSF
rh IL-3

6.4 Competition for high and low affinity ^{125}I-CSF binding

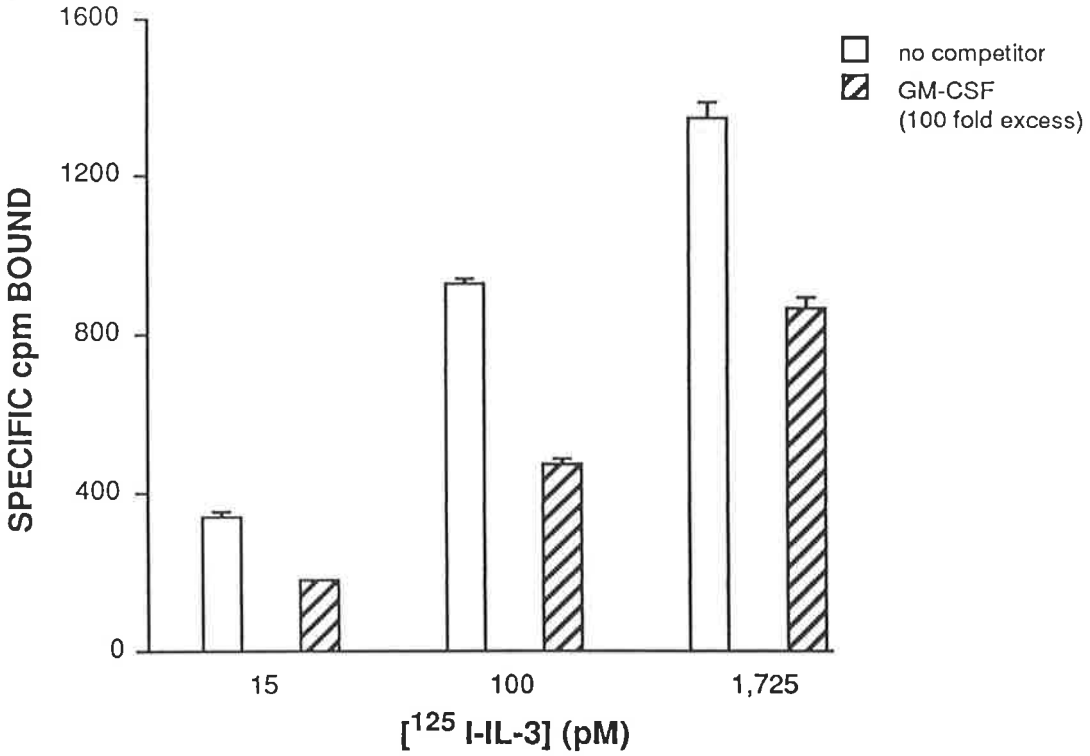
The competition experiments shown in Fig. 6.4 and 6.5 were performed using concentrations of radioligand of 100pM. This concentration, while exceeding the K_D values for high affinity binding for the two factors (Table 6.1), falls within or at the end of the steep initial component of their respective binding curves (Fig. 6.1 and 6.2). The competition observed is therefore predominantly for high affinity ^{125}I-CSF binding. To examine competition for the two affinity classes in greater detail, monocytes were incubated in different concentrations of ^{125}I-IL-3 with or without competitors, and specific binding determined (Fig. 6.6). GM-CSF inhibited 47% of ^{125}I-IL-3 binding at a radioligand concentration of 15pM. A similar proportion of binding was inhibited at a concentration of ^{125}I-IL-3 of 100pM (49%). In contrast, only 35% of binding was inhibited when radioligand was used in the low affinity range (1.7nM). A comparison of the number of counts bound for radioligand concentrations of 100pM (452cpm) and of 1.7nM (476pM) shows no significant increase in absolute competition despite large increases in total binding (100pM: 925cpm; 1.7nM: 1346cpm). These data suggest that competition by GM-CSF for ^{125}I-IL-3 binding to monocytes is largely for the high affinity binding sites.

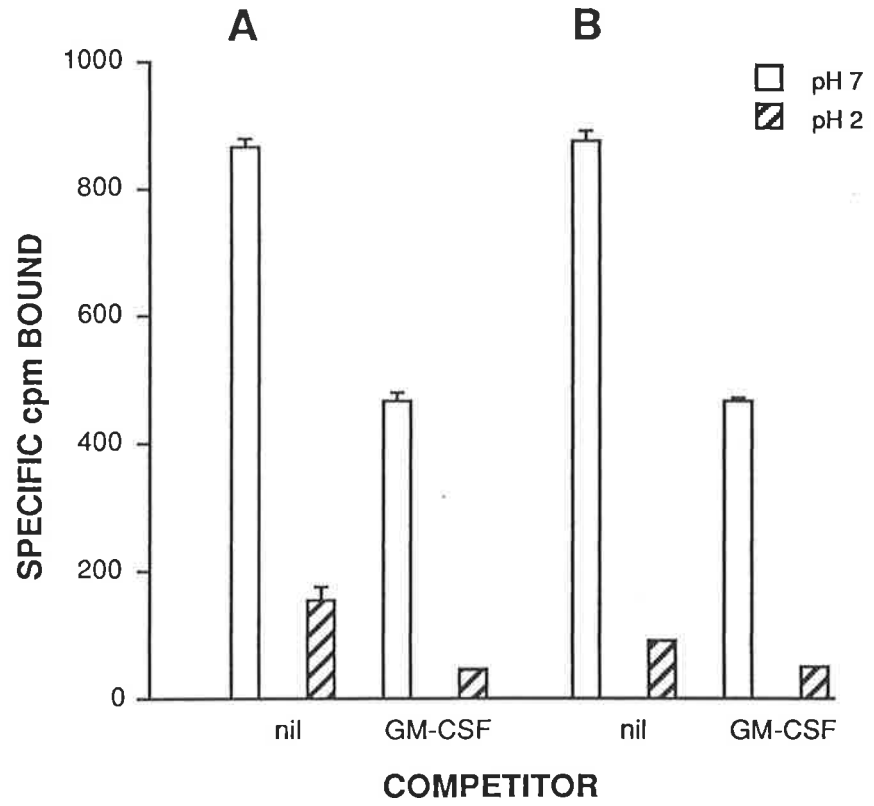
6.5 Mechanisms of competition

The work of Walker et al (1985A,B) on murine bone marrow cells showed that alterations in radioligand binding may result from CSF-induced heterologous receptor down-regulation. While the competition experiments reported here were performed at 4^{o}C, it was important to establish that CSF-receptor internalisation was not occurring. In the experiment shown in Fig. 6.7, monocytes were incubated overnight at 4^{o}C with 100pM ^{125}I-IL-3 without (Fig. 6.7A) or with (Fig. 6.7B) 0.1% sodium azide. Competitor cytokines at 100-fold excess concentration were included in some tubes. Bound ligand was then separated from free

Figure 6.6 Competition for high and low affinity binding sites for ^{125}I-IL-3. Monocytes were incubated for 18 hours at 4°C in the presence of 15 and 100pM ^{125}I-IL-3 (high affinity range) and 1,725pM ^{125}I-IL-3 (low affinity range). GM-CSF at 100-fold excess concentration was included in some tubes. Results are expressed as specific cpm bound (mean ± SD for 2 replicates), after subtraction of nonspecific binding at each point.

Figure 6.7 Dissociation of surface-bound ^{125}I-IL-3 from monocytes by acid washing. Monocytes were incubated for 18 hours at 4°C with 100pM ^{125}I-IL-3 with or without 10nM GM-CSF. Incubations were performed without (A) and with (B) 0.1% sodium azide. The cells were then centrifuged through a cushion of FCS at 4°C, the pellets were resuspended in binding medium without or with sodium azide, and aliquots exposed to either pH 7 binding medium (open bars) or pH 2 medium (hatched bars) for 2 minutes at 4°C. Cell-associated radioactivity was measured after a final centrifugation through FCS. Results are expressed as specific cpm bound (mean ± SD for 2 replicates). Identical results were obtained for ^{125}I-GM-CSF binding.


1600
1200
800
400
0
SPECIFIC cpm BOUND
no competitor
GM-CSF
(100 fold excess)
15
100
1,725
[125 I-IL-3] (pM)


A
B
1000
800
600
400
200
0
SPECIFIC cpm BOUND
pH 7
pH 2
nil
GM-CSF
nil
GM-CSF
COMPETITOR

ligand by centrifugation through FCS at 4°C, and aliquots of cells exposed to pH 7 or pH 2 media for 2 minutes at 4°C. Cell associated radioactivity was measured after a final centrifugation through FCS. The results show significant inhibition of ^{125}I-IL-3 binding by GM-CSF irrespective of the presence of sodium azide. Further, >82% of cell-associated radioactivity was acid dissociable, indicating that it was surface-bound. Identical results were seen with ^{125}I-GM-CSF binding (data not shown).

While IL-3 and GM-CSF show little primary sequence homology (Yang et al, 1988), there appear to be similarities in the predicted secondary and tertiary structures of these molecules, particularly at their C-termini (S. Leach, personal communication). The partial competition for binding between IL-3 and GM-CSF raised the possibility that the C terminal region might interact with a shared binding site on the monocyte membrane. To investigate this possibility, synthetic peptides comprising the N-terminal residues (1-53) and the C-terminal residues (54-127) of GM-CSF were used as competitors for ^{125}I-CSF binding. A peptide comprising nearly all of the natural GM-CSF molecule (residues 14-127) was used as a control (Fig. 6.8). While peptide 14-127 was almost as good a competitor for both ^{125}I-GM-CSF and ^{125}I-IL-3 binding as rhGM-CSF, peptide 51-53 and 54-127 showed no significant competition for either ligand.

DISCUSSION

The presence of high affinity binding sites for IL-3 and GM-CSF (Fig. 6.1, 6.2 and Table 6.1) was predicted from the results of functional experiments with these cytokines (Chapters 3 and 4). The apparent dissociation constants (K_D) represent concentrations of ligand at which 50% of surface receptors are occupied at equilibrium (Cuatrecasas et al, 1976). K_D values for IL-3 (8-38pM) and for GM-CSF (5-39pM) high affinity binding are similar, and compare well with half maximal biological responses at concentrations of CSF of

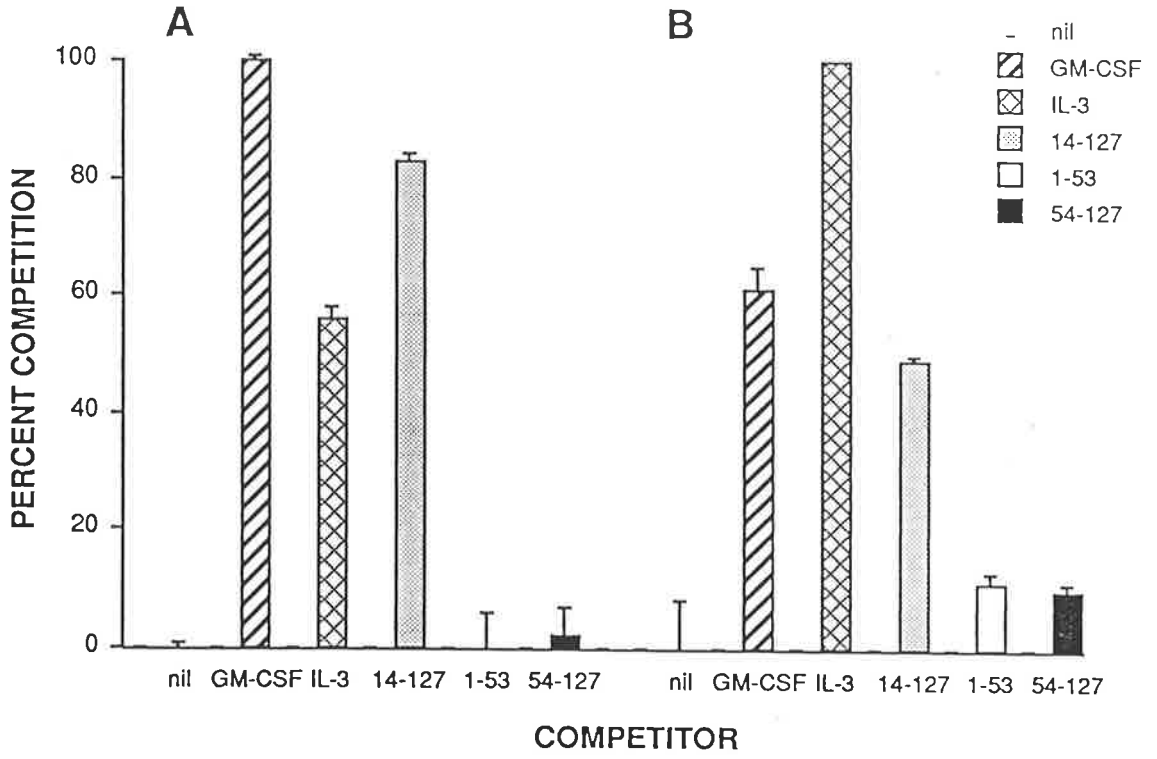


Figure 6.8 Competition for ^{125}I-GM-CSF (A) and ^{125}I-IL-3 (B) binding to monocytes by synthetic GM-CSF peptides. Monocytes were incubated for 18 hours at 4°C with 100pM ^{125}I-GM-CSF or ^{125}I-IL-3 with or without competitors. Peptides were used at 10,000-fold excess concentration (10^{-6}M) and unlabelled cytokines at 1,000-fold excess concentration (10^{-7}M). Results are expressed as percent competition (mean ± SEM for 3 replicates).

approximately 6pM (monocyte survival, Fig. 3.5A) and 20pM (adhesion, Fig. 4.4, Fig. 5.1, Fig. 5.2). These results are in keeping with numerous other reports, showing that CSF-induced biological responses are seen at levels of receptor occupancy as low as 10% (Nicola, 1987; Nicola et al, 1988). Also highlighted in Table 6.1 is the remarkably low number of high affinity binding sites seen on these cells. Only 266±128 high affinity sites for IL-3, and 31±11 sites for GM-CSF were seen per cell.

