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CHARACTERISTICS AND FUNCTIONS OF HUMAN T LYMPHOCYTE SUBPOPULATIONS SEPARATED ON THE BASIS OF THEOPHYLLINE SENSITIVITY OF E ROSETTE FORMATION

by

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Date 16th March 1985 Signed

Thesis submitted to the University of Adelaide for the degree of Master of Clinical Science.

JULY, 1984.

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STATEMENT

This thesis contains no material which has been accepted for any other degree or diploma in any University and does not contain any material previously published or written by another person, except where due reference is made to such material in the text.

July, 1984.

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SUMMARY

CHAPTER 1. INTRODUCTION AND CLASSIFICATION OF T LYMPHOCYTES

The development of T lymphocytes from lymphoid stem cells in the foetal yolk sac to mature T cells is traced with their concurrent acquisition of cell surface antigenicity. These T lymphocytes are subdivided into functional subgroups consisting of the initiator lymphocyte, the helper lymphocyte, the cytotoxic lymphocyte and the suppressor lymphocyte.

CHAPTER 2. IMMUNOLOGICAL FUNCTION OF T CELL SUBSETS.

The T suppressor lymphocyte forms part of the suppressor system in humans. It has also been shown that there is an adherent cell suppressor system effected by a steroid and radio-resistant monocyte; a prostaglandin related suppressor system and a mitogen induced suppressor system. Cytotoxic T lymphocytes are sensitised and only kill cells carrying the antigen to which they are sensitised. They also mediate in delayed hypersensitivity.

The helper T lymphocyte provides inducer and helper function in interactions between T and B cells, T cells and macrophages, and between T cells themselves. Natural killer (NK) cells have been demonstrated in peripheral blood and shown to have the morphology of large granular lymphocytes with a low avidity for sheep red blood cells. N.K. cells can spontaneously lyse certain tumour cells in vitro and may provide early resistance against tumour growth.

CHAPTER 3. MARKERS OF T LYMPHOCYTES.

The sheep erythrocyte receptor defines human T cells and receptors for the Fc fragment of IgG and IgM have helped in delineating T lymphocytes into $T\gamma$ and $T\mu$ subsets. However, it has been shown that $T\gamma$ cells can undergo transition to $T\mu$ cells proving that sequential expression of Fc - IgG and Fc - IgM receptors on the same T cell is possible.

Other markers include the use of anti-TH_1 antisera and reactivity with antibodies found in Juvenile Rheumatoid Arthritis (JRA). Theophylline sensitivity of some T-rosettes led to further separation of T cells into theophylline sensitive and theophylline resistant T lymphocytes, with a proportion of cells only rosetting in the presence of theophylline thus making them theophylline dependent.

The cytoplasmic Gall body first described in 1936 has been subsequently shown to predominate in helper T lymphocytes. The advent of monoclonal antibodies has further defined the various lymphocyte subsets.

CHAPTER 4. MATERIALS AND METHODS

This study was undertaken to evaluate an apparently simple and rapid means of identifying the suppressor and helper subsets for clinical use based on the premise that theophylline resistant lymphocytes have predominantly helper function and theophylline sensitive cells suppressor function. Red Cross donor blood was used for the main part of the study. In addition, five patients with systemic lupus erythematosus were followed over varying periods to assess the changes in theophylline sensitive populations and to correlate these with other criteria of disease activity. The Tsens population was also assessed in 19 patients with other autoimmune and miscellaneous conditions.

The following tests were carried out:-

Separation of lymphocyte subpopulation and functional assays

1. Harvesting of peripheral T lymphocytes.
2. Separation of theophylline sensitive and theophylline resistant T lymphocytes.
3. Presence of Gall bodies.
4. May-Grumwald-Giemsa stain.
5. Determination of acid α-nepthyl acetate esterase activity (ANAE).
6. B cell differentiation assays using co-cultures of Tsens, Tres, adherent cells and EFRC depleted cells.
7. Determination of intracytoplasmic Ig production.
8. Indirect immunofluorescence technique for determining monoclonal antibody profile using the following monclonal antibodies: OKT3, OKT4, OKT8, OKMI, Leu 2A and Leu 3A.
9. Determination of spontaneous plaque forming cell numbers.
10. Determination of percentage of theophylline sensitive T lymphocytes.

CHAPTER 5. RESULTS

5A. CHARACTERISTICS OF THEOPHYLLINE SENSITIVE AND THEOPHYLLINE RESISTANT LYMPHOCYTES

The effect of theophylline treatment on the reactivity of T lymphocytes with OKT monoclonal antibodies

There were no significant changes in cell viability or the proportion of cells reactive with OKT3, OKT4, OKT8 and OKM1 monoclonal antibodies.

Characteristics of Tsens and Tres subsets compared to total T lymphocytes.

Tsens showed a significant depletion of OKT3 + cells compared to Ttot, as well as a lower percentage of cells containing Gall bodies or positive staining for ANAE. There was enrichment for Fcγ receptors and

OKM1 positive cells. Tres was enriched for ANAE positive cells but did not differ significantly from Ttot in other respects.

Comparison of Tsens and Tres fractions

Marked differences between these two fractions were demonstrated. Tsens contained three times as many cells with $Fc\gamma$ receptors as cells with $Fc\mu$ receptors whilst the ratio of $Fc\gamma/Fc\mu$ receptor bearing cells in Tres was 1:3. Reactivity of Tres cells with OKT monoclonal antibodies showed a predominantly helper phenotype, and a large number of cells contained Gall bodies. Tsens contained a high proportion of OKM1 positive cells, and did not show a suppressor phenotype with regard to OKT8 and Leu 2A.

5B. FUNCTIONAL ASSAYS ON Tsens AND Tres SUBSETS

There was generally poor generation of immunoglobulin in B cells in the earlier cultures. However, Tres did show optimal helper function when co-cultured with B cells in a ratio of 1:1.25 using pokeweed mitogen. Tsens showed a lack of help though no dose related suppressor effect could be demonstrated.

5C. PERCENTAGE OF Tsens LYMPHOCYTES IN NORMAL CONTROLS

These were determined on blood from Red Cross blood donors as well as from laboratory staff. In 19 subjects, a range from 2% to 47% was obtained with a mean of 17.5% (S.D. $\pm$ 11.5).

Percentage of Tsens lymphocytes in active/inactive SLE

Patients with active SLE had a mean of 7.9 $\pm$ 11.3% of Tsens cells which was significantly lower than in control subjects, and theophylline inducible cells were also demonstrated. However, patients with inactive SLE did not differ from the control subjects in respect of Tsens cells.

Percentage of Tsens lymphocytes in other miscellaneous diseases

A wide variation in Tsens percentages was obtained in a group of miscellaneous conditions including alcoholic liver disease, chronic lymphocytic leukemia, myasthenia gravis and dilantin treated epileptics.

5D. LONGITUDINAL STUDIES IN FIVE PATIENTS WITH SLE

Five patients with SLE were followed over periods ranging from five months to a year. There was a demonstrable fall in Tsens lymphocytes during clinical relapses with a subsequent rise shown during prednisolone therapy in some but not all of the patients.

CHAPTER 6. DISCUSSION

The selective loss of ability of a proportion of T lymphocytes to form rosettes with sheep red blood cells in the presence of theophylline was used to separate a theophylline sensitive group from theophylline resistant lymphocytes. An attempt was then made to ascertain the degree of homogeneity of these fractions with regard to putative markers for helper and suppressor function. The results suggest that the Tres fraction was enriched for cells with putative helper markers but that the Tsens fraction was enriched for cells with some suppressor markers but also contained a large number of cells of monocytic lineage. However the two fractions were not homogeneous with respect to Fc receptor status, presence of Gall bodies, or antigens defined by monoclonal antisera. Thus the use of theophylline sensitivity as a means of estimating the sizes of helper and suppressor populations does not seem advisable.

ACKNOWLEDGEMENTS

The clinical and laboratory data collected for this thesis was obtained during my tenure as a Lecturer in Medicine in the Department of Medicine, The University of Adelaide at the Queen Elizabeth Hospital, Woodville, South Australia. This position allowed an unique opportunity to combine clinical medicine with correlative laboratory science. I am grateful to the various members of the Department of Medicine who have guided and assisted me, thus contributing to this aspect of my educational experience. In particular I would like to express my thanks to Miss S. Whitington, Miss M. Swinkels, Mrs. K. Teague, Miss P. Cowled, Mrs. A. Pollard and Mr. A. Milton for their assistance in the various immunological procedures, and Miss J. Hawkes for her able assistance in some of the culture work as well as in the A.N.A.E. stains.

I must express my gratitude to Dr. R. Beale of the Blood Transfusion Centre for allowing me access to Red Cross donor blood, as well as the various volunteers who helped establish the normal ranges for the Tsens fraction. Thanks are also due to Dr. Tony Leong for his help in electron microscopy of the Gallbody and Dr. H. Zola of the Flinders Medical Centre and Miss H. Moore who helped in the Leu-monoclonal antibody work as well as in arranging for the use of the fluorescein activated cell sorter (F.A.C.S.) in some of the analysis.

The illustrations were done by Ms. I. Cronin, and photography of the graphs and electron microscopy were done by Mr. J. Hadaway, Mr. A. Drew, Mr. B. Popowycz and Mr. C. Hentschke of the Clinical Photography Department and to them I express my greatest appreciation. I am also indebted to the secretarial staff of the Department of Medicine who have helped in the various stages of preparing this manuscript, especially Mrs. S. Rumball and Miss B. Moss. Mrs. E. Jobson undertook to prepare

this final manuscript and to her I must express my grateful thanks. I would like to record a special note of gratitude to Dr. R. Ratnaike for his guidance in arranging this thesis. My family has helped in the various stages of preparation of this work and to them I must express my heartfelt thanks.