In addition to high affinity binding, both IL-3 and GM-CSF displayed low affinity binding to monocytes as indicated by the complex biphasic Scatchard plots (Fig. 6.1, 6.2). Analysis of the binding data by LIGAND confirmed a statistical preference for a two-site binding model, yielding K_D values and receptor numbers for the low affinity site as shown in Table 6.1 Cuatrecasas et al (1976) have described a number of causes of non-linear Scatchard plots other than the presence of low affinity sites. These include a failure to adequately control for non-specific binding at high ligand concentrations, inaccurate estimation of free ligand concentrations, the presence of negative cooperativity between receptors, the presence of ligand-ligand interactions leading to self-isomerization, and heterogeneity of the radioligand.

In the experiments reported here, non-specific binding was directly measured at each concentration of radioligand used, by including tubes containing a 100 fold excess of unlabelled CSF. Free ligand concentrations were corrected for maximal binding capacity, the specific radioactivity of the labelled protein was accurately determined by self-displacement analysis, and analysis of radiolabelled ligand on SDS-PAGE showed no evidence for self-isomerization (data not shown). In addition, the binding of ^{125}I-GM-CSF to neutrophils and monocytes from the same donor was measured concurrently (Fig. 6.3). This yielded a straight Scatchard plot and single receptor class for neutrophils, but a curvilinear plot and two receptor classes for monocytes. Although most of the experiments

reported here were performed at 4°C, the experiments performed in parallel with neutrophils took place at 24°C for 1 hour. In these experiments also, two classes of monocyte receptors were seen. Taken together, these results suggest that the non-linear Scatchard plots are not the result of an experimental artifact, and indicate instead the presence of both high and low affinity receptors for IL-3 and GM-CSF on these cells.

The dual binding site model presented here contrasts with data previously reported by Park et al (1989A) on the binding of IL-3 to human monocytes, which showed a single class of binding sites (124±4 sites per cell) with a K_D of 1.1 x 10^{-10}M. However, the methods used in the two studies can be distinguished by the cell populations employed. Park et al used 1 day old leukocyte layers to prepare monocytes and purified the cells on Percoll gradients. In contrast, the studies reported here employed cells less than 4 hours old purified by counter-current elutriation. The influence of methods of purification and cell maturity on monocyte phenotype could therefore explain the differences seen. Previous reports of a single class of binding sites for GM-CSF on human monocytes (450±75 sites per cell) were based on autoradiographic data (Gasson et al, 1986) or were presented without binding curves or Scatchard plots (Park et al, 1986; DiPersio et al, 1988), making a direct comparison with this work difficult. In addition, the methods used by each of these groups differed from those employed here.

Low affinity binding of CSF to haemopoietic cells has been reported by several authors. Walker and Burgess (1985A) showed high and low affinity binding to murine bone marrow cells, peritoneal exudate neutrophils and to the murine cell line WEHI-3B(D^+) in studies with mGM-CSF. Park et al (1989B) showed high and low affinity sites for IL-3 and GM-CSF on human acute non-lymphocytic leukemia (ANL) cells and on the human cell line KG-1. The same cell types showed only high affinity sites for G-CSF, and other cell lines, including HL-60, JM-1 and U937 showed only high affinity sites for IL-3 and GM-CSF. No

previous reports could be found of low affinity CSF binding to a primary cell type in man. The pattern of binding of CSF to myeloid cells is clearly heterogeneous, and these studies emphasise the dangers inherent in extrapolation from one cell/CSF combination to another.

The low concentrations of IL-3 and GM-CSF needed to stimulate monocyte survival and adhesion are consistent with an effect through binding to the high affinity receptors. While higher concentrations of CSF are needed to enhance monocyte ^{3}H-thymidine uptake (Fig. 3.5B), it remains to be established if this effect is mediated by the low affinity binding site. Low affinity binding sites for GM-CSF have recently been identified on the monkey kidney cell line COS (Cocita-Baldwin et al, 1989), where a K_D of 1.13±0.3nM was recorded. While this is of similar affinity to the low affinity site seen on monocytes, its significance across species is difficult to determine. In addition, it is not clear whether expression of the receptor on this cell line is associated with a biological response to GM-CSF. Low affinity GM-CSF receptors are also found on human placenta, and a GM-CSF-binding protein has recently been cloned from a human placental library and expressed in FDCP-1 cells (D. Gearing, personal communication). This receptor is of very low affinity (K_D 5.8nM) yet is capable of transduction of a proliferative signal given sufficiently high concentrations of CSF. These studies provide support for the contention that the low affinity CSF receptors on monocytes may be functional, and that the stimulation of ^{3}H-thymidine uptake in monocytes at high concentrations of CSF may be transduced via the low affinity receptors.

Of particular interest in these experiments was the demonstration of competition between ^{125}I-IL-3 and ^{125}I-GM-CSF for high affinity binding. In the experiment demonstrated in Fig. 6.5 it can be seen that up to 40% of ligand binding was inhibitable by the heterologous cytokine. The specificity of this interaction is demonstrated in Fig. 6.4, where other purified recombinant cytokines fail to act as competitive inhibitors. These include rhM-CSF, rhIFN-γ, and rhTNF-α, all known to have biological effects on human monocytes, and rhG-

CSF and rhIL-1 in addition. These experiments were performed using concentrations of radioligand of 100pM, which, while exceeding the K_D values for high affinity binding, are still well below the K_D values for low affinity sites on these cells. Examination of the binding curves (Fig. 6.1, 6.2 insets) shows that 100pM falls within or at the end of the steep initial component. The competition seen at this concentration of radioligand is therefore likely to be predominantly for high affinity sites. This is confirmed in Fig. 6.6, where competition is seen for 15 and 100pM ^{125}I-IL-3 binding to monocytes, but no further competition is seen when radioligand concentrations are increased to 1,725pM, suggesting that the low affinity site is non-competable.

Careful examination of the curves in Fig. 6.5 shows that IL-3 exhibited greater efficiency at competing for high affinity radioligand binding at low concentrations than did GM-CSF. This finding was unexpected, given the equivalent affinities of binding for the two radioligands. Recently, however, it was reported that *E-coli*-derived GM-CSF showed much higher affinity for binding to human neutrophils than did CHO derived material (Kelleher et al, 1988). The major difference between the two lay in the fact that *E-coli*-derived CSF is non-glycosylated. In the experiments shown here, the IL-3 and GM-CSF used for radiolabelling were both glycosylated products. However, non-glycosylated *E-coli*-derived IL-3 was used as a competitor cytokine, a fact which may explain the differential function of IL-3 and GM-CSF as competitors in Fig. 6.5. In subsequent kinetic binding experiments (Chapter 7) a glycosylated CHO-derived IL-3 preparation was used as a competitor, in order to be able to compare IL-3 and GM-CSF as competitors more equally.

The presence of cross-competition for binding between IL-3 and GM-CSF requires further comment. Previous work on murine bone marrow cells (Walker et al, 1985A) demonstrated partial inhibition of ^{125}I-mGM-CSF binding by mIL-3 at 20^{o}C and 37^{o}C but not at 4^{o}C. The inhibition was interpreted as resulting from down-regulation or modification of the

GM-CSF receptor, and the authors have subsequently proposed a model of CSF receptor trans-downmodulation on murine bone marrow cells to explain the findings (Walker et al, 1985B). Several other examples can be found of modulation of receptor expression by heterologous agents. LPS, phorbol esters and chemotactic peptides have all been shown to influence expression of receptors for G-CSF, GM-CSF and M-CSF on a variety of cell types (Nicola, 1987), and more recently, IL-1α and TNF-α were shown to trans-downmodulate the receptor for epidermal growth factor (EGF) at 37^{o}C, despite showing no competition for ^{125}I-EGF binding at 4^{o}C (Bird et al, 1989). Finally, IL-4 binding to T and B cell lines resulted in a reduction in surface expression of high affinity IL-2 receptors, as determined by ^{125}I-IL-2 binding experiments and by cross-linking (Fernandez-Botran et al, 1989). This example may fall into a different category to those mentioned above, for although the authors concluded that the phenomenon represented IL-2 receptor trans-downmodulation, the persistance of competition at 4^{o}C suggests a more direct interaction between IL-2 and IL-4 at the cell surface. The competition experiments reported here were performed at 4^{o}C either in the presence or absence of 0.1% sodium azide. Under these conditions, >82% of bound radioactivity was acid-dissociable (Fig. 6.7), indicating that it was surface-bound and not internalised. These findings suggest that the observed competition between ^{125}I-IL-3 and ^{125}I-GM-CSF for binding to human monocytes is not a result of receptor trans-downmodulation.

Competition for ^{125}I-CSF binding at 4^{o}C is becoming apparent in other cell types. For example, IL-3 and GM-CSF compete for binding to human eosinophils and IL-3 and GM-CSF compete for binding to basophilic leukemia cells (Lopez et al, 1989, and personal communication). In addition, GM-CSF and IL-3 compete for binding to the KG-1 leukemia cell line (Gesner et al, 1988; Park et al, 1989A), and GM-CSF competed for IL-3 binding in each of 5 cases of ANL tested (Park et al, 1989B). In the converse experiment, however, only 2 of the 5 cases showed significant competition for GM-CSF binding by IL-3.

The explanation for the partial competition between IL-3 and GM-CSF for high affinity binding to monocytes is unclear, but the data would support at least two separate models. A non-random distribution of CSF receptors on the cell membrane, with association between specific high affinity IL-3 and GM-CSF binding sites, is one such model. Alternatively, there may be three types of high affinity receptors on monocytes for the binding of IL-3 and GM-CSF. One receptor type binds only IL-3, a second is specific for GM-CSF, and the third is a common receptor, capable of binding both cytokines. Attempts were made to test the latter hypothesis in the synthetic GM-CSF peptide experiments (Fig. 6.8). These experiments arose out of the observation that while IL-3 and GM-CSF show little primary sequence homology, the close tandem linkage of their respective genes on the long arm of chromosome 5 suggests that they have evolved from a common ancestral gene (Yang et al, 1988). In addition, similarities in the predicted secondary and tertiary structures of GM-CSF and IL-3, particularly at their C-termini raise the possibility that the C-terminal region of IL-3 and GM-CSF may interact with a common binding site on monocytes. The experiment shown in Fig. 6.8, in which synthetic N-terminal and C-terminal peptides of GM-CSF were assessed for their capacity to compete for ^{125}I-GM-CSF and ^{125}I-IL-3 binding, was designed to test this hypothesis. Neither peptide significantly inhibited ^{125}I-CSF binding, although the (near) full length peptide (14-127) caused significant inhibition. These results should be interpreted with caution as the experiments did not control for the influence of tertiary structure on the conformation of the proposed binding site. The use of synthetic peptides with point mutations or deletions in the C-terminus may therefore yield further information.

Unlike binding to high affinity sites, low affinity ^{125}I-CSF binding was not inhibited by the non-cognate ligand (Fig. 6.6). This concurs with data from human placenta, where low affinity binding of GM-CSF is not competable by IL-3. As might be expected, binding of ^{125}I-GM-CSF to the cloned low affinity GM-CSF receptor, which was derived from a

human placental library, also fails to show competition by IL-3 (D. Gearing, personal communication). These findings add a further layer of complexity to an already complicated binding picture, and have implications for any model of CSF receptors which might be proposed. The experiments reported in Chapter 7 attempt to further define this receptor system.

SUMMARY

In this chapter, IL-3 and GM-CSF were shown to bind with both high and low affinity to human monocytes. An average of 266 high affinity IL-3 binding sites, and 31 high affinity GM-CSF binding sites were seen per cell, and K_D values were shown to correlate well with concentrations of CSF required for a biological response. IL-3 and GM-CSF showed partial, but not complete, competition for binding to the high affinity sites under conditions shown to prevent receptor internalisation. Linkage of unique CSF receptors, or the presence of a third common CSF receptor were models proposed to explain these findings. The low affinity CSF binding sites were present in greater numbers than the high affinity sites (IL-3: 1976 per cell; GM-CSF: 381 per cell) and showed mean K_D values 30-50 times higher. Unlike the high affinity sites, however, low affinity CSF binding did not show cross-competition.

CHAPTER 7

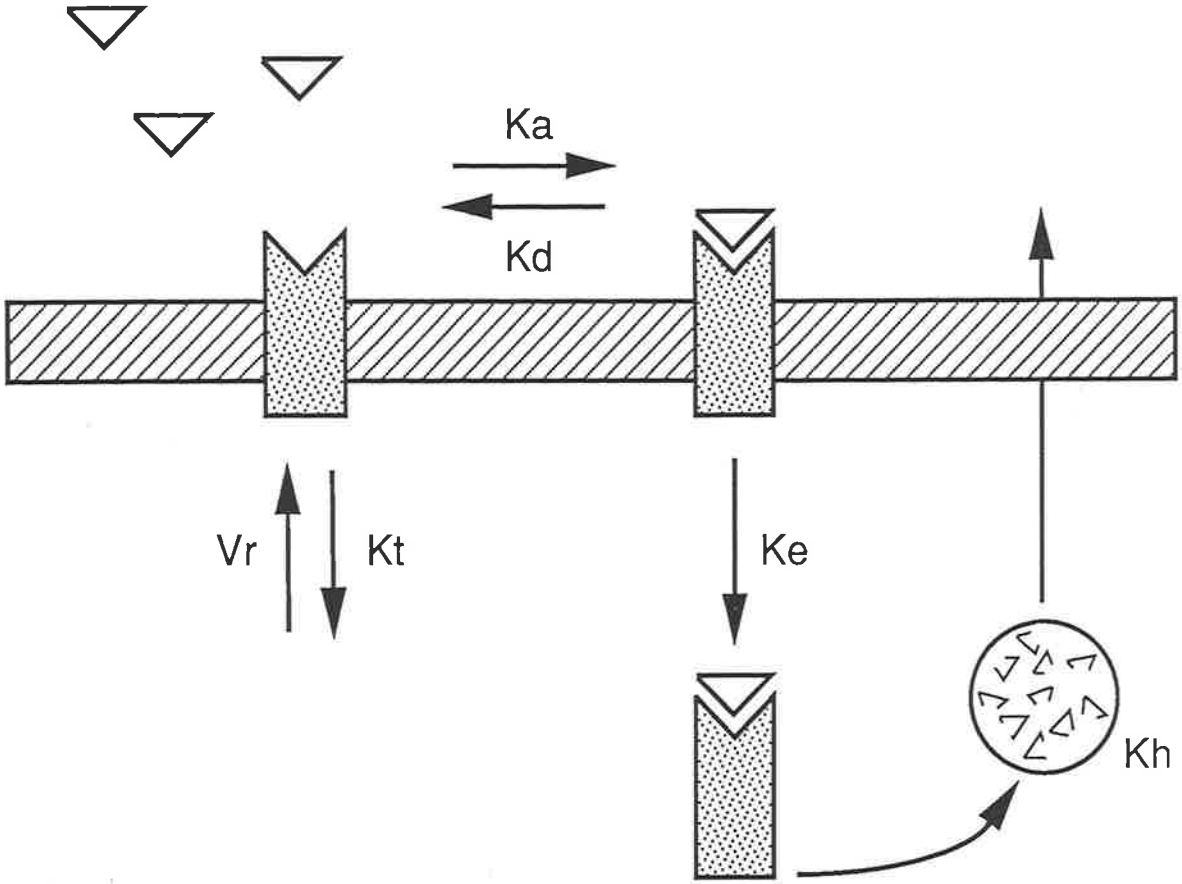
KINETIC BINDING STUDIES WITH ^{125}I-IL-3 AND ^{125}I-GM-CSF

INTRODUCTION

The data presented in chapter 6 regarding CSF receptor number, affinity and specificity were gathered under equilibrium binding conditions. Although universally employed, the validity of this approach has recently been questioned (Guilbert et al, 1986; Nicola et al, 1988), in part because of the nearly irreversible binding of some ligands at 4°C, and the lack of relevance of some affinity data so gathered to binding at physiological temperatures. An alternative approach to the study of ligand-receptor interactions involves separate measurements of each of several processes which contribute to the final complex formation and subsequent processing. In this kinetic model of binding (Fig. 7.1), each process is described by a rate constant (K value), allowing comparisons to be made between different processes or ligand-receptor systems. As well as allowing determination of an affinity constant for binding at 37°C (K_D), kinetic binding studies provide details of association and dissociation rate constants (Ka and Kd respectively) and of receptor-ligand complex internalisation (Ke), ligand degradation (Kh) and unoccupied receptor turnover (Vr and Kt).

A kinetic approach to binding studies was considered relevant to the subject of this thesis for several reasons. Firstly, although apparent K_D values for high affinity sites derived from the equilibrium data appeared to correlate well with biological effects, independent corroboration was desirable. Secondly, the demonstration of internalisation of radioligand under appropriate conditions would provide further support for the contention that the ^{125}I-CSF binding sites were in fact receptors. Thirdly, by including competitor cytokines in the binding experiments, further information on the nature of competable and non-competable sites might be revealed. Finally, the differential rates of monocyte stimulation seen in early phase adhesion (Fig. 4.2) might be explainable in terms of more rapid association of ^{125}I-GM-CSF with its binding site, or by differences in receptor-ligand processing.

Figure 7.1 The kinetic model of receptor-ligand interactions. The open triangles represent ligands, the stippled bars represent receptors, and the cross-hatched area is the plasma membrane. The circle represents a lysosome which is degrading internalised ligand. The rate constants shown are: K_a, association rate constant; Kd, dissociation rate constant; Vr, rate of insertion of new receptors into the membrane; K_t, rate of internalisation of unoccupied receptors; Ke, rate of internalisation of receptor-ligand complexes; Kh, rate of ligand degradation.


Ka
Kd
Vr
Kt
Ke
Kh

In this chapter, data are presented describing association, dissociation and internalisation of ^{125}I-IL-3 and ^{125}I-GM-CSF in monocytes. The experiments were performed with specific focus on the high affinity receptor class. Concentrations of radioligand of 200pM were used in order to maximise high affinity binding, and to minimise binding to low affinity sites. However, it is conceded that some low affinity binding was likely to be occurring at this concentration, and so the results may be in part influenced by this fact. Heterologous competitors were included in some experiments in an attempt to distinguish kinetic parameters for competable and non-competable binding sites.

RESULTS

7.1 Theoretical background

The data presented in this chapter have been analysed by KINETIC on the basis of the relationships described by McPherson (1985) and summarised below.

The association of radioligand with its receptor is a second order process depending on the concentration of ligand (L) and of receptor (R) as described below:

$$[R] + [L] \xrightarrow{Ka} [RL]$$

where Ka is the association rate constant.

By using a ligand concentration in vast excess of receptor concentration (i.e. by ensuring less than 10% of ligand is bound at equilibrium) the reaction can be treated as a pseudo-first order process, and the first order rate equation can be used. The simplified first order rate equation for association to a single site is shown below:

$$[L_t] = [L_e] . (1 - e^{-Kobs.t})$$

where L_t and L_e are the amount of ligand bound at time = t and at equilibrium respectively, and where Kobs is the observed association rate constant.

The dissociation of a radioligand is, in contrast, a first order process depending solely on the concentration of complex. Thus:

$$[RL] \xrightarrow{Kd} [R] + [L]$$

where Kd is the dissociation rate constant, and

$$[L_t] = [L_o].e^{-Kd.t}$$

where L_o is the concentration of bound ligand at t = o.

From Kobs and Kd, the actual association rate constant, Ka, can be calculated:

$$Ka = (Kobs - Kd) / [L]$$

and the kinetic dissociation constant revealed:

$$K_D = Kd / Ka$$

The internalisation rate constant, Ke, was calculated from the relationship described by Wiley and Cunningham (1981):

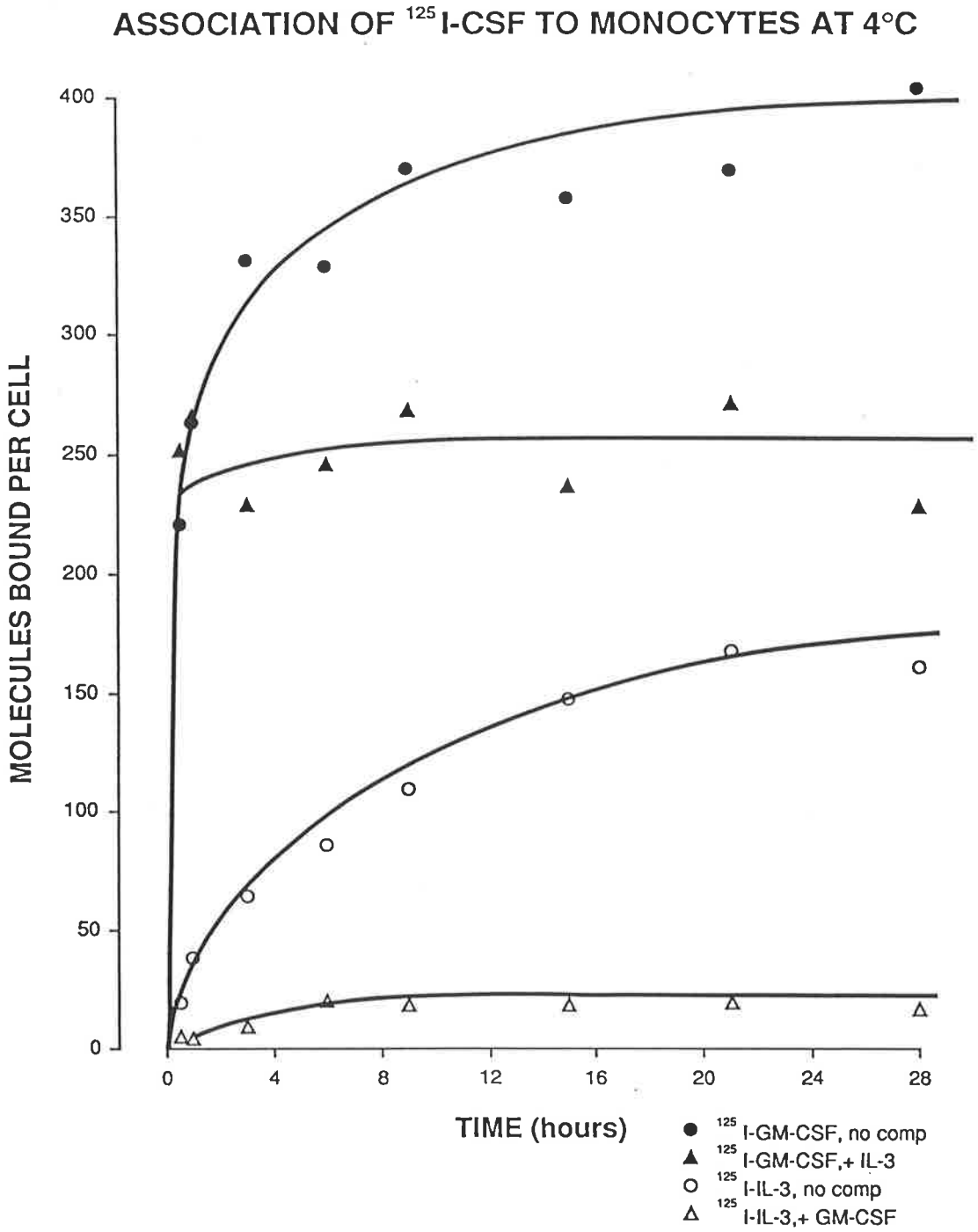
$$t.Ke = [LR]in / [LR]sur$$

where [LR]in and [LR]sur are the concentrations of ligand-receptor complexes inside and on the surface of the cells respectively. This relationship holds true only if there is no degradation of the internalised ligand, and so only time points within the first 30 minutes of incubation have been used to calculate Ke.

7.2 Association Kinetics at 4°C

A time course of association of ^{125}I-IL-3 and ^{125}I-GM-CSF to monocytes at 4°C is shown in Fig. 7.2. Although both ligands were used at a concentration of 200pM, ^{125}I-GM-CSF

Figure 7.2 Association of ^{125}I-CSF to monocytes at 4°C. Purified monocytes were incubated with 200pM radioligand at 4°C, with or without competitor cytokines. Specific cell-associated radioactivity was determined at the indicated times after centrifugation through FCS. Maximum cpm bound were less than 4.5% of total cpm added. Each point is the mean of two replicates.


ASSOCIATION OF 125I-CSF TO MONOCYTES AT 4°C
MOLECULES BOUND PER CELL
400
350
300
250
200
150
100
50
0
0
4
8
12
16
20
24
28
TIME (hours)
125I-GM-CSF, no comp
125I-GM-CSF,+ IL-3
125I-IL-3, no comp
125I-IL-3,+ GM-CSF

showed higher levels of binding than ^{125}I-IL-3, and approached equilibrium more rapidly. Inclusion of unlabelled GM-CSF in some tubes with ^{125}I-IL-3 resulted in near complete inhibition of ^{125}I-IL-3-binding, while IL-3 inhibited approximately 40 percent of ^{125}I-GM-CSF binding. In terms of absolute cpm, the degree of inhibition was similar for both ligands.

Binding data were analysed by KINETIC (McPherson, 1985) using a weighted non-linear curve fitting technique. Observed association rate constants (Kobs) for two experiments performed at 4^{o}C are shown in Table 7.1. Mean values for ^{125}I-GM-CSF association are approximately 10-fold greater than for ^{125}I-IL-3 (0.02 min^{-1}, 0.0024 min^{-1} respectively). Half times for association (t1/2) were 35 minutes (^{125}I-GM-CSF) and 4.8 hours (^{125}I-IL-3).