Finally, I would like to express my deepest gratitude and humble thanks to my supervisor and teacher, Professor A.G. Wangel who has so patiently guided me through the whole thesis, both in scientific technique as well as in preparation of the manuscript and without whom this thesis could not have been completed.

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CHAPTER 1. INTRODUCTION AND CLASSIFICATION OF T LYMPHOCYTES

1A. INTRODUCTION

Immunology deals with the mechanisms whereby an organism recognised substances as foreign and mounts responses designed to eliminate or neutralise them with a minimum of injury to itself. Specific immunological response is restricted primarily to chordates and is mediated via lymphocytes.

There are two main categories of lymphocytes - those produced in the bone marrow but subsequently processed in the micro-environment of the thymus and thus called T-lymphocytes, and the remainder of the bone marrow derived lymphocytes considered analogous to lymphocytes from the avian bursa of Fabricius, bursa-equivalent or B-lymphocytes.

Some cells which morphologically are lymphocytes do not have the characteristics of T or B lymphocytes and are called "null" cells or third population lymphocytes. With the advent of immunological markers it has been shown that the T-lymphocyte population is not homogenous but consists of subpopulations which appear to have different and specific roles in the immune response. T-lymphocytes constitue 55% - 60% of the total lymphocytes in the circulation and over 90% of the cells in the thymus, whilst most of the lymphocytes in the bone marrow are B cells. Once the immune system has developed, there is a constant turnover of lymphocytes and it has been estimated that approximately 10^{11} new lymphocytes are generated in the adult human daily.

1B. LYMPHOCYTE ONTOGENY AND DIFFERENTIATION.

In man, cells are derived from lymphoid stem cells which arise in the foetal yolk sac, migrate to the liver by 4 to 5 weeks of gestation and thereafter reside in the bone marrow.

At 6 to 8 weeks of gestation, the epithelial thymus begins to form from the third and fourth pharyngeal pouches. Pre-T lymphocytes differentiate into mature T cells under the influence of the epithelial thymus and a hormone-thymopoietin while migrating from the thymic cortex into the central medullary area and finally by 12 weeks of gestation, into the circulation and peripheral lymphoid tissues. (Scheid 1978).

1C. ACQUISITION OF CELL SURFACE ANTIGENICITY

Profound changes in cell-surface antigens mark the stages of T-cell ontogeny in man and have been reviewed by Reinherz and Schlossman (1980a). The earliest lymphoid cells within the thymus bear antigens shared by some bone marrow cells but lack mature T-cells antigens. They account for about 10% of thymic lymphocytes and are reactive with two monoclonal antibodies, anti-OKT9 and anti-OKT10. With maturation, thymocytes lose OKT9, retain OKT10 and acquire a thymocyte-distinct antigen defined by anti-OKT6. They also then express antigens defined by anti-OKT4 and anti-OKT5. These $OKT4^+$, $OKT5^+$, $OKT6^+$ and $OKT10^+$ thymocytes account for about 70% of the total thymic cells. With further maturation thymocytes lose the OKT6 antigen, acquire OKT1 and OKT3 antigens and segregate into $OKT4^+$ and $OKT5^+$ subsets. Immunologic competence is acquired at this stage but is not fully developed until these thymic lymphocytes are exported. Outside the thymus, the $OKT4^+$ subset represents the circulating inducer-helper lymphocytes forming 55-60% of peripheral T-cells and the $OKT5^+$ subset, the cytotoxic/suppressor group represented in 20 to 30% of T-cells.

1D. TYPES OF T-LYMPHOCYTES

Classification of T-lymphocytes into major functional subgroups has been aided by the discovery of a variety of cell surface markers and the advent of monoclonal antibodies directed at stable cell antigens has further enhanced this characterisation.

In the murine system the best understood allo-antigens are Thy-1, which is common to all T cells but absent from B cells, Lyt-1, Lyt-2, 3 and the Ia family of antigens (Snell 1978). Mitogen studies show that the functional activity of the cell is pre-programmed once it has differentiated to the stage of an Lyt-1+ or Lyt-2, 3+ cell. All these antigens show characteristic distribution patterns in different lymphocyte populations and these patterns correlate with known functional groupings. This suggests that the antigens themselves play a role in functional behaviour, perhaps conveying instructions that guide cell interactions.

Current information suggests that there are four major categories of T lymphocytes: the initiator cell (T1), the helper cell (Th), the cytotoxic effector cell (Tc) and the suppressor cell (Ts) (Snell 1978). In addition, natural killer (NK) cells express some T cell markers (West 1977).

(i) The Initiator Lymphocyte (T1).

The initiator lymphocyte is the least firmly categorised of the four classes of T cells. It appears to act first in the chain of responses initiated by exposure to antigen. In the murine system initiator lymphocytes (T1) are operationally defined as T cells from thymus or spleen, which when sensitised on allogenic fibroblasts and injected into the foot pad of isogeneic hosts, recruit from lymphocytes circulating through the nodes those with graft-versus-host potential for the priming alloantigen. The spleen is several times richer in T1 than thymus but T1 are not demonstrable in the lymph nodes. Sensitisation of T1 cells occurs within 4 to 6 hours, requires protein but not DNA synthesis, and once established, is not destroyed by irradiation with 2000 rads (Cohen 1976). The T1 are susceptible to anti-Thy-1 plus complement and are absent in irradiated, bone marrow-reconstituted mice and hence are T cells. Unlike most T-cells, they are adherent to nylon wool and their number is reduced by adult thymectomy suggesting that they are a short lived population. Cohen et al

(1976) suggest that the function of T1 is to patrol the tissues and detect invading immunogens. The remarkably short time required for priming is in keeping with this function. They also found that T1 gave rise to memory cells with initiator capacity and specificity for the priming alloantigen.

(ii) The Helper Lymphocytes (Th)

Helper cells (Th) are a subpopulation of T lymphocytes which play an obligatory accessory role in most though not all immune responses, both humoral and cellular. They contribute to antibody formation by B lymphocytes, to the generation of cytotoxic T cells, to development of delayed hypersensitivity, and probably also to the generation of suppressor cells.

In the murine system, the Th subpopulation is of the phenotype Lyt-1^+, Lyt-$2,3^-$. The Fc receptor (FcR) may be one surface marker which could distinguish Th subclasses. When T cells were characterised functionally, it was found that the T cell collaborating with B cells in antibody production was FcR^-, while cells that collaborated with T effector cells were FcR^+ (Stout et al 1976). This would divide helper cells into two groups according to the type of cell which they help, and hence, cells with helper function in antibody production have been labelled "helper cells" and cells with helper function in cytotoxic T cell responses have been named "amplifier cells" (Stout 1976). It has also been suggested that specific cells are involved in help of B cells of a particular allotype. (Herzenberg 1976). Regulation of antibody production and cell mediated cytotoxicity are clearly two major functions of helper cells.

(iii) The Cytotoxic Lymphocytes (Tc)

Cytotoxic lymphocytes cause cell mediated lysis in vitro. Tc cells are probably effector mechanisms in most instances of allograft rejection and probably play a role in the graft-versus-host reaction and in delayed hypersensitivity. Substantial evidence exists that the

principal function of this lymphocyte is the elimination of virus-infected cells. These T cells are long lived in mice and have the surface phenotype Lyt1$^-$, Lyt2, 3$^+$. They are resistant to anti-Ig plus complement, are susceptible to anti-lymphocyte serum (ALS) and remain after adult thymectomy. They can be distinguished from Ts by means of another cell surface marker, la-4, the murine equivalent of HLA-D, found on suppressor cells (Murphy 1976).

(iv) The Suppressor Lymphocyte (Ts)

The discovery of the suppressor lymphocyte (Ts) (Baker et al 1970) was an important step in understanding the regulation of the immune response and it is now widely accepted that such cells play a primary role in the regulation of many immunologic responses to all types of antigens. Some oncogenic viruses turn on suppressor cells in some strains of mice, thereby thwarting the host's defences (Kumar et al 1976). As a T cell, the Ts is Ig-negative and Thy-1 positive. It is not removed by thymectomy and hence is long lived but susceptible to ALS and bears the Lyt1$^-$, Lyt2, 3$^+$ surface antigenic phenotype in mice (Feldman 1977).

However, cells with non-specific suppression have been characterised in mice undergoing graft versus host reaction which have an Lyt-1$^+$, Lyt-2, 3$^+$ phenotype and to complicate the issue, Watanabe et al (1977) have identified a suppressor cell of Lyt-1$^+$, Lyt2, 3$^-$ phenotype.

Several investigators have reported that the Ts is positive for Ia antigen and there was subsequent identification of a locus, Ia-4 in the I-J subregion of the H-2 complex whose end product is a marker for Ts. It is also present in soluble suppressor factors. It has been shown that Ia although present on activated Ts, is absent from the Ts precursor.

It is thus evident that subpopulations of suppressor lymphocytes also exist. The Ts population is particularly abundant in the spleen. This abundance in the spleen rather than lymph nodes might have significance in the transplantation phenomenon as it might account for the greater relative

success of organ grafts with immediate drainage into the blood stream as compared with skin grafts which drain via lymph and lymph nodes.

The classification of T lymphocytes in the above fashion utilises cell surface antigenic markers as well as functional differences between the types. A further method of classification based on cell dynamics has been propounded.

(v) Lymphocytes distinguished by their circulatory pattern - The intestinal and nodal lymphocyte pools

Several authors (Cahill et al 1977, Scollay et al 1976) using radio-labelled lymphocytes from various lymphoid organs have shown that lymphocytes can be separated into two groups on the basis of their circulatory patterns - an intestinal pool and a nodal pool. The nodal pool circulates mainly from the blood via lymph nodes to the thoracic duct lymph and possibly the spleen. The intestinal pool circulates from the blood to the small intestine and Peyer's patches. Both appear morphologically to be small lymphocytes. No direct correlation has been demonstrated between these two populations and subpopulations of T lymphocytes classified according to their functional properties.