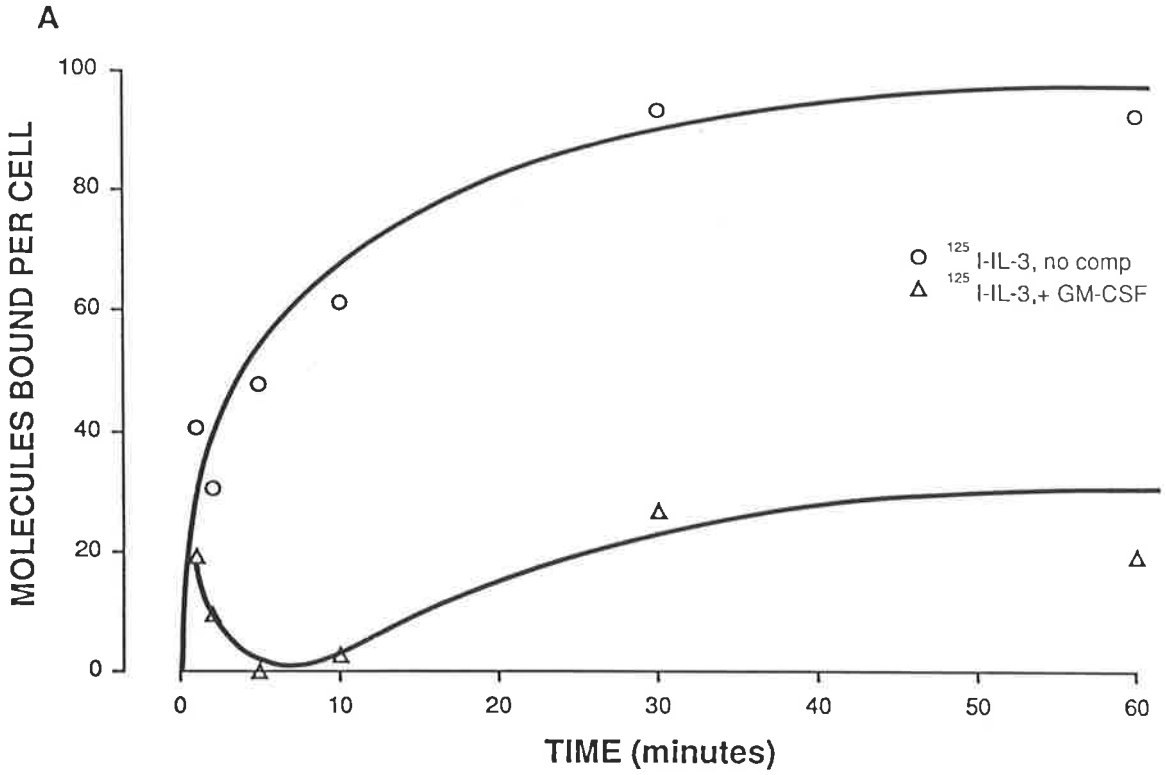
The presence of near-maximal values for non-competable ^{125}I-GM-CSF binding at the first time point (Fig. 7.2) precludes an accurate assessment of Kobs for this fraction of binding sites. Nevertheless, it is apparent from Fig. 7.2 that non-competable binding reaches plateau earlier than its competable counterpart, implying a more rapid association rate.

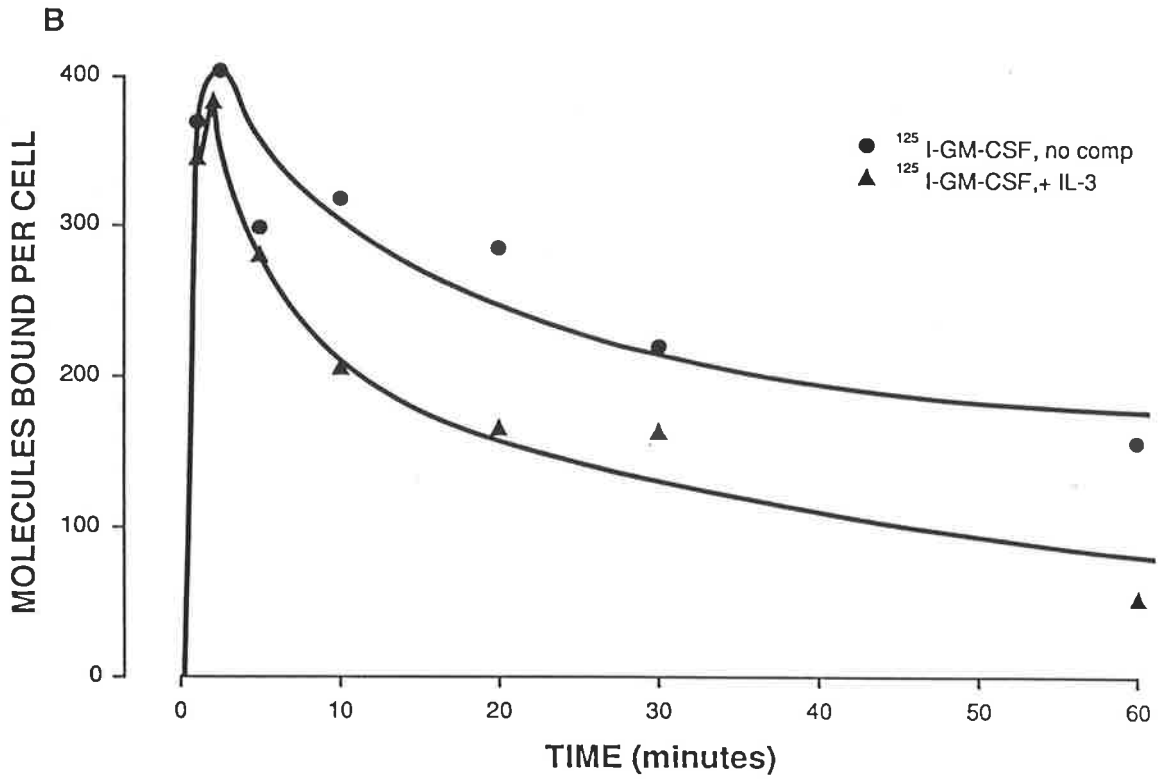
7.3 Association Kinetics at 37^{o}C

The association of both ligands to monocytes at 37^{o}C is shown in Fig. 7.3. ^{125}I-IL-3 showed rapid association, with plateau at approximately 100 molecules per cell after 30-60 minutes incubation. ^{125}I-GM-CSF binding was almost complete at 1 minute, peaked at 2 minutes, and then showed a decline. Receptor internalisation was prevented in this experiment by including 0.1% sodium azide in the binding medium. The decline in ^{125}I-GM-CSF binding is therefore most likely due to dissociation from the receptor, and not to internalisation and subsequent degradation of the ligand. Heterologous CSF were added to

Figure 7.3 Association of ^{125}I-IL-3 (A) and ^{125}I-GM-CSF (B) to monocytes at 37°C. Procedures were as described for Fig. 7.2, but with incubation at 37°C in the presence of 0.1% sodium azide. Competitor cytokines were added simultaneously with radioligand. Maximum cpm bound were less than 1% of total cpm added. Each point is the mean of two replicates.

ASSOCIATION OF ^{125}I-CSF TO MONOCYTES AT 37°C



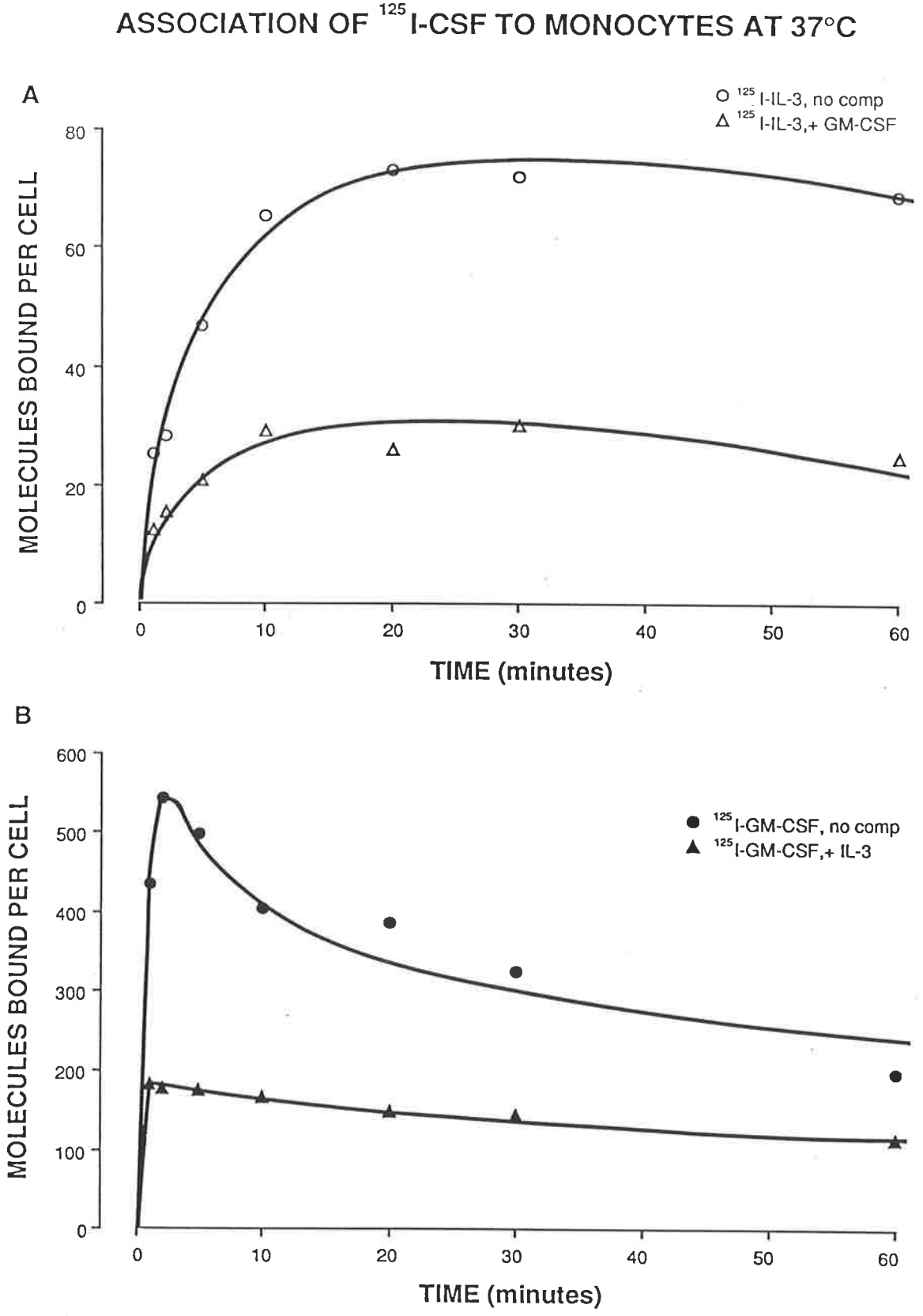


some tubes simultaneously with the radioligands. The IL-3 and GM-CSF preparations used as inhibitors in these experiments were both glycosylated, as were the radioligands, removing glycosylation state from consideration of the variables involved (Kelleher et al, 1988). The patterns of inhibition seen mirrored the binding patterns of the two radioligands, with rapid inhibition of ^{125}I-IL-3 binding by GM-CSF (Fig. 7.3A) and a more gradual inhibition of ^{125}I-GM-CSF binding by IL-3 (Fig. 7.3B).

Fig. 7.4 shows a second experiment, where monocytes were preincubated with the competitors for 15 minutes at 37^{o}C prior to the addition of radioligand. Using this protocol, competition was immediately evident for both combinations of ligands. Observed association rate constant (Kobs) for these experiments are shown in Table 7.1. Mean half times (t1/2) for association are 2.5 minutes for ^{125}I-IL-3 and 40 seconds for ^{125}I-GM-CSF. Values for ^{125}I-GM-CSF binding are approximate only, owing to the difficulty in measuring binding at time intervals of less than one minute. Association rates are 50-100 fold greater in these experiments than for the same ligands at 4^{o}C, demonstrating the temperature dependence of CSF-receptor interactions.

As with binding at 4^{o}C, association of non-competable ^{125}I-GM-CSF binding at 37^{o}C was too rapid to permit an accurate estimate for Kobs for this fraction, with maximal binding seen at the first time point measured (1 minute, Fig. 7.4B). Non-competable binding was, however, clearly more rapid than competable binding. For ^{125}I-IL-3, observed association rates of 0.52 min^{-1} and 0.27 min^{-1} were obtained for non-competable and competable binding respectively for the experiment shown in Fig. 7.4A. These data show that the competable receptor phenotype is associated with slower ligand association.

Figure 7.4 Association of ^{125}I-IL-3 (A) and ^{125}I-GM-CSF (B) to monocytes at 37°C. Procedures were as described for Fig. 7.3, but with preincubation of cells with competitors for 15 minutes at 37°C, prior to addition of radioligands. Maximum cpm bound were less than 9% of total cpm added. Each point is the mean of two replicates.


ASSOCIATION OF 125I-CSF TO MONOCYTES AT 37°C
A
O 125I-IL-3, no comp
Δ 125I-IL-3,+ GM-CSF
MOLECULES BOUND PER CELL
0
20
40
60
80
0
10
20
30
40
50
60
TIME (minutes)
B
● 125I-GM-CSF, no comp
▲ 125I-GM-CSF,+ IL-3
MOLECULES BOUND PER CELL
0
100
200
300
400
500
600
0
10
20
30
40
50
60
TIME (minutes)

7.4 Dissociation Kinetics at 37°C

To measure rates of ligand dissociation, 200pM radioligand was incubated with monocytes overnight at 4°C to achieve equilibrium, and unbound radioactivity was removed by centrifugation through an FCS cushion. The cells were resuspended in cold medium containing 0.1% sodium azide, aliquotted into separate tubes, and a temperature shift to 37°C performed. Non-specific binding was measured at each time point, as with all other binding experiments reported here. ^{125}I-IL-3 showed a gradual dissociation over 40 minutes, with a Kd value of 0.022 min^{-1} (Fig. 7.5A and Table 7.1). Dissociation of ^{125}I-GM-CSF, as predicted from Fig. 7.3 and 7.4, was more rapid, with a precipitous fall in cpm bound and plateau at 20-40 minutes (Kd 0.21 min^{-1}).