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CHAPTER 2. IMMUNOLOGICAL FUNCTION OF T CELL SUBSETS

2A. CYTOTOXIC/SUPPRESSOR LYMPHOCYTE

This T cell subset contains a mature population of cells with suppressor functions. After concanavalin A activation it will suppress autologous proliferative response to alloantigens. This same subset suppresses B cell immunoglobulin production and in man this subset reacts with OKT5 anti serum and appears to be analogous to the murine Lyt2, 3^+ subset, which mediates both cytotoxic and suppressor functions, and the $OKT4^+$ subset is analogous to the murine $Lyt1^+$ subset which provides for inducer-helper function.

Suppression is basically a T lymphocyte function designed to regulate the magnitude of any individual component of the immune response. The T suppressor lymphocyte however, forms only one aspect of the suppressor system in normal blood. Rice (1979) has demonstrated three suppressor systems within human blood that modulate T cell lymphoproliferation. These are the adherent cell suppressor system which appears to be effected by a steroid and radio-resistant monocyte; the prostaglandin related suppressor system, also mediated by a monocyte but with different properties, and sensitivity to indomethacin; and the mitogen induced suppressor system activated by pre-incubation with concanavalin A. Stockinger et al (1979) have also produced evidence for a 7S-immunoglobulin producing B cell that functions as a suppressor cell under certain conditions and antigen specific inhibition can be obtained by transfer of these B cells. The initial event in suppression appears to be the activation of a suppressor-inducer cell (Paul 1983). This then acts on a suppressor-precursor cell which subsequently develops into an activated suppressor cell. This cell secretes a factor that can bind the antigen that initiated the response, although not causing suppression on its own. It is not clear whether the helper T lymphocyte or the B lymphocyte is the target of the suppressive activity.

Loss of suppressor activity has been noted in auto-immune diseases like systemic lupus erythematosus (Bresnihan 1977, Kaufman 1979) auto-immune haemolytic anaemia and active chronic hepatitis (Hodgson 1978). There is evidence that auto-antibodies in the sera of these patients are directed against the suppressor group either selectively eliminating them or modulating their functional properties (Sagawa 1979, Morimoto 1980). Conversely, the presence of excessive numbers of activated suppressor cells can result in severe immunodeficiency (Waldmann 1974). Increased numbers of activated suppressor cells have been demonstrated in patients after Epstein-Barr and cytomegalovirus infections (Johnsen 1979). In chronic graft-versus-host disease, persistent circulating suppressor cells can cause prolonged immunologic incompetence (Reinherz 1979a).

In some patients with acquired hypogammaglobulinaemia, excess circulating suppressor T cells can be demonstrated (Waldmann 1974) and to support the fact that this was the main cause for hypogammaglobulinaemia, treatment with methyl prednisolone results in a reversal of the immuno-deficiency. The modulation of IgE responses by suppressor T cells in man also seems well established (Geha 1979). Peripheral lymphocytes of individ-uals with high IgE levels have been shown to secrete significantly more IgE than lymphocytes from normal individuals. This increased IgE secretion can be suppressed by normal donor lymphocytes (Becker 1978).

The cytotoxic T lymphocytes are sensitised and possess specific receptors that allow them to bind to their target cell and then to mount an attack leading to the destruction of the target cell. These T cells appear to play an important role in cellular immunity to viruses and other intra-cellular pathogens. Their suggested role in the immune serveillance against cancer may be through its ability to eliminate cells infected with oncogenic viruses (Kiessling 1976).

2B. INDUCER/HELPER LYMPHOCYTE

This subset provides inducer and helper function in interactions between T cells, between T cells and B cells, and between T cells and macrophages. (Reinherz 1980a). It is also required for optimal development of cytotoxicity by the OKT5$^+$ cells. Several studies have shown that this subset alone is required to induce B cells to proliferate and differentiate into immunoglobulin-containing plasma cells. It secretes lymphocyte mitogenic factor which induces proliferation of all major lymphocyte subclasses and T-cell helper factors which initiate B cell immunoglobulin synthesis (Reinherz 1980b). The regulatory effects of the helper subset is not restricted to lymphoid cells alone as it has been shown that it can modulate erythroid stem-cell production in vitro and is possibly important in haemopoietic differentiation (Cline 1979). Similarly a variety of other factors including osteoclast-activating factor and soluble factors inducing fibroblast proliferation and collagen biosynthesis have been shown to be derived from antigen stimulated T cells.

As these immunologic functions are acquired only at the latest stage of intrathymic ontogeny, premature release of immunologically incompetent cells or aberrations of T-cell maturation could lead to immuno-deficiency. Some patients with acquired agammaglobulinaemia lack the OKT4$^+$ subset and thus are incapable of triggering B cell synthesis of immunoglobulin (Reinherz 1980c).

2C. NATURAL KILLER CELLS (NK)

Human natural killer cells are present in the peripheral blood, have the morphology of large granular lymphocytes, and express low avidity for sheep red blood cell receptors, Fc receptors for IgG and complement receptors for split products of C3 (Herberman 1979). These features imply that part of NK cells belong to the T cell system and could be immature T cells. Ferrarini et al (1980) have compared the third

population lymphocytes with the $T\gamma$ population and shown many similarities on morphological grounds at the electron microscopy level, as well as similar histochemistry. The third population or "null" cells have been demonstrated to include immature B cells, immature T cells and non lymphoid cells, and are clearly not homogeneous but consist of several subpopulations with regard to cell surface markers. Jondal and Merrill (1981) have shown that in the active NK cell population accounting for 6% of non-adherent lymphocytes, 60% had FcR for IgG and 17% had Fc receptor for IgM, and also a fraction lacking both types of receptors. Ng and Co-workers (1981) have shown that null cells with high natural killer activity, isolated by a simultaneous double rosetting procedure expressed receptor for IgG Fc in 70% of cells as well as monkey erythrocyte receptors in 60 - 90%, but lacked receptors for the IgM Fc fragment. Zarling and Kung (1980) have shown that the majority of NK cells are $OKMI^+$, and OKMI monoclonal antibody with complement causes marked diminution of NK cell activity. Recent evidence points to NK cells being able to spontaneously lyse certain tumour cells in vitro and to provide early resistance against tumour growth.

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CHAPTER 3. MARKERS OF T LYMPHOCYTES

3A. THE SHEEP ERYTHROCYTE RECEPTOR

The formation of rosettes by human peripheral lymphocytes with sheep erythrocytes (SRBC) in the cold has been shown to be a specific marker of human T cells (Lay 1971). This rosette forming ability is a specific function of viable T lymphocytes only, and surface immunoglobulins or complement do not play a role in this. Inhibition of rosette formation by various agents has allowed further characterisation of the site of the SRBC receptor on T lymphocytes (Pyke 1975). Anti-human lymphocyte serum (ALS) and specific anti-thymocyte serum was shown to inhibit E-rosette formation whereas antisera raised to B cells failed to do so. Antisera directed against other known surface components represented on both B and T cells such as β2-microglobulin, I and i antigens did not influence rosette formation, suggesting that these membrane components are not involved in the binding of SRBC. The HL-A antigens were also shown to be unlikely as the site of SRBC binding as they were also present on B cells and they were not trypsin-sensitive, whereas E-rosette formation was. E-rosettes are inhibited by high concentrations of cyclic adenosine monophosphate and thus by agents that increase it at intra cellular level, such as theophylline (Jondal 1976).

3B RECEPTORS FOR THE Fc FRAGMENT OF IgG/IgM

Moretta et al (1976) demonstrated the possibility of recognising different sets of human T lymphocytes with receptors for the Fc fragment of IgG (Tγ) or IgM molecules (Tμ). A single T cell carried either one or the other receptor for the specific immunoglobulin or had no detectable receptor - T "null". Later, T cells with receptors for other Ig subclasses have been described. Lum (1979) and Sjoberg (1980) showed that a small percentage of T cells (5%) have receptors for IgA. The ability of specific

T cells to bind Ig of different classes makes possible their physical separation in vitro.

In the mouse, T cells depleted of the $T\gamma$ fraction contain helper cells and precursors of cytotoxic effectors or cell-mediated lympholytic (CML) responses (Stout et al 1976), whereas $T\gamma$ enriched cells contain the differentiated cytotoxic effector cells of CML, but not helper T cells. In the human, the Fc-IgG receptor negative but Fc-IgM receptor positive ($T\mu$) cells provide help in a B cell immunoglobulin-producing assay (Moretta 1977). $T\gamma$ cells act as suppressor cells. Some $T\gamma$ lose their Fc-IgG receptor spontaneously in culture at $37^{o}C$, whereas most lose the receptor after interaction with immune complexes.

Pichler et al (1978) showed that $T\gamma$ cells after 48 hour cultivation and prior interaction with immune complex, underwent transition to Fc-IgM receptor positive cells, proving that sequential expression of Fc-IgG and Fc-IgM receptors on the same T cell is possible. They also observed that on various occasions some T cells could express both receptors.

The frequent observation that $T\gamma$ cell numbers in healthy individuals varies widely may reflect the instability of the Fc-IgG receptor. Therefore a high $T\gamma$ percentage in an immunodeficient patient may not necessarily reflect an excessive suppressor function but may merely reflect a lack of critical IgG-Fc interaction due to low serum IgG levels in these patients (Moretta 1977).

Moretta (1981) subsequently showed that in mixed lymphocyte cultures, peripheral T cells could progressively lose $Fc\mu$ and acquire $Fc\gamma$ receptors, resulting in a decrease in number of $T\mu$ cells. Thus it can be seen that the Fc-IgG and Fc-IgM receptor may not be definite markers for T cell subsets under some conditions and that expression of these receptors may occur at different functional stages.