Determination of the rate constants for dissociation allows the calculation of the actual association rate constants (K_a) for ligand binding, according to the formula outlined in Section 7.1. This yields a K_a value for ^{125}I-IL-3 binding to monocytes of 1.3 x $10^9 M^{-1}$ min^{-1}, and for ^{125}I-GM-CSF binding of >4 x $10^9 M^{-1}$ min^{-1}. Calculation of kinetic dissociation constants using the formula:

$$K_D = Kd / Ka$$

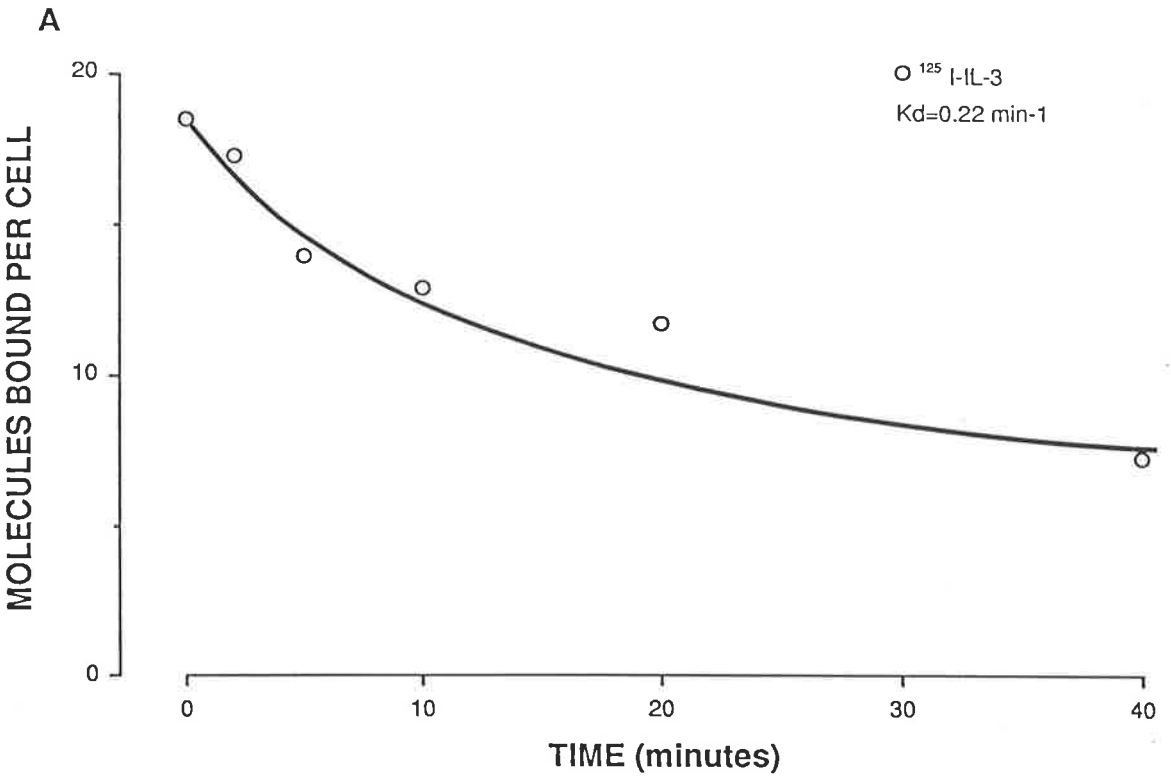
yields values of 17pM for ^{125}I-IL-3 and <53pM for ^{125}I-GM-CSF. K_D values derived from equilibrium binding experiments (Chapter 6) were 8-38pM for ^{125}I-IL-3 and 5-39pM for ^{125}I-GM-CSF, showing good concordance with the kinetically derived values.

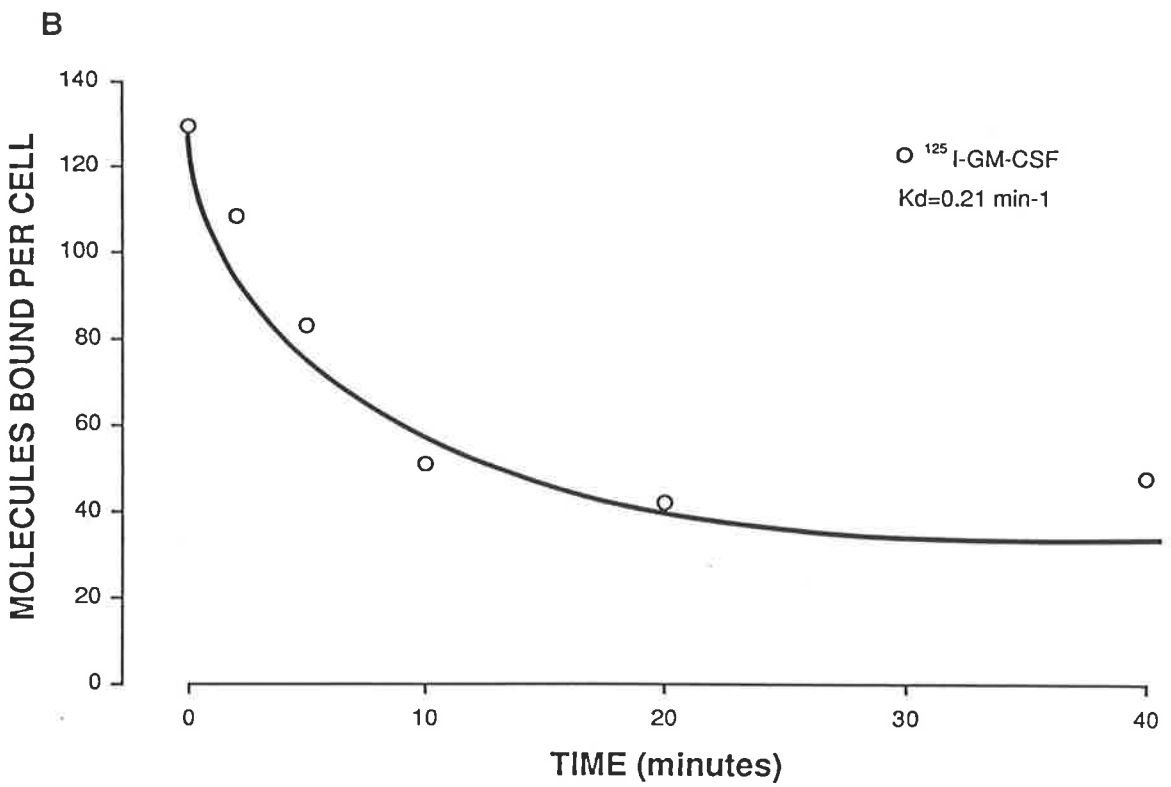
7.5 Internalisation of CSF-receptor complexes

For measurement of internalisation, ^{125}I-CSF were pre-equilibrated with monocytes overnight at 4°C as described in section 7.3, but with the omission of sodium azide. Following a temperature shift to 37°C, aliquots of cells were removed at regular time points,

Figure 7.5 Dissociation of ^{125}I-IL-3 (A) and ^{125}I-GM-CSF (B) from monocytes at 37°C. Monocytes were incubated at 4°C for 18 hours with 200pM ^{125}I-CSF, centrifuged through FCS, and resuspended in binding medium with 0.1% sodium azide. Specific cell-associated radioactivity was measured at the indicated times after a temperature shift to 37°C at t=0. Each point is the mean of two replicates.

DISSOCIATION OF ^{125}I-CSF FROM MONOCYTES AT 37°C





exposed to either pH 2 or pH 7 medium for 2 minutes at 4°C, and centrifuged through FCS. Total cell associated radioactivity was defined as that present after a pH 7 wash, while internalised radioactivity was that present after washing at pH 2. Surface-bound radioactivity was derived by subtracting pH 2 from pH 7 cpm at each time point. Internalisation of ^{125}I-IL-3 is shown in Fig. 7.6A, where pH 2-resistant binding shows a rapid rise after 2 minutes at 37°C, with plateau at 20 minutes. Surface bound radioactivity shows a coincident decline, reflecting a combination of dissociation to the medium, and internalisation into the cells. The ratio of internalised:surface bound radioactivity is plotted against time in Fig. 7.6B, yielding the internalisation rate constant, Ke (Wiley and Cunningham, 1981). Ke values for two experiments with ^{125}I-IL-3 are shown in Table 7.1, with a mean ± SD of 0.05±0.002 min^{-1}. Similar results were obtained with ^{125}I-GM-CSF internalisation (Fig. 7.7, and Table 7.1). Ke values were remarkably constant, despite differing degrees of competition in these experiments (^{125}I-IL-3 : Exp. 1:95% competition; Exp. 2 : 18% competition; ^{125}I-GM-CSF : 55% competition). These findings suggest that competable and non-competable receptors do not differ markedly in their rate of internalisation.

Figure 7.6 Internalisation of ^{125}I-IL-3 in monocytes. Monocytes were incubated with 200pM ^{125}I-IL-3 at 4°C for 18 hours, and processed as described for Fig. 7.5. Cells were resuspended in azide-free medium, warmed to 37°C, and washed at pH 7 or pH 2 for 2 minutes at 4°C at the indicated times. Cell-associated radioactivity was measured after a final centrifugation through FCS. The pattern of internalised (●) surface bound (○) and total cell-associated radioactivity (□) with time is shown in A. Derivation of the internalisation rate constant (K_e) is shown in B. Each point is the mean of two replicates.

INTERNALISATION OF ^{125}I-IL-3 IN MONOCYTES

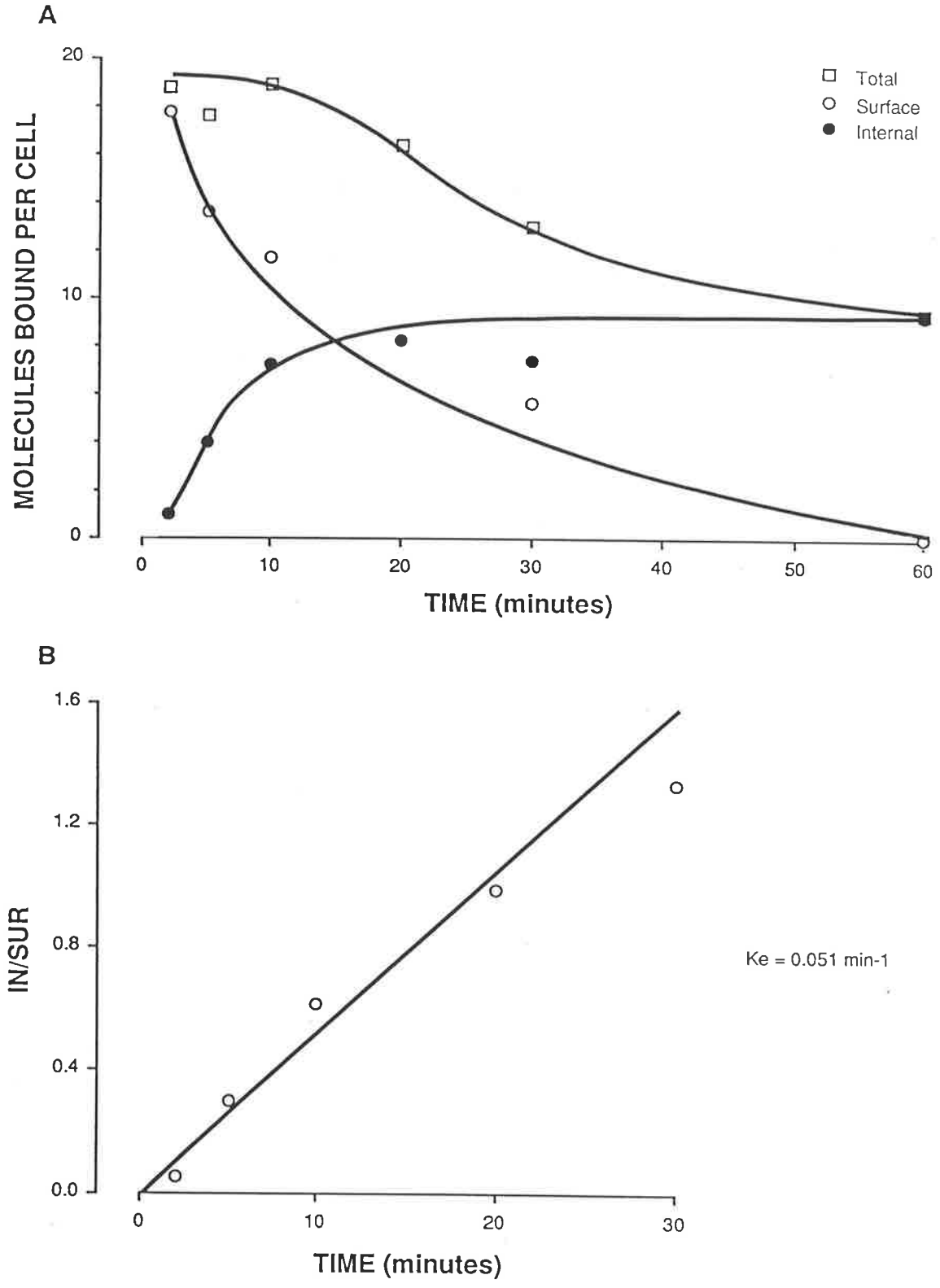
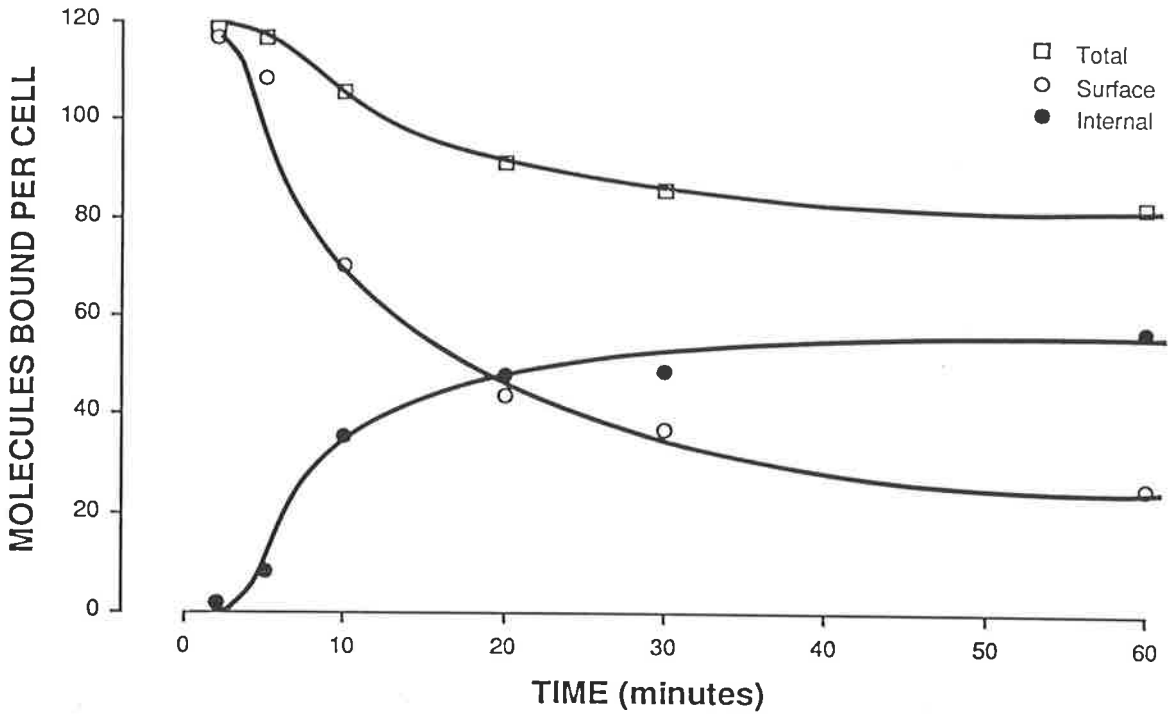


Figure 7.7 Internalisation of ^{125}I-GM-CSF in monocytes. Experimental procedures were as outlined for Fig. 7.6. A, changes in internalised (●), surface bound (○) and total cell-associated radioactivity (□) with time. B, derivation of K_e for ^{125}I-GM-CSF.

INTERNALISATION OF ^{125}I-GM-CSF IN MONOCYTES

A



B

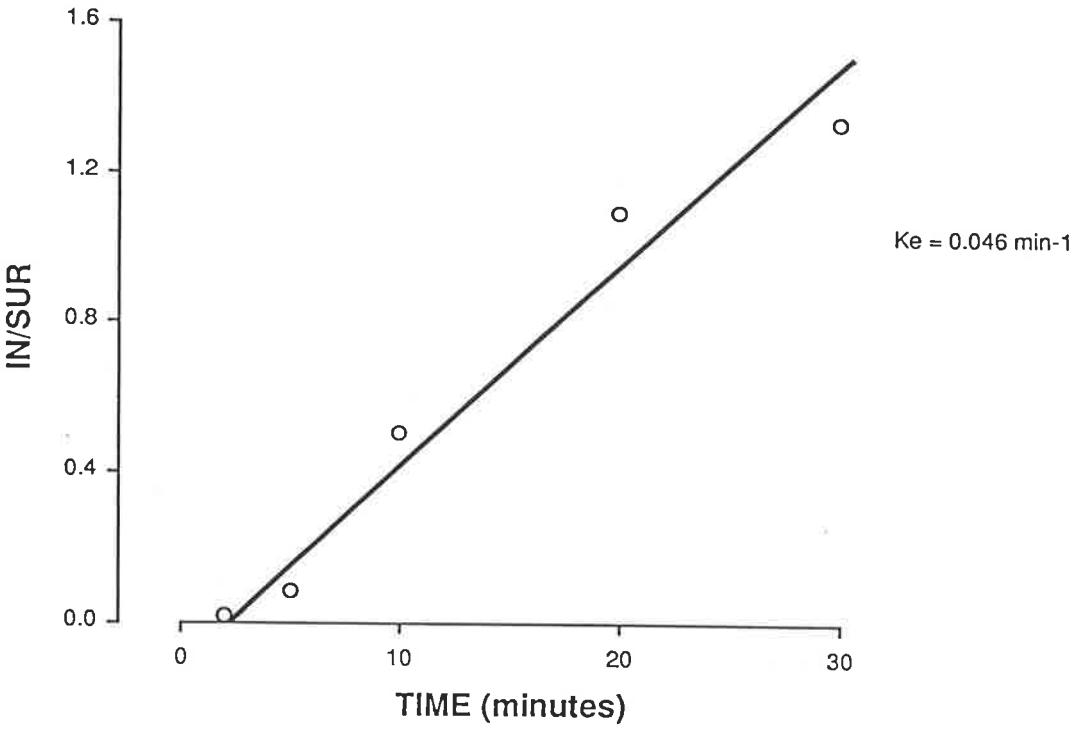


Table 7.1 Kinetic parameters* for ^{125}I-CSF interaction with monocytes.

^{125}I-IL-3

Temp (°C)	Kobs (min^{-1})	Kd (min^{-1})	Ka (M^{-1} min^{-1})	Ke (min^{-1})
4°	1) 0.0042 2) 0.00069 0.0024±0.0018**	-	-	-
37°	1) 0.18 2) 0.38 0.28±0.1**	0.022	1.3 x 10^9	1) 0.051 2) 0.048 0.050±0.002**

^{125}I-GM-CSF

Temp (°C)	Kobs (min^{-1})	Kd (min^{-1})	Ka (M^{-1} min^{-1})	Ke (min^{-1})
4°	1) 0.029 2) 0.010 0.020±0.010**	-	-	-
37°	1) >1.0 2) >1.0	0.21	>4 x 10^9	1) 0.046

* Determined as described in the text.

** Mean ± SD for two experiments.

DISCUSSION

The experiments reported in this chapter have allowed calculation of kinetic K_D values for the binding of IL-3 and GM-CSF to monocytes of 17pM and <53pM respectively. The value for IL-3 is consistent with data from equilibrium studies, where high affinity K_D values ranged from 8-38pM. The kinetically-derived K_D value for GM-CSF must be regarded as approximate only, owing to the difficulty encountered in measuring very fast rates of association. Nevertheless, the value is comparable to those obtained by equilibrium methods (5-39pM). These findings are consistent with the concentrations of IL-3 and GM-CSF needed to achieve a biological response in monocytes, and provide further support for the existence of very high affinity receptors for these growth factors.

Analysis of the association and dissociation rate constants for IL-3 and GM-CSF is of particular interest. Both growth factors showed a marked temperature dependence for association, with Kobs values at 37°C 50-100 fold greater than at 4°C. Such temperature dependence seems to be a feature of CSF binding in general (Park et al, 1989A) but is not seen with many other cytokines including IL-1 and IL-4 (Park et al, 1987, and Downer et al, 1985). A comparison of observed association rates for the two cytokines shows Kobs values 8 fold (4°C) and at least 3.5 fold (37°C) greater for GM-CSF than for IL-3. In addition, GM-CSF dissociates more rapidly than does IL-3. Neither dissociation curve shown in Fig. 7.5 is that of a simple first order process, and KINETIC showed a better fit for the data using a biexponential model. The improvement in fit was not, however, statistically significant, and Kd values for a mono-exponential model have therefore been used (IL-3: 0.022 min^{-1}; GM-CSF: 0.21 min^{-1}). Similar complex dissociation kinetics have been previously observed for other cell surface receptor systems (Park et al, 1986), and their explanation remains unclear. However, it is apparent that low affinity CSF-binding sites generally show more rapid dissociation than do their high affinity counterparts, although rates of association

tend to be similar (N. Nicola, personal communication). While it is difficult to be certain how much low affinity binding took place in the experiments reported here, the biphasic dissociation curves would be consistent with the presence of low affinity as well as high affinity CSF-receptor complexes.

Account is taken of the differing Kd values for IL-3 and GM-CSF and of ligand concentration in the calculation of the actual association rate constants (Ka, Table 7.1), yielding a value for GM-CSF association which is at least 3 fold greater than for IL-3. These data show that while association of both factors at 37°C is rapid, GM-CSF association is particularly so. This finding may explain the more rapid activation of monocytes induced by GM-CSF in the early phase adhesion experiments (Fig. 4.2).

The demonstration of CSF internalisation by monocytes (Fig. 7.6 and 7.7) is important for several reasons. Firstly, while no single property can distinguish receptor from non-receptor interactions, a number of features of true receptor-ligand interactions have been described (Cuatrecasas et al, 1976). These include reversibility and saturability of binding, expression of binding sites in an appropriate tissue distribution and of appropriate affinity for biological effects, inhibitability of binding by analogues and/or antagonists, and the demonstration of receptor-ligand complex internalisation. While no data is at present available regarding the use of analogues or antagonists for CSF binding to monocytes, each of the other criteria listed above has been fulfilled for this interaction. The high affinity binding sites for IL-3 and GM-CSF on monocytes are therefore most likely true receptors. Secondly, the Ke data derived for IL-3 and GM-CSF internalisation are very similar (0.05 min^{-1}, 0.046 min^{-1}, respectively). Previously published values for IL-3 internalisation are limited to tumour cell lines (Nicola et al, 1988; Murthy et al, 1989; Gesner et al, 1988). Nicola showed a range of Ke values for IL-3 on different murine cell lines of 0.032-0.067 min^{-1}, and Murthy showed internalisation in the murine line $B6SU_tA$, with t1/2 of 15 minutes and plateau at 50

minutes. Gesner showed internalisation of IL-3 in the human tumor cell line KG-1, where 80% of the ligand was inside the cells by 20 minutes. Internalisation of GM-CSF in murine cell lines is similar to internalisation of IL-3 (Nicola et al,1988; Ke 0.01-0.065 min^{-1}). A comparison of Kd and Ke values for human monocytes at 37°C (Table 7.1) shows that for IL-3, internalisation of a surface bound molecule is at least twice as likely as dissociation from the receptor. In contrast, for GM-CSF, dissociation is at least 4 times more likely than internalisation.

Expression of rate constants for each process in terms of half-times (t1/2) allows a picture to be built of the interaction of each CSF with the cell. Thus, GM-CSF associates rapidly (t1/2 = 40 seconds) but often transiently (t1/2 for dissociation = 3.3 minutes), with a slower rate of complex internalisation (t1/2 = 15 minutes). IL-3 associates less rapidly than GM-CSF (t1/2 = 2.5 minutes) and is more likely to stay bound to the receptor (t1/2 for dissociation = 31.5 minutes). IL-3 internalisation is, however, of similar efficiency (t1/2= 14 minutes) to GM-CSF.

A further point of interest which emerges from these experiments is the level of binding seen with the two growth factors. In Figures 7.2, 7.3, 7.4 and 7.5, binding of IL-3 and GM-CSF was measured concurrently to the same sets of donor cells. In each case, despite equal concentrations of ligand used, higher binding was noted for GM-CSF. In addition, in most cases the number of molecules bound per cell exceeded the estimates given from Scatchard analysis of high affinity sites (Table 6.1). This adds to the evidence suggesting that low affinity sites may also be binding ligand in these experiments. Alternatively, the estimates of high affinity site number, especially for GM-CSF, may be conservative. The occupancy of a greater number of receptors by GM-CSF than by IL-3 at equivalent molarities may also help explain the more rapid biological action of the former cytokine.

Each of the experiments reported here was performed with some tubes containing heterologous ligands, in order to distinguish the characteristics of competable and non-competable binding more fully. Because of variable degrees of inhibition of radioligand binding, and low cpm recorded in some experiments, not all the data is interpretable. However, two major points emerge. Firstly, non-competable association appears to be more rapid than competable association (Fig. 7.2, ^{125}I-GM-CSF; Fig. 7.4, ^{125}I-IL-3 and ^{125}I-GM-CSF). In the case of ^{125}I-IL-3 binding at 37°C (Fig. 7.4A) the two classes of receptor were shown to have Kobs values of 0.52±0.09 min^{-1} and 0.27±0.07 min^{-1} (M ± SEM; non-competable and competable binding respectively), with approximately 54% competition by GM-CSF recorded at 60 minutes. Secondly, rates of internalisation do not vary widely, despite large differences in degrees of competition. Thus Ke for ^{125}I-IL-3 Exp. 1 was 0.051 min^{-1} (>90% competition by GM-CSF) and for Exp. 2 was 0.048 min^{-1} (18% competition by GM-CSF) and for ^{125}I-GM-CSF was 0.046 min^{-1} (55% competition by IL-3). These data suggest that competable and non-competable receptors internalise at similar rates when complexed with their ligands.

One aspect of the binding of ^{125}I-GM-CSF to monocytes at 37°C was puzzling. While association was extremely rapid, this was followed by a decline in cell-associated radioactivity with time (Fig. 7.3 & 7.4) prompting questions as to its mechanism. Both ^{125}I-GM-CSF, and the unlabelled GM-CSF used as a competitor to determine non-specific binding were glycosylated, suggesting that differences in association rates between ligand and competitor were unlikely to be present. In additon, in Fig. 7.4, competitor was present for 15 minutes at 37°C prior to the addition of radioligand, allowing ample time for saturation of binding sites. These facts make it unlikely that the pattern seen resulted from inaccurate estimations of non-specific binding. A loss of cells by adhesion to the incubation tubes might also lead to a declining pattern of binding, especially in the presence of a pro-adhesive stimulus such as GM-CSF. However, monocytes adhere poorly to siliconised

glass, and do not adhere at all in the presence of sodium azide. Similarly, a loss of ^{125}I-GM-CSF from the reaction mixture by adsorption to the glass tube would seem unlikely in the presence of binding medium containing 0.5% BSA. Finally, the inclusion of sodium azide in the binding medium was designed to prevent receptor-ligand complex internalisation and subsequent degradation. While the potential for internalisation under these conditions was not directly addressed, similar conditions have been used by other groups to prevent CSF-receptor complex internalisation (Park et al, 1989A).