(i) Morphology of Tγ/Tμ Cells

Grossi et al (1978) showed that Tμ cells were small lymphocytes with a nucleus occupying almost the whole cell with a thin rim of basophilic cytoplasm. In contrast the Tγ cells were larger with abundant cytoplasm containing azurophilic granules. The nuclear chromatin in Tγ cells is homogeneously distributed. Under the electron microscope Tμ cells had a smooth cell surface with a small Golgi apparatus, rare mitochondria and monoribosomes dispersed uniformly. The Tγ cells, however, had a relatively rough surface with microvilli, an extensive Golgi apparatus, rough endoplasmic reticulum and numerous mitochondria occurring in clusters. Grossi also showed that up to 95% of the Tμ cells were esterase positive with this activity confined to a single large spot or sometimes two such vesicles with positive staining. In contrast, 90% of the Tγ cells were esterase-negative.

(ii) Functional aspects of Tγ/Tμ cells

Moretta et al (1976) showed that the Tγ and Tμ cell subsets had different patterns of response to the T cell mitogen phytohaemagglutinin (PHA), but similar response patterns to concanavalin A.

The Tμ population of cells provided help in B cell immunoglobulin synthesis, whilst Tγ were unable to provide help and suppressed immunoglobulin synthesis. The suppressor effect was abrogated by irradiation.

Hayward et al (1978) however, demonstrated that suppression did not always require the presence of Tγ cells. The Tμ subpopulation was also shown to have suppressor activity in co-cultures after they had been incubated with concanavalin A for 24 hours. Hayward and his co-workers concluded that Tμ cells can either help or suppress B lymphocyte differentiation whereas the Tγ subset either suppresses or is without effect. Stockinger et al (1979) have also shown suppressor activity in a 7S immunoglobulin-producing B cell.

Herzenberg et al (1976) provided further insight into the role of suppressor cells in antibody responses in the allotype suppression system. It was shown that the suppressor cells removed the helper activity required for antibody production by limiting the amount of available helper activity, whereas helper cells exerted direct control over IgG antibody production.

The maternal-foetal immunological relationship and tolerance has been another model studied in an attempt to understand the subtle balance between suppression and help of lymphocyte activity. Murgita (1977) showed that alpha-foeto-protein (AFP) a tumour associated embryonic substance and normally produced by foetal hepatocytes, is capable of inducing the formation of highly efficient suppressor cells, with the capacity to inhibit helper T cells. This is in keeping with the fact that thymus derived lymphocytes from newborn humans can function as suppressor cells with regard to the proliferative capacities of the lymphocytes from their mothers. It was suggested that AFP serves as an agent to protect the mammalian foetus from the graft-versus-host reaction through the selective induction of suppressor T cells.

3C. THE GALL BODY

Edward A. Gall first described the lymphocyte granule in 1936 which now bears his name (Gall 1936). He described it as -

> "occurring exclusively within lymphocytes ..... and it was noted almost entirely in wet preparations. These spherules are markedly refractile, appear solid black at one focus and have a colourless glassy sheen at another in the unstained state. Ordinarily a single granule and much less often two or three are present in one lymphocyte. These intracellular inclusions are actively motile."

He found that an average of 35% of peripheral lymphocytes contained this body. It has since been claimed that Gall bodies are present mainly in helper lymphocytes (Marcus 1981). In this connection

it is of interest that Gall found the percentage of lymphocytes containing the granules to be low in infectious mononucleosis. Recent work has shown that suppressor cells are induced in Epstein-Barr virus infections and helper cells are diminished. Johnsen et al (1979) were able to demonstrate a functional lack of T helper activity during acute infectious mononucleosis and showed that the T lymphocytes seen in these patients were an expansion of T suppressor cells. The human Ia-like antigen HLA-DR antigen was identified on up to 75% of peripheral lymphocytes in these patients and these cells could suppress proliferation of normal B lymphocytes in vitro, suggesting that they were mainly suppressor T lymphocytes. This suppressor predominance in acute infectious mononucleosis thus overcomes the otherwise unlimited proliferation that the Epstein-Barr virus can induce in infected B cells.

Hempelmann (1953) attempted to elucidate the nature of this body and based on its optical properties and histochemical reactions concluded that it contained lipoid material and was birefringent. However, more recently Marcus and Hurtubise (1981) have studied this elusive body further and claim that

> "this ephemeral particle is confined mainly to helper T lymphocytes and correlates with most and perhaps all of the recently recognised cytoplasmic focal acid esterase activity in T cells. At present the nature and function of Gall granules are a mystery."

They showed that no B cell had Gall granules, in contrast to T-helper cells of which 48% contained the body and in which 88% of all Gall bodies resided. They concluded that the Gall granule probably correlates entirely with focal lymphocyte acid esterase activity and was a simple and good marker for T-helper lymphocytes.

Dr. Gall was honoured by the naming of a minor planet to commemorate his many achievements in pathology. Because there already was an asteroid named 148 GALLIA, his friend the distinguished atronomer Dr. Paul Herget, named it 1661 GRANULE.

3D. CHARACTERISATION OF HUMAN T-CELL SUBSETS USING ANTI-TH_1 ANTISERA

A further means of defining T cell subpopulations was described by Evans and co-workers (1977) utilising rabbit antiserum raised against highly purified human T cells and rendered T cell-specific by absorption with autologous B lymphoblastoid cells. 60% of peripheral T cells were reactive with this antiserum (TH_1^+) and the un-reactive group was designated TH_1^-. The TH_1^+ cells were found to proliferate in response to alloantigens in the mixed lymphocyte culture and also secreted lymphocyte mitogenic factor. The TH_1^- subclass however, could be triggered by specific antigens, e.g. tetanus toxoid and mumps, to proliferate, but did not respond in mixed lymphocyte cultures or secrete lymphocyte mitogenic factor.

Anti-TH_2 antiserum was evolved by immunising rabbits with human thymocytes. The strongly reactive TH_2^+ subset was shown to contain the cytotoxic effector cell in cell mediated lympholysis as well as the immuno-regulatory suppressor cell. The 70-80% T cells which were un-reactive to TH_2 antisera were shown to be heterogeneous and had inducer and feedback regulator activity.

Subsequent development of monoclonal antibodies to define T cell restricted surface antigens showed that the OKT_4 monoclonal antibody which was proposed as defining helper T cells, was limited to the TH_2^- subpopulation. This was further confirmed by Rubinstein and co-workers (1981) when $T\mu$ cells were found to be enriched in the TH_2^- subset as defined by an autoantibody derived from a patient with acquired agammaglobulinaemia.

3E. T CELL SUBSETS DEFINED BY JRA ANTISERA

Strelkauskas et al (1978) utilised naturally occurring auto-immune antibodies found in patients with juvenile rheumatoid arthritis (JRA), to isolate two subpopulations of T cells. It was found that the sera of children with severe JRA contained antibodies which were reactive with subclasses of T cells but not B cells. The JRA + cells forming 30% of T cells responded to Con A stimulation but poorly to PHA and did not

help in the synthesis and secretion of Ig by B cells, but exerted an immunoregulatory influence and it seemed to overlap with the TH_1^+ subset and Fcγ receptor bearing populations previously defined. The JRA^- subset accounting for 70% of T cells, however, proliferated well in response to PHA but not Con A and greatly enhanced the secretion of Ig by B cells, thus demonstrating helper activity. The demonstration that this naturally occurring antibody had the ability to influence T cell activity suggests that autoantibodies reactive with suppressor T cells and produced during active autoimmune disease may be responsible for the overproduction of immunoglobulins seen.

Reinherz and Strelkauskas (1979b) undertook a correlative study to compare the TH_2^+ and JRA^+ subsets which, though similar in size, were shown to define different subpopulations based on their mitogenic responsiveness. It was shown that 40% of the TH_2^- subset was JRA^+ whereas only less than 5% of the TH_2^+ subset was JRA^+. Based on these findings, three phenotypically distinct subsets were identified in man - the TH_2^+ JRA^-, TH_2^- JRA^+ and TH_2^- JRA^- T cell subsets.

3F. THEOPHYLLINE SENSITIVE AND THEOPHYLLINE RESISTANT T CELLS

The binding of unsensitised sheep erythrocytes to T lymphocytes has been established as a T cell characteristic since the first observation by Lay and Mendes (1971) that some human lymphoid cells bound sheep erythrocytes to form rosettes in the cold. It has since been shown that human cells bind other heterologous erythrocytes including those from swine and rhesus monkey (Lohrmann 1974), in addition to autologous erythrocytes. This rosetting can be modified by various agents like anti-lymphocyte serum and specific anti-thymocyte serum, B-adrenergic agonists, prostaglandins and cyclic AMP (Pyke 1975). Limatibul et al (1978) showed that the drug theophylline, a phosphodiesterase inhibitor capable of increasing cyclic 3'5' adenosine monophosphate (c-AMP) within the cell, could also alter the rosetting ability of some T cells. They also demonstrated that

sustained elevation of lymphocyte c-AMP levels was required to prevent the re-expression of E-rosetting ability.

Based on the modulation of the E-rosetting ability by theophylline, they were able to delineate three subpopulations of T lymphocytes.

(a) Theophylline sensitive T cells (Tsens) which lost the ability to form E-rosette following theophylline treatment.

(b) Theophylline resistant T cells (Tres) which were unaffected by the drug.

(c) Theophylline dependent cells which acquired the ability to form E-rosettes following incubation with theophylline.

The effect of theophylline was shown to be dose-dependent, and optimal at 3mM concentration, whilst concentrations greater than 10^{-2}M theophylline impaired cell viability. It was reversible and temperature dependent, occurring only at 37°C incubation, suggesting that the drug required cellular metabolic processes mediated through cyclic nucleotides.

The major E rosetting populations in peripheral blood were shown to be either theophylline-sensitive or theophylline-resistant whilst thymocytes were shown to be theophylline-resistant.

Shore and Gelfand (1978) using this method of separation of T cell subsets showed that the theophylline-sensitive cells completely suppressed plaque forming cells (PFC), whereas theophylline-resistant cells augmented. They also showed that the majority of Tsens cells (91%) carried Fc receptors for IgG whereas 86% Tres cells were found to have Fc receptors for IgM.

Birch and Polmar (1981) showed that Tsens cells comprised 20% of the total T cells while Tres comprised 80% of T cells. They also demonstrated that adenosine and impromidine, which are H_2-histamine agonist, were able to increase the percentage of Fc receptors for IgG in the Tres population resulting in a loss of their helper activity in B-lymphocyte differentiation.

It has been shown that there is a receptor for histamine of the H_2 type on the surface of suppressor cells (Plaut 1975) and histamine has been shown to be a potent activator of suppressor cells in man. Cimetidine, a known H_2-histamine blocking agent has been shown to abrogate suppressor cell function (Osband 1981).

3G. MONOCLONAL ANTIBODIES

The advent of monoclonal antibodies has been an important advance in the identification of antigenically distinct immuno-regulatory human T-lymphocyte subsets. Köhler and Milstein (1975) were the first to show that cultured mouse myeloma cells could be fused to normal spleen cells from animals immunised with sheep red cells and that the hybrid cells produced large amounts of homogeneous antibody to SRBC. Since then, hybridomas have come into extensive use for production of large amounts of homogeneous antibody to a variety of antigens including cell surface constituents.

The basic method involved in producing a monoclonal antibody to T cell surface antigens is exemplified by the hybridoma technique described by Kung and Goldstein (1979) for generating monoclonal antibodies against surface determinants of human T cells. Eight week old female mice were immunised intraperitoneally with human T cells at 14 day intervals. Four days after the third immunisation, the spleens were removed and single cell suspensions prepared. Cell fusions were then carried out using splenocytes and myeloma cells, which were then cultured in selective medium. Only one out of 2×10^5 spleen cells actually form a viable hybrid with a myeloma cell and up to 500 hybrids can be generated from each spleen. Hybridomas secreting T cell specific monoclonal antibodies were then repeatedly cloned and later injected into the peritoneum of mice whereby the ascites so produced contains high titres of the specific antibody.

Ledbetter and Evans (1981) utilising monoclonal antibodies, have

redefined the two subpopulations previously defined by αTH_2 antisera. The mouse analogue Lyt2,3 was found to be defined by the Leu2a/Leu2b monoclonal antibody expressed on the suppressor subset. Leu3 defined the human helper/inducer subset and Leu-1 was the human analogue of the murine Lyt-1 antigen.

Janossy and Kung (1980) analysed the human T lymphocyte subsets reacting with the OKT range of monoclonal antibodies and defined various markers. The helper T cells were shown to react with the OKT4 monoclonal antibody, while the suppressor-cytotoxic T cell was recognised by OKT5 and OKT8 antibodies. The distribution of these subsets was analysed in the lymphohaemopoietic organs and gut. $OKT4^+$ cells of the inducer type were shown to predominate in the thymic medulla, blood and T cell traffic areas such as tonsillar paracortex and intestinal lamina propria. $OKT8^+$ cells of the suppressor-cytotoxic type, however, constituted the larger part of the T cell population in normal human bone marrow and gut epithelium.

In the human thymus 78% of all cells were $OKT4^+$. The intra-epithelial T cells of the human gut consisted mainly of $OKT8^+$ cells. OKT3 was shown to label almost all T cells and OKMI to label human monocytes.

Reinherz et al (1982) have further characterised three additional monoclonal antibodies viz. anti-T4A, anti-T4B and anti-TQ1, which were all reactive with the OKT4 positive human helper-inducer T subset. Anti-TQ1 reacted with 70 to 85% of $T4^+$ lymphocytes but also stained 50% of T cells in the cytotoxic/suppressor subset, as well as a fraction of normal B and null lymphocytes. They showed that the $OKT4^+$ $TQ1^+$ subset was responsible for maximal proliferation in autologous MLR reactions whereas the $OKT4^+$ $TQ1^-$ subset provided the main T cell help for B cell immunoglobulin production in a pokeweed-driven system. Thus the $OKT4^+$ subset was further divided into two subpopulations with the major T helper function restricted to only

15 to 25% of this population. Anti-TQ1 reactivity with part of the OKT8 subset suggests that this population is also heterogeneous.

Beverley and Callard (1981) utilising the UCHTI monoclonal antibody which is an IgGi mouse anti-T cell monoclonal antibody showed that two very different phenotypic E-rosetting T cells could be identified. The E rosetting UCHTI$^+$ cells responded well to T cell mitogens PHA and Con A and provide help for an in vitro specific antibody response. In contrast the E rosette + UCHTI - population had no other markers for T cells, failed to respond to T cell mitogens and were shown to be highly active natural killers. They thus concluded that not all E-rosetting cells were in fact T cells.

CHAPTER 4. MATERIAL AND METHODS

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CHAPTER 4

4A AIMS OF THE STUDY

T lymphocyte subsets have been shown to have different cell surface markers as well as different mitogen responsiveness in studies done in vitro. Their identification and study becomes important in diseases where the pathogenesis may be due to an imbalance in numbers or function of these subpopulations. The recent literature abounds with numerous methods of identifying these subsets, but some of the methods are time consuming and too difficult to reproduce to be applicable for clinical use. The recent advent of monoclonal antibodies has contributed significantly to defining lymphocyte subsets but the correlation of, for instance, OKT-defined markers and other surface markers like Fc receptor status is not a simple one Reinherz et al (1980d).

There may thus be a place for a simple and rapid means of identifying the suppressor and helper subsets in a given patient. One such method can be theophylline modulation of ERFC ability, first described by Limatibul and co-workers (1978). The present study concerns the characteristics of theophylline sensitive and theophylline resistant T lymphocytes in normal blood donors. The characteristics studied include monoclonal antibody profile, Fc receptor status with respect to IgG or IgM, the presence or absence of Gall bodies, the esterase content as determined by ANAE staining and ability to aid or suppress immunoglobulin production in B cells.

In addition, longitudinal studies were carried out in five patients with systemic lupus erythematosus over periods ranging from five months to a year, in an attempt to correlate the changes in theophylline sensitive and theophylline resistant populations with disease activity as judged by other indices.

4B MATERIALS

1. Red Cross donor blood was used for the major part of the analysis of theophylline sensitive and theophylline resistant T lymphocytes. Blood packs of 300-450 ml. were obtained twice weekly from the Red Cross Blood Transfusion Centre, Adelaide.
2. Five patients with systemic lupus erythematosus from the different medical units of The Queen Elizabeth Hospital, were followed up over periods ranging from five months to a year.
3. Single studies were done in 19 other patients with auto-immune diseases as well as other miscellaneous conditions.

Disease	Number of Patients
Systemic lupus erythematosus	5
Alcoholic liver disease	4
Chronic lymphatic leukaemia	2
Scleroderma	1
T cell lymphoma	1
Active chronic hepatitis	1
Sarcoidosis	1
Graves' disease	1
Myasthenia gravis	1
Wegener's granulomatosis	1
Epilepsy (Dilantin treated)	1

4C METHODS

1. HAEMATOLOGICAL EXAMINATIONS

1.1 The erythrocyte sedimentation rate (ESR) was determined by the method of Westergren with an established normal range of 2-7mm/hour.

1.2 Fresh unstained suspensions of T lymphocytes suspended in RPMI medium + 10% FCS were examined under the phase microscope to determine the presence of Gall bodies.

1.3 Cytocentrifuged preparations of rosetted lymphocytes were stained with the May-Grumwald-Giemsa stain.

2. IMMUNOLOGICAL METHODS

2.1 Native DNA binding activity

Native deoxyribonucleic acid (n-DNA) binding activity was measured by radio-immunoassay with the Amersham I^{125} kit (Radiochemical Centre, Amersham,England) based on the ammonium sulphate precipitation assay first described by Wold et al (1968).

Method

50µl of a 1:10 dilution of the patient's serum was added to 50µl of I^{125} labelled DNA and mixed and allowed to stand at 37°C for one hour. This was then placed in a 4°C chamber overnight. Separation of free and bound DNA was achieved by adding 100µl of saturated ammonium sulphate solution and mixing vigorously and this was centrifuged at 1000G at 4°C for 15 minutes. The supernatant was aspirated and the radioactivity in the precipitate was measured in a gamma counter. The normal DNA binding by this technique was 0-25 units/ml.

2.2 Antinuclear factor titres

Antinuclear antibodies were sought by indirect immunofluorescence using 6μ cryostat sections of unfixed rat liver and rat liver fixed in absolute alcohol for three minutes at 20°C. This was incubated with a 1:10 dilution of the patient's sera for 30 minutes and counterstained with FITC conjugated anti-human gammaglobulin (Wellcome Laboratories) used at a dilution of 1:30.

Nuclear staining was classified as homogeneous, rim pattern, fibrillar, speckled or nucleolar pattern.

3. SEPARATION OF LYMPHOCYTE SUBPOPULATIONS AND FUNCTIONAL ASSAYS

3.1 Harvesting of peripheral T lymphocytes

Human peripheral blood lymphocytes from normal adult donors were isolated from heparinised venous blood by Ficoll-Hypaque density gradient centrifugation. The mononuclear cell fraction was harvested, washed thrice with PBS and then suspended in RPMI 1640 medium supplemented with 10% heat inactivated foetal calf serum (Commonwealth Serum Laboratories, Australia).

Adherent cells were removed by incubation of these cells in plastic petri dishes at 37°C for 60 minutes. The non-adherent lymphocytes were then siphoned off and the adherent cells removed by scraping the base of the plastic dishes with a plastic policeman and resuspending it in medium.