If the decline in ^{125}I-GM-CSF binding is therefore to be explained in terms of dissociation from the receptor, the question arises as to why other ^{125}I-GM-CSF molecules in the medium do not rapidly associate with the vacant site, leading to the development of a dynamic equilibrium. One explanation is that the receptor may be refractory to further binding for a period following dissociation, possibly through the mechanism of binding-induced allosteric change. Alternatively, the process of binding and dissociation may lead to the conversion from high to low affinity status. This hypothesis will be developed further in the General Discussion, where a model attempting to reconcile all of the functional and receptor data on monocytes will be proposed. Whatever the explanation for the shape of the ^{125}I-GM-CSF association curve, however, it is clear that it differs quite markedly from that of ^{125}I-IL-3.

SUMMARY

In this chapter, the kinetic parameters for ^{125}I-IL-3 and ^{125}I-GM-CSF interactions with monocytes have been determined. ^{125}I-GM-CSF showed very rapid association at 37^{o}C, with a t1/2 of only 40 seconds. The pattern of binding with this ligand was complex, with a decline in overall cell-associated radioactivity after 2 minutes of incubation, despite the presence of sodium azide. ^{125}I-IL-3 showed slower association, with a t1/2 at 37^{o}C of 2.5

minutes. The different rates of association correlate well with the more rapid activation of monocytes by GM-CSF than by IL-3. Dissociation rates for the two ligands also differed markedly, with Kd values for ^{125}I-GM-CSF approximately 10 fold greater than for ^{125}I-IL-3. The kinetically derived K_D values of 17pM and <53pM (^{125}I-IL-3 and ^{125}I-GM-CSF respectively) correlated well with equilibrium-derived data, and with the concentrations of CSF needed for a biological response. Rates of internalisation were similar for the two cytokines, with t1/2 of 14-15 minutes. A comparison of cross-competable and non-cross-competable binding showed a more rapid rate of association of ligand to the latter class of receptors, but similar rates of internalisation for the two groups.

CHAPTER 8

GENERAL DISCUSSION

The experiments described here have been performed using highly purified peripheral blood monocytes from normal donors. By using such primary cells, rather than 'monocytoid' tumour cell lines, it was intended that the findings would be more relevant to monocyte biology *in vivo*. However, this approach demanded the establishment of methods of cell separation which could not only give high monocyte purity, but also an acceptable yield. The adhesion and radioligand binding experiments were made possible by the technique of countercurrent elutriation, which allowed not only the preparation of larger numbers of monocytes than adhesion-dependent methods, but also the delivery of cells which had not been activated by surface contact (Dougherty et al, 1988; Parker et al, 1988; Haskill et al, 1988; Eierman et al, 1989). This situation is likely to have contributed particularly to the discovery of the early phase of CSF-stimulated adhesion.

The major finding in Chapter 3 was that IL-3 and GM-CSF maintained monocyte numbers in long term *in vitro* culture, and stimulated increased cell size, protein content and antigen expression. The maintenance of cell numbers at concentrations well below those required for stimulation of ^{3}H-thymidine uptake (Fig. 3.5) indicated that this phenomenon resulted primarily from enhanced monocyte survival. This finding is in keeping with previously published data concerning the role of CSF in monocyte and macrophage survival. Thus, M-CSF is a survival factor for murine BMM and human monocytes (Tushinski et al, 1982, 1985; Becker et al, 1987) and GM-CSF promotes survival in a proportion of murine PAM (Lin et al, 1989). CSF have also been shown to promote survival of immature haemopoietic cells and mature granulocytes (Metcalf et al, 1982, 1986B; Lopez et al, 1986; Begley et al, 1986) suggesting that this may be a general function of haemopoietic growth factors on myeloid cells. The interpretation of the CSF-stimulated ^{3}H-thymidine uptake seen in monocyte cultures is open to discussion, but as already argued in Chapter 3, is most likely a result of proliferation within a subpopulation of cells. Whether these are monoblasts released prematurely from the bone marrow, and capable of limited division before terminal

differentiation, or whether some mononuclear phagocytes remain for long periods with proliferative potential is unclear. However, a number of reports support the latter contention. CSF-driven proliferation is seen *in vitro* in a number of different murine mononuclear phagocyte populations (Tushinski et al, 1982, 1985; Chen et al, 1988A,B; Lin et al, 1989), and intraperitoneal injection of either IL-3 or GM-CSF in mice leads to enhanced mitotic activity in peritoneal macrophages (Metcalf et al, 1986A, 1987). In addition, other animal studies have described the *in situ* proliferation of macrophages in association with experimentally induced synovitis (Loewi, 1969), infection with *Listeria monocytogenes* (North, 1969) and methylcholanthrene-induced sarcomas (Evans et al, 1984). Proliferation has also been studied in human pulmonary alveolar macrophages obtained from smokers and a non-smoker by broncho-alveolar lavage (Golde et al, 1974). A labelling index of between 0.35 and 1.25% was seen in the smokers, together with a much higher macrophage recovery. This compared with an index of 0.35 in the single non-smoker studied. Because of differences in the experimental methods used (in particular the length of exposure to ^{3}H-thymidine), these findings are difficult to relate directly to those reported in Chapter 3. However, the proportion of cells proliferating was similarly low in the two studies. The extent to which mononuclear phagocyte proliferation in man is biologically relevant is unclear, but it should be noted that a small growth fraction may be more significant in long-lived cells such as tissue macrophages than in cells of short half life.

CSF-stimulated ^{3}H-thymidine uptake required concentrations of cytokines of at least 60pM (Fig. 3.5), concurring with another recent publication, where enhanced ^{3}H-thymidine uptake in human monocytes was seen with concentrations of GM-CSF of 100pM but not 50pM (Koyanagi et al, 1988). Data presented for IL-3 by the same authors suggested that this cytokine was active at lower concentrations (10pM). However, it was unclear from their data whether this was significant, because no statistical analysis was presented. At least two studies suggest that concentrations of this magnitude are achievable *in vivo* at the

inflammatory site, at least for GM-CSF (Williamson et al, 1988; Xu et al, 1989). In addition, both GM-CSF and IL-3 have been shown to adsorb to the connective tissue matrix laid down by bone marrow stromal cells *in vitro* (Gordon et al, 1987; Roberts et al, 1988A), and the responsible element appears to be heparan sulphate. The retention of biological activity by the bound CSF (Roberts et al, 1988A) raises the possibility that local production of growth factors may result in the compartmentalised control of myeloid cell function or proliferation by conversion from a soluble to a substrate-bound form. Furthermore, this phenomenon may allow the development of localised regions of high CSF concentrations, thereby providing a proliferative signal to neighbouring macrophages.

The findings presented in Chapter 4 show that IL-3 and GM-CSF also influence the functional state of human monocytes. Both CSF stimulated two phases of adhesion, distinguishable not only by their timing, but also in their dependence on protein synthesis. Early phase adhesion was not inhibited by cycloheximide, and control experiments confirmed that the drug was indeed active in inhibiting protein synthesis at this time (Fig. 4.9; Table 4.1). These findings were not surprising, given the rapidity of the adhesion response. In contrast, late phase stimulated adhesion was abolished by cycloheximide added 5 hours prior to harvest, with no effect on cell viability in this time. This was also consistent with the time course of adhesion (Fig. 4.1) which showed a delay of 6-9 hours prior to the onset of the late phase.

In contrast to these differences between early and late phase adhesion, both phases of stimulated adhesion were LFA- but not MAC-1-dependent, suggesting a role for LFA-1 or p150/95 in these phenomena. The partial inhibition of late phase adhesion by anti-ICAM-1 confirmed that LFA-1 was involved, but since the inhibition was incomplete, it seems likely that p150/95 is important as well. Unstimulated adhesion was only partially LFA-dependent at both time points, and was independent of MAC-1. These findings concur with previous

reports of the partial dependence of unstimulated monocyte adhesion on LFA-1 and p150/95, but not MAC-1 (see Chapter 4).

Changes in the surface expression of LFA complexes in general correlated poorly with stimulated adhesion. No changes in expression were seen at 30 minutes of culture, despite the development of enhanced adhesion, and upregulation at 21 hours was seen most strongly for MAC-1. In addition, IL-4 stimulated increases in all LFA complexes at 21 hours of culture in the face of inhibition of adhesion. The regulation of monocyte adhesion may therefore depend more on the distribution of LFA-1 and p150/95 molecules on the cell surface, or on their functional state, rather than on changes in their overall expression. The publications referred to in Chapter 4 concerning the irrelevance of changes in neutrophil MAC-1 expression to MAC-1-dependent stimulated adhesion (Vedder et al, 1988; Buyon et al, 1988) and the stimulation of novel binding characteristics in monocyte MAC-1 complexes (Altieri et al, 1988A,B) provide a possible model for such functional change.

One limitation of the MAb blocking studies used in Chapter 4 is the potential for inhibition of adhesion by indirect means. Numerous publications illustrate the capacity for adhesion to perturb monocyte function (Dougherty et al, 1988; Parker et al, 1988; Haskill et al, 1988; Navarro et al, 1989) and there is evidence that the type of substrate may determine the functional outcome (Eierman et al, 1989). The implication in these reports is that integrins may act as signalling proteins, rather than simply functioning as passive attachment molecules, and there is some direct evidence to support such a conclusion in T cells (Dunn et al, 1989). The possibility therefore exists that MAb 60.3 inhibits monocyte adhesion by 'signalling' the cell to become, in some way, less adhesive. Such an hypothesis must be able to explain the fact that different alpha-chain MAb inhibit adhesion in different cell types (anti-MAC-1 in neutrophils; anti-LFA-1 in monocytes). Mentzer et al (1987) have investigated possible mechanisms by which such 'signalling' might occur, but found little

evidence in its support. Inhibition of unstimulated monocyte adhesion to HUVEC was seen both with F(ab')2 fragments of anti-LFA-1 antibodies and with whole antibody, suggesting that Fc receptor-mediated binding and subsequent cell signalling was not responsible for inhibition of adhesion. The same authors also found that some, but not all anti-LFA-1 MAb inhibited adhesion, showing that simple binding of MAb to LFA-1 does not necessarily trigger detachment.

The rapidity of early phase stimulated adhesion may provide a mechanism for the local control of monocyte-endothelial attachment and diapedesis *in vivo*. The production of GM-CSF by a wide variety of cell types, in contrast to the restricted production of IL-3 (see Chapter 1), the identification of GM-CSF at the inflammatory site (Xu et al, 1989), and the more rapid stimulation of adhesion seen with this cytokine (Chapter 4) suggest that GM-CSF may be of most importance in this process. The possibility that CSF-stimulated monocyte adhesion may be pathogenetic in atherogenesis is an intriguing one, and deserves further study. The late phase of stimulated adhesion may be important in monocyte migration through the extracellular space, and to the localisation of monocytes at the inflammatory site (see Chapter 4). Alternatively it may contribute to the roles of IL-3 and GM-CSF in the stimulation of adhesion-dependent effector function, such as tumour lysis.

It is of interest to compare some aspects of monocyte adhesion with those of neutrophils. The latter cells show only slight stimulation of adhesion with GM-CSF *in vitro* (Devereux et al, 1989; Obrist et al, 1989) but injection of GM-CSF *in vivo* causes a similar reversible margination of both neutrophils and monocytes in the pulmonary vasculature. Human neutrophils do not respond functionally to IL-3 *in vitro* (Lopez et al, 1988A) and lack IL-3-R (Lopez et al, 1989). In the case of monocytes, the adhesion response is somewhat slower with IL-3 than with GM-CSF, but with time is equally as great (Chapter 4). These findings may provide a clue to understanding the apparent redundancy in the stimulation of

monocyte adhesion by CSF. It may be an advantage to the host to be able to control monocyte adhesion selectively, without perturbing neutrophil function. Sequential production of first GM-CSF and second IL-3 at the inflammatory site may help to explain the initial influx of neutrophils, followed by accumulation of monocytes in the subacute phase. Further distinctions can be drawn between the two cell types in their mechanism of adhesion, in that the LFA-dependent component of neutrophil-endothelial attachment is MAC-1 mediated (Vedder et al, 1988), while monocyte adhesion depends on LFA-1 and p150/95. Whether these differences can explain the shorter t1/2 of neutrophils in the circulation, and the presence of a marginating pool of neutrophils but not apparently of monocytes (Johnston, 1988) remains to be seen.

The observed stimulation of monocyte adhesion by IL-3 and GM-CSF, and the inhibition of the same function by IL-4, are findings which are all the more important for their relative isolation. Amongst the cytokines, IFNγ has been shown to stimulate murine PEM to basement membrane proteins, and to stimulate homotypic adhesion in human monocytes (Shaw et al, 1989; Mentzer et al, 1986), while the effect of IL-1 in enhancing monocyte-endothelial attachment is through stimulation of endothelium rather than of monocytes. Although M-CSF may also stimulate human monocyte adhesion (Fig. 4.6) no information could be found indicating a role for other cytokines in this process. It is also important to note the recent publication describing the presence of only very low levels of IFNγ in synovial fluid and tissue in RA (Firestein et al, 1987), a finding which surprised the authors, in view of the morphologic and histochemical evidence of T cell activation in the RA synovium. Relatively high concentrations of M-CSF and GM-CSF were detected by the same group, however (Firestein et al, 1988; Xu et al, 1989), suggesting that CSF may be the more important soluble mediators in this disease.

The literature on cytokine inhibitors of human monocyte adhesion is also sparse. One report describes the production of a leukocyte adhesion inhibitor (LAI) from activated endothelial cells, and its partial characterisation as a heat- and acid-stable but pepsin-sensitive molecule of Mr 14-24KD (Wheeler et al, 1988). This factor inhibited the adhesion of neutrophils and monocytes to IL-1-stimulated HUVEC, and its action was on the leukocyte rather than on the endothelial cell. IL-4 has a number of features in common with LAI. It is a polypeptide of Mr 20KD when glycosylated, is very acid stable due to the presence of 3 disulphide bonds (W. Windsor, personal communication), and inhibits monocyte adhesion to HUVEC by an action on the monocyte (Chapter 5). It appears, however, that LAI is identical to another recently described monokine, 'neutrophil activating factor' (M. Gimbrone, personal communication), suggesting that LAI and IL-4 are not one and the same.