Human T lymphocytes were then isolated by adding to the non-adherent cells, 1.5% SRBC solution to give an optimal ratio of 50 SRBC:1 lymphocyte, and 40% FCS. This was then centrifuged at 150g for 5 minutes at 4°C and incubated on an ice bath for one hour (overnight rosetting on an ice bath was occasionally used). The rosetting pellet was then gently resuspended and layered on a Ficoll-Hypaque gradient and centrifuged at 450g for 30 minutes.

The non-rosetting cells in the interface were collected separately and washed with PBS - this formed the B cell fraction for the subsequent cultures. The rosetted pellet was treated with 1.5 ml. of glass distilled water for 20 seconds to lyse the SRBC, washed and resuspended in RPM1 1640 with 10% FCS. This formed the T cell fraction.

3.2 Separation of theophylline sensitive and theophylline resistant T lymphocytes

The T lymphocytes were incubated with 3mM theophylline solution for 60 mins. at $37^{o}C$. The cells were then rosetted with SRBC as described above, in the presence of theophylline. The fraction found to retain the ability to rosette was termed theophylline resistant lymphocytes (T res) whereas the cells which lost the ability to form rosettes were termed theophylline sensitive lymphocytes (T sens). These fractions were then separated by a further Ficoll-Hypaque density centrifugation.

3.3 B cell differentiation assay

The non-T non-adherent peripheral blood lymphocytes were suspended at a concentration of 2.5×10^{6}/ml. in RPMI 1640 supplemented with 10% FCS. This was incubated with varying concentrations of T res and T sens subsets in the presence of pokeweed mitogen at a final concentration of 1:400. In addition 5% of adherent cells were added to each culture well. The final mixtures were incubated at $37^{o}C$ for six days in a humidified 5% CO_2 atmosphere. At the end of incubation the cells were harvested and its viability determined by trypan blue dye exclusion. Cytocentrifuge preparations were then prepared using 2×10^{5} cells with 20μl of chicken red blood cells (CRBC) at a concentration of 0.5×10^{6}/ml as indicator cells, and 20% FCS. These smears were then air dried.

3.4 Determination of intracytoplasmic immunoglobulin production

The cytocentrifuge preparations were fixed by immersing in 3% paraformaldehyde solution for 10 mins and then ice cold acetone for 5 mins. This was then stained with fluorescein-conjugated polyvalent anti-human immunoglobulin antiserum (Wellcome Reagents Ltd., England) for 30 mins and then mounted in Coons-glycerol before examination with fluorescence microscopy. The number of intracytoplasmic immunoglobulin containing cells were counted per 150-200 CRBC for each slide.

3.5 Indirect immunofluorescence technique for determing monoclonal antibody profile.

The lymphocytes to be studied were suspended in RPMI 1640 supplemented with 10% heat inactivated foetal calf serum at a concentration of 5 x 10^6/ml. 200µl of reconstituted monoclonal antibody solution (Ortho Pharmaceutical Corporation, Immunobiology Division, Raritan, New Jersey 08869) were used in each case, viz. OKT3, OKT4, OKT8 and OKM1. This was mixed and incubated on an ice bath for 30 minutes. The suspension was then washed twice with ice cold PBS-Azide solution. 100µl of fluorescein-labelled goat anti-mouse immunoglobulin was then added to the cell pellet, mixed and allowed to stand a further 30 mins. in ice. This was again washed twice with ice cold PBS-Azide. The remaining cell pellet was then resuspended in 5µl 90% glycerol mounting medium and examined under incident ultra-violet light.

3.6 Determination of percentage of theophylline sensitive T lymphocytes

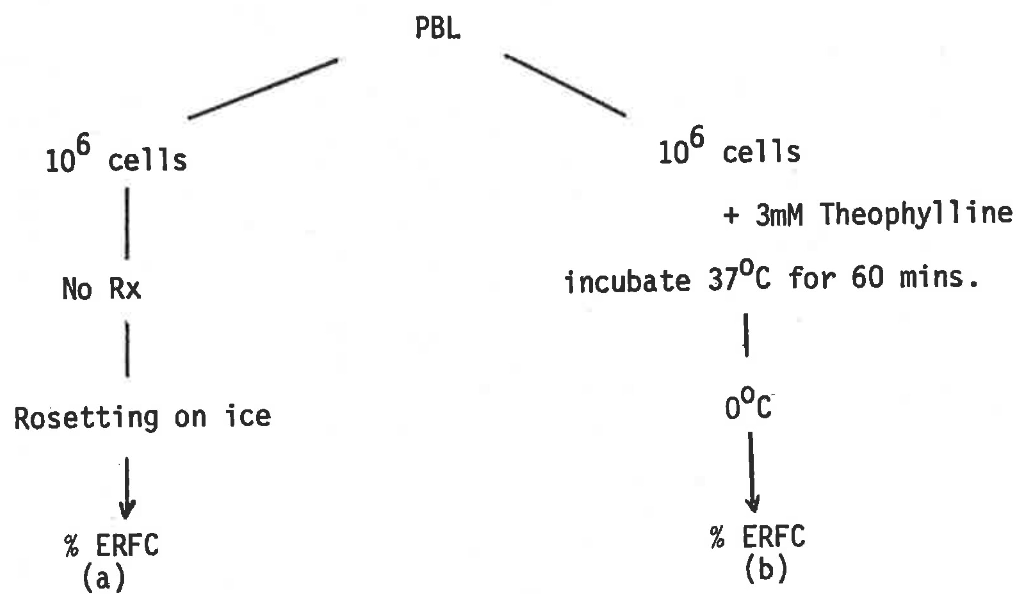
Selective loss of the ability of T lymphocytes to rosette with SRBC after exposure to theophylline as described by Limatibul and Shore (1978) was utilised in this test to identify the theophylline sensitive population.

Method

Peripheral blood lymphocytes (PBL) were suspended at 2×10^6/ml in RPMI 1640 medium supplemented with 10% foetal calf serum (FCS). Into each of two test tubes, 0.5 ml. of suspension was pipetted and to one of them 0.5 ml. of 6mM theophylline solution was added. This tube was then incubated at 37°C for 60 mins.

A 1.5% SRBC solution was prepared meanwhile by suspending thrice washed SRBC in PBS. To each of the tubes containing theophylline treated and untreated lymphocytes was added 300 µl. of 1.5% SRBC solution and either 333µl or 666µl of FCS to give a final concentration of 40% FCS in each tube. This was then centrifuged gently at 150g for 5 mins and stood on an ice bath for 60 mins. The rosetting mixture was then gently resuspended and a drop of this mixture was placed on a glass slide and stained with 3% of acridine orange. The number of rosette forming cells was then counted under incident ultraviolet light with a Zeiss fluorescence microscope using a HBO-50 mercury vapour lamp, a BG12 excitation filter and a 500mµ barrier filter. Lymphocytes with three or more sheep erythrocytes attached were considered a rosette and a minimum of 150-200 mono-nuclear cells were counted. The percentage of theophylline-sensitive T lymphocytes was calculated by subtracting the percentage of theophylline treated lymphocytes forming E rosettes from the percentage of E rosettes in the lymphocyte suspension not exposed to theophylline (Figure 1).

METHOD TO DETERMINE PERCENTAGE OF THEOPHYLLINE SENSITIVE T LYMPHOCYTES



a - b = % Theophylline sensitive lymphocytes

FIGURE 1.

3.7 Determination of spontaneous plaque forming cell numbers

Method

Spontaneous plaque forming cells (SPFC) were determined using SRBC coated with Staph protein A (SPA-SRBC) according to the method of Gronowicz (1976). Peripheral blood mononuclear cells (MNC) were separated by Ficoll-Hypaque density gradient centrifugation and incubated for 60 mins at 37°C in RPMI 1640 with 10% FCS to allow shedding of passively absorbed immunoglobulin. No attempt was made to remove adherent or phagocytic cells in these estimations.

SPA-SRBC were prepared by coupling three times washed senescent SRBC with SPA as described by Gronowicz (1976). SPA and the washed SRBC were allowed to interact for ten minutes at room temperature before chromic chloride ($Cr.Cl_3$) at 1:150 dilution and at pH5 was added in small aliquots with constant vortexing (Goding 1976). This mixture was then agitated at 37°C for 7 mins and then washed three times in HBSS and used at a concentration of 25%.

To 25μl of SPA-SRBC suspension were added 25μl of antisera at a previously determined optimum dilution - antihuman IgG, IgA, IgM - IgGAM - (Dako, Copenhagen), 100μl of the mononuclear suspension containing 2×10^5 cells with the morphological appearance of lymphocytes, and 50μl of fresh guinea pig complement diluted 1:9. The complement had been absorbed three times with washed SRBC. The reagents were mixed in micro-titre wells and the mixture transferred to Cunningham Chambers which were sealed with paraffin wax and incubated for 30 minutes at 37°C with a further 30-60 minutes at room temperature.

Plaques which appeared as circular areas of SRBC lysis with a central lymphoid cell, were counted at 20 x magnification using a binocular dissecting microscope.

3.8 Determination of acid-α-naphthyl acetate esterase (ANAE) activity

ANAE activity was determined according to the method described by Knowles et al (1978). Air dried cytocentrifuge preparations of the cell fractions were fixed in cold Bakers formol calcium (pH 6.7) for 10 minutes at $4^{o}C$ and washed in distilled water for 20 minutes. The slides were then incubated for 4 hours at room temperature in a mixture of 1.2 ml. hexazotised pararosaniline, 1.2 ml. of freshly prepared 4% sodium nitrate in distilled water, 40 ml. of 0.067 M phosphate buffer (pH 5.3) and 10 mg. of α-naphthyl acetate in 0.4 ml. acetone following adjustment with 2M sodium hydroxide to pH 5.8. Hexazotised pararosaniline was prepared by combining 1 gm. pararosaniline in 20 ml. distilled water with 5 ml. of concentrated (12 N) HCL, gently warming the mixture and then filtering. Following incubation the slides were rinsed in distilled water, counter-stained with 1% toluidene blue for 30 minutes and rinsed with running water. They were then dehydrated in increasing concentrations of alcohol, cleared in xylol and mounted in pix solution. When counting, cells displaying one to three large discrete cytoplasmic dots were scored positive whilst cells with no staining or scanty dust like cytoplasmic staining were regarded as negative. Monocytes showed abundant cytoplasmic staining and were counted separately.