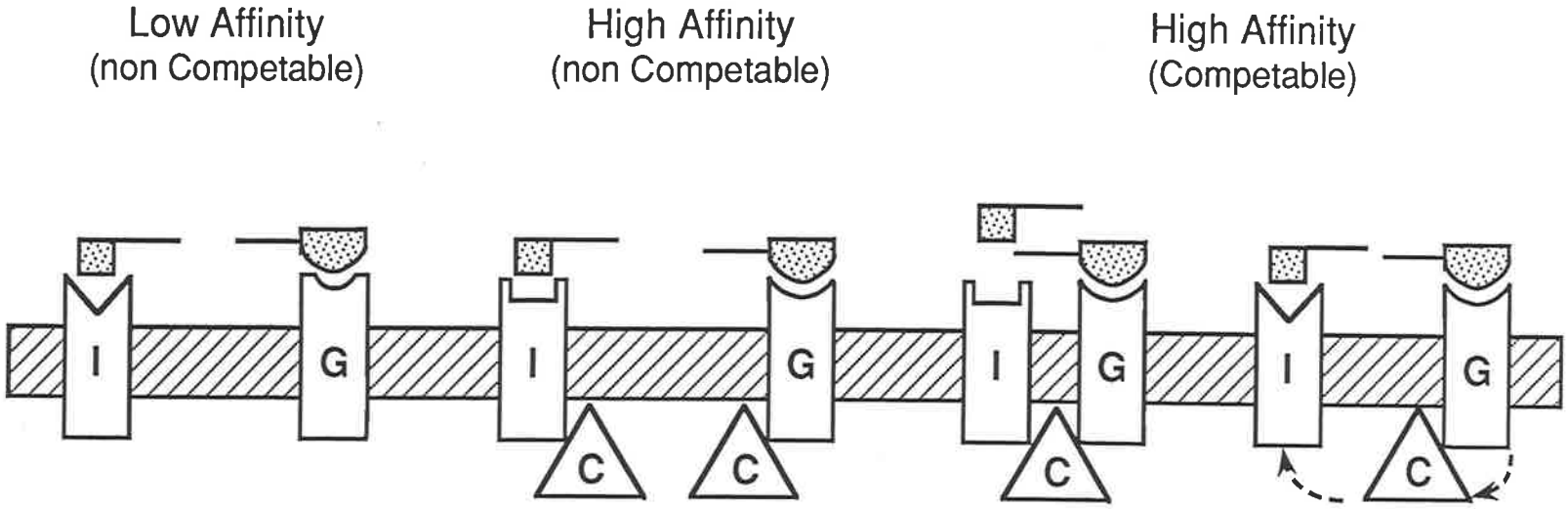
High concentrations of PMA may also inhibit monocyte-endothelial attachment (Kamp et al, 1989) and work in this laboratory has shown the induction of a hypo-adhesive state in HUVEC treated with transforming growth factor β (TGF-β). This decreased adhesiveness is manifest for neutrophils (Gamble et al, 1988) but not for monocytes (JR Gamble, personal communication).

The experiments described in Chapters 6 and 7 of this thesis were undertaken in order to help understand the mechanisms by which IL-3 and GM-CSF stimulate monocytes. A number of novel findings concerning the binding of these CSF to their receptors resulted, and are summarised here. IL-3 and GM-CSF bound to a small number of very high affinity receptors (K_D 5-39pM) and to a larger number of low affinity receptors (K_D 513-1120pM). The affinity constants for the high affinity sites were determined both by equilibrium and by kinetic methods, and close agreement was seen. These values also correlate closely with the concentrations of CSF needed for biological responses. It is unclear at present whether the low affinity sites are capable of cell signalling in monocytes, but as already discussed in

Chapter 6, functional low affinity receptors for GM-CSF are found on human placental cells, and a cloned low affinity GM-CSF receptor is also capable of transducing a proliferative signal when expressed in the murine cell line FDCP-1. It is of interest that proliferation in monocytes seems to require much higher concentrations of CSF than do cell survival or functional stimulation, and that no plateau is seen in this response even at CSF concentrations as high as 600pM (Chapters 3, 4 and Koyanagi et al, 1988). Colony formation in bone marrow cells also requires higher CSF concentrations than do other functions, raising the possibility that low affinity receptors specifically mediate the proliferative response. This implies either the need for much larger numbers of occupied receptors to achieve proliferation, or a different system of second messengers for low and high affinity-receptor-mediated stimulation. Evidence in support of the latter hypothesis arises from the work of Lopez et al (1988B) which demonstrates the presence of different intracellular signals in non-proliferative and proliferative cells stimulated with GM-CSF.

The partial competition for binding between IL-3 and GM-CSF indicated an interaction between the CSF or their receptors, but this interaction was quite different from that previously described on mouse bone marrow cells (Walker et al, 1985A,B). Specifically, the interaction was limited to IL-3 and GM-CSF and did not also involve G-CSF or M-CSF, it was bidirectional rather than hierarchical, and it was seen under conditions shown to prevent receptor internalisation. The receptor trans-downmodulation model is therefore not adequate to explain these data, and an alternative model for IL-3 and GM-CSF receptors on monocytes is proposed in Figure 8.1. In this model, discrete IL-3 and GM-CSF binding proteins act as low affinity receptors for their respective CSF, but association of either protein with a third connecting subunit induces a change to high affinity status. This may occur through an allosteric change in the CSF binding site, as has been previously proposed in the regulation of epidermal growth factor (EGF) receptor affinity by soluble mediators (Schlessinger, 1988). Alternatively, the connecting subunit may also be found on the cell

Figure 8.1 A model for IL-3 and GM-CSF receptors on monocytes. I, IL-3 unique binding protein; G, GM-CSF unique binding protein; C, connecting chain.


Low Affinity
(non Competable)
High Affinity
(non Competable)
High Affinity
(Competable)
I
G
I
G
I
G
I
G
C
C
C
C

surface, and provide additional binding sites for ligand interaction. In order to explain the preponderance of low affinity sites, the connecting subunit must be present in limiting concentrations, and the regulation of turnover of this subunit may therefore control the responsiveness of the cell to stimulation. Association of one IL-3-binding and one GM-CSF-binding protein via a connecting subunit results in competition for CSF binding, and two possible mechanisms for competition are illustrated. In the first, competition is achieved through steric hindrance, and in the second, binding of one CSF molecule leads to the dissolution of the trimolecular receptor complex, with retention of high affinity status for the occupied receptor, but conversion to low affinity status for the unoccupied receptor.

This model accommodates much of the available data. It allows for high and low affinity binding, for partial competition for high affinity binding only, and for the retention of the highly individual association and dissociation kinetics outlined in Chapter 7. An alternative model invokes the presence of a separate CSF-binding protein with a common binding site for the two ligands, in addition to high and low affinity sites specific for the individual CSF. While this remains possible, it is a more complex model and requires at least one common epitope on the two CSF molecules.

The existence of the connecting subunit is at present unproven. Two pieces of evidence, however, support its inclusion in this model. Firstly, in cross-linking experiments with ^{35}S-IL-3 and ^{125}I-GM-CSF on KG-1 cells, one group has found unique CSF binding proteins of Mr 69KD for IL-3 and 95KD for GM-CSF, together with a high Mr protein of about 175KD in each case. The 175KD band is fully inhibited by the cognate CSF (as is the smaller band appropriate for that ligand) but only partially inhibited by the non-cognate CSF (SC Clark, personal communication). This is the pattern one would expect if the p175 were a connecting subunit, as only those p175 molecules in a trimolecular receptor complex would be inhibited. In the alternative, the p175 might represent a common CSF receptor. In this

case, however, it should be fully inhibited by the non-cognate CSF, and therefore this representation would seem less likely. Secondly, the cloned GM-CSF receptor already referred to has only a very short intracellular domain, with little potential for tyrosine kinase activity (D Gearing, personal communication). However, studies on mechanisms of signal transduction of GM-CSF in human neutrophils have revealed the rapid appearance of tyrosine phosphorylation in a number of membrane proteins upon stimulation with GM-CSF, suggesting that the GM-CSF-R is associated with a tyrosine kinase (Gomez-Cambronero et al, 1989D). Similar conclusions for the IL-3 receptor arise from studies on the binding of mIL-3 to various murine cell lines. Binding of IL-3 results in the rapid appearance of a number of phosphotyrosine-containing proteins within the cell (Isfort et al, 1988), one of which is intimately associated with the IL-3 binding protein (Isfort et al, 1988; Sorensen et al, 1989). These reports provide evidence that a second protein is associated with the IL-3 and GM-CSF binding proteins, and therefore support the inclusion of the connecting subunit in the receptor model proposed here. In addition, it seems possible that the connecting subunit may function as a signal transducer with tyrosine kinase activity, allowing for the type of rapid cell stimulation which was described in Chapter 4.

The receptor model also provides an explanation for the similarities seen between IL-3 and GM-CSF in their stimulation of monocytes. The two CSF behave identically in their stimulation of monocyte expression of TNFα (Cannistra et al, 1988A) and M-CSF (Vellenga et al, 1988; Ernst et al, 1989), while neither stimulates G-CSF expression (Vellenga et al, 1988). In addition, both enhance the growth of HIV in human monocytes, and stimulate tumouricidal and microbicidal function (Koyanagi et al, 1988; Perno et al, 1989; Cannistra et al, 1988A; Wang et al, 1989). To these reports can be added the information contained in Chapters 3 and 4 of this thesis, which show very similar activity for the two CSF. Of particular interest in this regard is the failure of IL-3 and GM-CSF to show synergy or additive effects when used at optimal concentrations (Fig. 4.5). The

transduction of signals via a common connecting subunit, whether in the bi- or tri-molecular configuration, would explain these findings. The fact that GM-CSF activates monocytes more rapidly than does IL-3 is also compatible with the model, being explainable in terms of differences in the kinetics of ligand interaction with the unique binding subunits. While the mechanism of CSF receptor internalisation in these cells is unknown, it is of interest that Ke values were very similar for IL-3 and GM-CSF, and for competable and non-competable binding (Chapter 7). This may indicate a role for the connecting subunit in internalisation as well as in its other proposed functions.

The last finding which the model must explain is the unusual on-off association pattern seen for GM-CSF but not for IL-3 (Chapter 7). Possible sources of experimental error in these experiments have been previously discussed, and would seem unlikely to be operative, although internalisation and subsequent degradation of ^{125}I-GM-CSF has not been totally excluded. The proposal was therefore made that GM-CSF binding is followed by dissociation, and a refractory period follows preventing further CSF molecules from binding. Such a process might occur by the dissolution of the GM-CSF-binding protein from its connecting subunit upon ligand binding, leading to its conversion to a low affinity site. Whether this would be associated with signal transduction and hence explain the more rapid stimulation of monocyte function by GM-CSF is unclear, and remains an area of great interest.

The complex model proposed to explain CSF-monocyte binding is not without parallel in other ligand-receptor systems. The IL-2 receptor comprises at least 2 chains, each of which binds IL-2 with low affinity when alone (Wang et al, 1987). Non-covalent association of the α and β chains results in a high affinity receptor, with binding domains for IL-2 on both chains, but with signal transduction occurring through the α chain only. Although IL-2 binding had previously been reported to be specific (Smith, 1987) a recent publication

showed a reduction in IL-2 binding to T and B cell lines after a 60 minute preincubation with IL-4 at 4°C (Fernandez-Botran et al, 1989). Although the potential for competition in the converse direction was not explored, the similarities between these findings and the ones reported here for CSF binding are obvious. High and low affinity binding sites are also seen for EGF, and these are capable of interconversion (Schlessinger, 1988). A similar pattern is seen for the TNF-R, which undergoes a protein kinase C-dependent conversion to low affinity status on appropriate stimulation (Scheurich et al, 1989). Both receptor systems have the added complexity of binding more than one ligand: the EGF receptor also binds transforming growth factor α, and the TNF-R binds both TNFα and TNFβ. A further variation on the theme is provided by the receptor for IL-6, which is a single chain protein of 80KD. Upon binding IL-6, this chain associates with a larger, non-ligand-binding glycoprotein of Mr 130KD, which is responsible for signal transduction (Taga et al, 1989). These multiple-subunit and multiple-ligand receptor systems contrast with the interaction between M-CSF and its receptor, which is specific for M-CSF, comprises a single chain only, and is of single affinity (Yeung et al, 1987).

Many questions about the interactions of IL-3 and GM-CSF with monocytes, and about the receptor model proposed, remain unanswered. For example, what advantage is conferred on the cell by the association of CSF receptors? Is this simply a 'marriage of convenience', brought about by a paucity of connecting or signal transduction chains, or does receptor association facilitate signal transduction in some way? Also worthy of consideration is whether all monocytes express all receptor classes, or whether there are subpopulations of cells expressing only competable or only low affinity sites. In fact there is some evidence to suggest that peripheral blood monocytes are heterogeneous in terms of physical properties and immunological function (Yasaka et al, 1981; Esa et al, 1986; Dransfield et al, 1988), suggesting that they may also vary in their expression of CSF receptors.

FUTURE WORK

It is clear that a great deal of *in vitro* work remains to be done, particularly in the areas of mechanisms of CSF-stimulated adhesion, and in the further characterisation of CSF receptors on monocytes. The biochemical characterisation and molecular cloning of the proposed connecting subunit would be a particular priority in such experiments. It would also be of great interest, however, to pursue the functional work contained in this thesis *in vivo*. The roles of IL-3 and GM-CSF in contributing to macrophage-associated pathology could be tested in animal models of atherogenesis, inflammatory arthritis and other diseases, with the potential for providing information of clinical importance.

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