CHAPTER 5. RESULTS

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CHAPTER 5. RESULTS

5A. CHARACTERISTICS OF THEOPHYLLINE SENSITIVE AND THEOPHYLLINE RESISTANT T LYMPHOCYTES

(i) The effect of theophylline treatment on reactivity of T lymphocytes with OKT monoclonal antibodies.

Incubation of total T lymphocytes with 3mM theophylline at 37°C for 60 minutes did not cause significant changes in the cell viability as determined by Trypan blue dye exclusion. This was 95% after theophylline incubation (Table 1). The E-rosetting ability however was significantly altered ($p < 0.01$) thus allowing their separation into Tsens and Tres lymphocytes. The reactivity of the T lymphocytes with OKT3, OKT4, OKT8 and OKM1 monoclonal antibodies were not significantly altered following theophylline exposure. 90% of the theophylline treated Ttot were OKT3^{+} compared to 85% before treatment, possibly reflecting a purer sample of T cells following a further Ficoll-Hypaque gradient separation.

(ii) Characteristics of Tsens compared to total T lymphocytes.

The Tsens population of lymphocytes was shown to be significantly depleted of OKT3^{+} cells with only 46% positive compared to 89% in the total T subset (Table 2). The number of cells containing Gall bodies (7%) as well as positive staining for ANAE (11%) was also significantly depleted compared to Ttot. There was however enrichment for cells with Fcγ receptor (55%) compared to 20% of the Ttot. A significant proportion (39%) of the Tsens were also OKM1 positive. Analysis of this subset with the Leu 2A and Leu 3A monoclonal antibodies did not appear to be discriminatory with regards to a helper or suppressor cell type.

COMPARISON OF CELL SURFACE MARKERS BEFORE AND AFTER THEOPHYLLINE TREATMENT OF TOTAL T LYMPHOCYTES

CHARACTERISTIC	% LYMPHOCYTES EXPRESSING CHARACTERISTIC		
	UNTREATED T_{tot}	THEOPHYLLINE-TREATED T_{tot}	STUDENT'S TEST
% ERFC	73 ± 8 *	50 ± 14	$p < 0.01$
OKT3	85 ± 8	90 ± 4	NS
OKT4	65 ± 7	59 ± 5	NS
OKT8	29 ± 8	24 ± 8	NS
OKM1	12 ± 11	12 ± 5	NS
CELL VIABILITY	96%	95%	

* Mean ± S.D. of 5 separate experiments

TABLE 1.

COMPARISON BETWEEN Tsens AND Ttot CELLS

CHARACTERISTICS	% lymphocytes expressing characteristic ± S.D.			
	n	Tsens	Ttot	p value
Presence of Gall body	20	7 ± 5	35 ± 4	<.01
ANAE +ve	6	11 ± 4	66 ± 8	<.001
Monocytes	5	31 ± 10	10 ± 5	<.05
$T\gamma$	10	55 ± 12	20 ± 15	<.001
$T\mu$	10	18 ± 11	33 ± 21	NS
OKT3	6	46 ± 21	89 ± 5	<.01
OKT4	6	26 ± 17	65 ± 10	<.01
OKT8	6	44 ± 25	28 ± 7	NS
OKM1	6	39 ± 19	11 ± 6	<.01
Leu 2A	5	29 ± 29	ND	-
Leu 3A	5	37 ± 16	ND	-

ND = not done

TABLE 2.

(iii) <u>Characteristics of Tres compared to total T lymphocytes</u> (Table 3).

The Tres subset contained 93% OKT3^{+} cells but did not appear to differ significantly with regard to Ttot in most of the characteristics. There was however a definite enrichment in A.N.A.E. positive cells (81%) in this subset, and half of them (49%) contained Gall bodies. 63% of Tres were Leu 3A positive suggesting a possible predominance of helper phenotype in this subgroup, while 31% were found to be Leu 2A positive. It is interesting to note that 17% of Tres cells were OKM1 positive whereas only 1% of these cells showed the A.N.A.E. staining pattern seen in monocytes.

(iv) <u>Comparison between the Tsens and Tres Fractions</u>

There were marked differences between these two fractions with regard to most of the characteristics studied (Table 4). Thus, Tsens contained three times as many cells with Fcγ receptors as cells with Fcμ receptors whilst the ratio of Fcγ/Fcμ receptors bearing cells in Tres was 1:3. Judging by reactivity with OKT monoclonal antisera, cells of helper phenotype were significantly more numerous in Tres; this also held true for helper phenotype determined with Leu 3A monoclonal antibody. However, cells of suppressor phenotype, i.e. OKT8 or Leu 2A were not significantly commoner in Tsens, but this fraction did contain a high proportion of cells which were OKM1 positive (39%) and showed the diffuse A.N.A.E. staining of monocytes (31%). The Tres fraction, on the other hand, contained virtually no monocytes judged by the pattern of A.N.A.E. staining although a small proportion of cells were OKM1 positive. Most of the cells containing Gall bodies and displaying discrete dot like A.N.A.E. staining were found in Tres. In some cells more than one Gall body was sometimes noted.

COMPARISON BETWEEN Tres AND Ttot CELLS

CHARACTERISTICS	% lymphocytes expressing characteristic ± S.D.			
	n	Tres	Ttot	p value
Presence of Gall body	20	49 ± 6	35 ± 4	NS
ANAE +	6	81 ± 4	66 ± 8	< .01
Monocytes	5	1 ± 0.8	10 ± 5	< .01
$T\gamma$	10	11 ± 4	20 ± 15	NS
$T\mu$	10	38 ± 23	33 ± 21	NS
OKT3	6	93 ± 4	89 ± 5	NS
OKT4	6	60 ± 17	65 ± 10	NS
OKT8	6	27 ± 10	28 ± 7	NS
OKM1	6	17 ± 11	11 ± 6	NS
Leu 2A	5	31 ± 8	ND	-
Leu 3A	5	63 ± 5	ND	-

ND = not done

TABLE 3.

COMPARISON BETWEEN Tsens AND Tres SUBSETS

CHARACTERISTICS	% lymphocytes expressing characteristic ± S.D.			
	n	Tsens	Tres	p value
Presence of Gall body	20	7 ± 5	49 ± 6	<.001
ANAE +ve	6	11 ± 4	81 ± 4	<.001
Monocytes	5	31 ± 10	1 ± 0.8	<.001
$T\gamma$	10	55 ± 12	11 ± 4	<.001
$T\mu$	10	18 ± 11	38 ± 23	<.001
OKT3	6	46 ± 21	93 ± 4	<.001
OKT4	6	26 ± 17	60 ± 17	<.002
OKT8	6	44 ± 25	27 ± 10	NS
OKM1	6	39 ± 19	17 ± 11	<.02
Leu 2A	5	29 ± 29	31 ± 8	NS
Leu 3A	5	37 ± 16	63 ± 5	<.005

TABLE 4.

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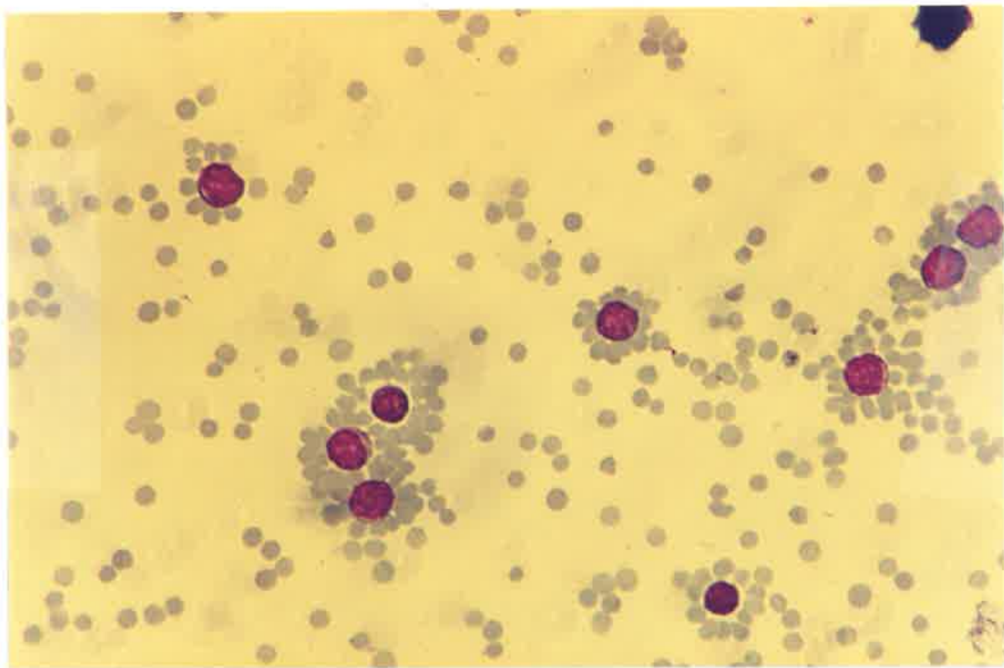

FIGURE 2. Tγ rosettes stained with May-Grumwald-Giemsa stain.

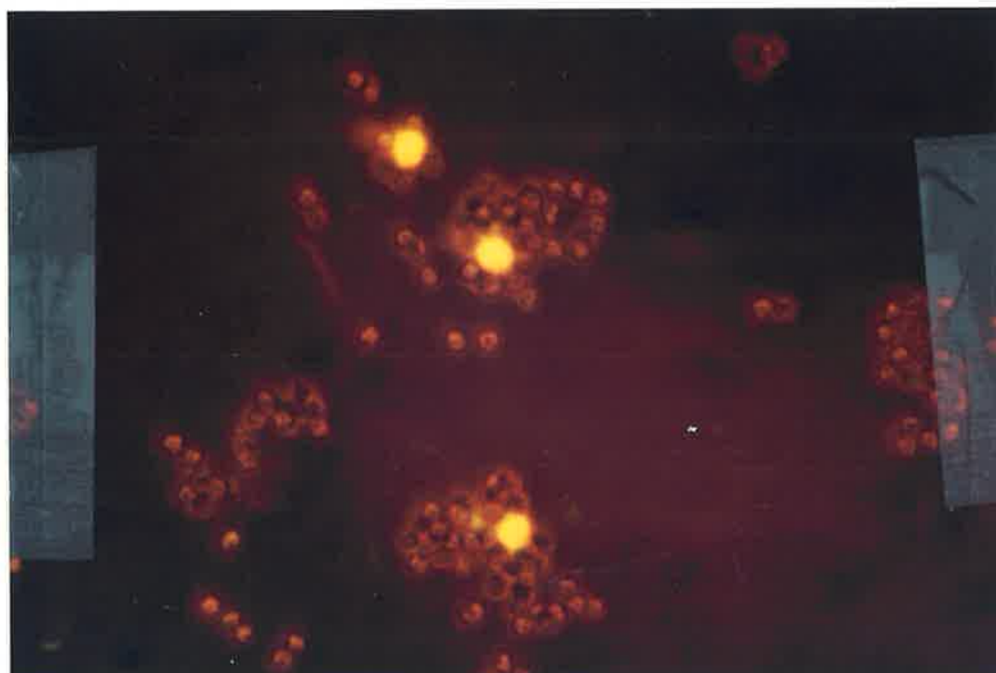

FIGURE 3 Tμ rosettes stained with Acridine Orange.

E/M x 30,000

Gall body
(arrowed)

Phase mic x 1360

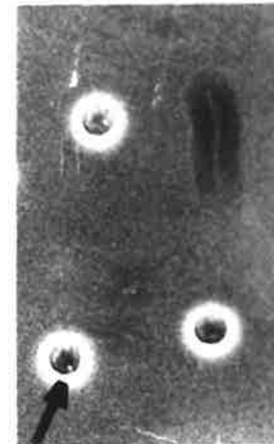

FIGURE 4. The Gall Body.

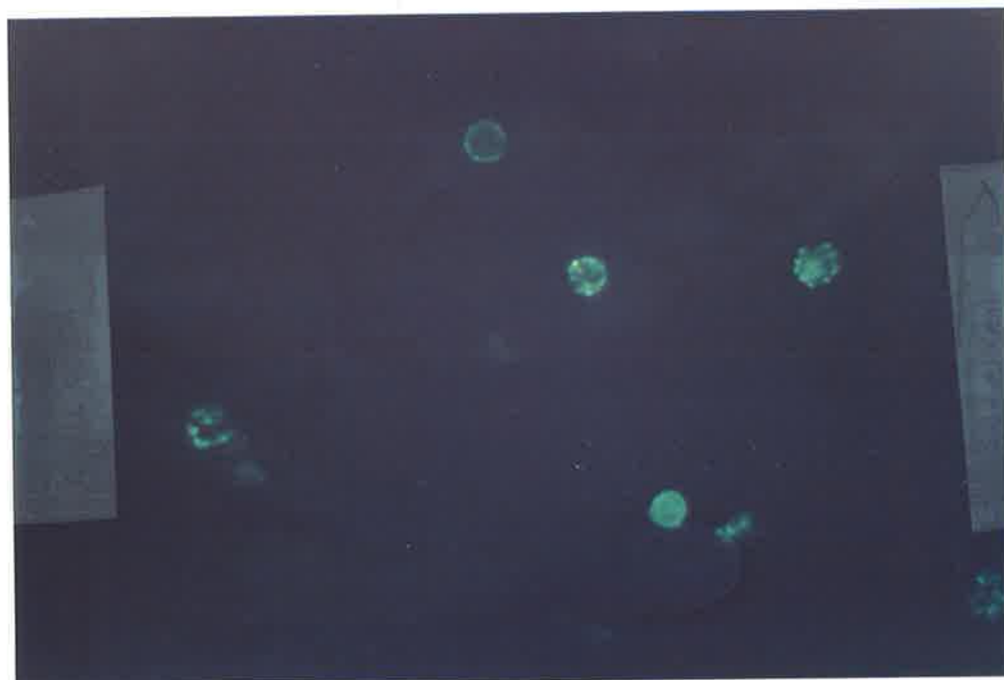

FIGURE 5. Indirect immunofluorescent staining of $OKT3^+$ lymphocytes.

5B. FUNCTIONAL ASSAYS ON THEOPHYLLINE SENSITIVE AND THEOPHYLLINE RESISTANT SUBSETS

The following experiments were performed to assess the functional nature of the Tsens and Tres subsets and an attempt was also made to determine optimal concentrations of each subset in providing this function in a pokeweed mitogen driven co-culture system.

(i) Measurement of intracytoplasmic immunoglobulin production in B cells co-cultured with Tres/Tsens lymphocytes. (Table 5).

There was poor generation of immunoglobulin production following six days of incubation in the presence of pokeweed mitogen and a 5% humidified CO_2 atmosphere. This could be the result of a number of factors including the failure of the medium (or FCS) to support immunoglobulin production, and the unstable carbon dioxide concentration in the incubator. Monocyte depletion was not carried out in these earlier experiments, and the presence of excess monocytes may have caused significant suppression of immunoglobulin production. The viability of the cultures was however, satisfactory after the six day incubation.

(ii) Intracytoplasmic immunoglobulin production in B cells co-cultured with Ttot/Tres/Tsens lymphocytes. (Table 6).

There was poor generation of intracytoplasmic immunoglobulin synthesis in these co-cultures under the same culture conditions as before. The viability of the cultures after a six day incubation period was fairly satisfactory but the non-generation of C_{Ig}+ cells may be attributable to some of the factors as suggested before e.g. unstable carbon dioxide concentrations, monocyte induced suppressor effect, or failure of FCS to enhance culture conditions. This experiment was repeated again. (See Table 7).

MEASUREMENT OF INTRACYTOPLASMIC IMMUNOGLOBULIN PRODUCTION IN B CELLS CO-CULTURED WITH Tres/Tsens LYMPHOCYTES IN THE PRESENCE OF POKEWEED MITOGEN

Tsens	Tres	B	VIABILITY	LIVE CELL COUNT	NO. C_{Ig}^{+} CELLS/ 100 CRBC	NO. C_{Ig}^{+}/ 10^6 LYMPHOCYTES
1.25×10^6	-	1.25×10^6	82%	0.36×10^6	-	-
1×10^6	0.25×10^6	1.25×10^6	89%	0.32×10^6	0	0×10^3
0.6×10^6	0.6×10^6	1.25×10^6	56%	0.54×10^6	0	0×10^3
0.25×10^6	1×10^6	1.25×10^6	43%	0.36×10^6	3	6×10^3
-	1.25×10^6	1.25×10^6	41%	0.30×10^6	6	12×10^3
-	-	2.5×10^6	53%	0.34×10^6	0	0×10^3

TABLE 5.

MEASUREMENT OF INTRACYTOPLASMIC IMMUNOGLOBULIN PRODUCTION IN B CELLS CO-CULTURED WITH Ttot/Tres/Tsens LYMPHOCYTES IN THE PRESENCE OF POKEWEED MITOGEN

Ttot	Tsens	Tres	B	PWM 1/400	VIABILITY	LIVE CELL COUNT	NO. C_{Ig}+/ 100 CRBC	No. C_{Ig}+ CELLS/ 10^6 LYMPHOCYTES
1.25×10^6	-	-	1.25×10^6	+	45%	0.76×10^6	0	0×10^3
-	-	1.25×10^6	1.25×10^6	+	49%	0.9×10^6	1	1×10^3
-	1.25×10^6	-	1.25×10^6	+	52%	0.9×10^6	0	0×10^3
1.25×10^6	-	-	1.25×10^6	+	57%	0.6×10^6	0	0×10^3
-	-	1.25×10^6	1.25×10^6	+	54%	0.61×10^6	0	0×10^3
-	-	-	1.25×10^6	+	28%	0.1×10^6	0	0×10^3
1.25×10^6	-	-	1.25×10^6	-	83%	0.8×10^6	0	0×10^3
-	-	1.25×10^6	1.25×10^6	-	78%	0.6×10^6	1	1×10^3

TABLE 6.

(iii) Repeat Assay of intracytoplasmic immunoglobulin production in B cells co-cultured with Ttot/Tres/Tsens lymphocytes. (Table 7).

It would appear that Tres can provide help for B cells as seen by the extent of intracytoplasmic immunoglobulin production, viz. 68 x 10^3 C_{Ig}+ cells/10^6 lymphocytes, compared to 4 x 10^3 C_{Ig}+ cells/10^6 lymphocytes when Tsens was incubated with B cells alone. There was thus almost total lack of help in the Tsens fraction. It is however, not clear why the Ttot cells had an even greater helper effect on B cells compared to Tres with 178 x 10^3 C_{Ig}+ cells/10^6 lymphocytes.

(iv) Co-culture experiment showing augmentation of PWM-driven spontaneous plaque forming cells by Tres and suppression by Tsens. (Table 8).

Augmentation of the PWM-driven PFC response was shown using the Tres subset as evidenced by 16,620 IgM producing lymphocytes compared to 1,780 IgM+ cells in the control with B cells only. There was some evidence for suppression when Tsens was co-cultured with B cells in the presence of Tres as IgM+ PFC dropped to 4,440.

(v) Determination of optimal Tres concentration in PWM-driven co-cultures (Table 9).

These results show that 10^6 Tres lymphocytes appeared to provide optimal helper function for intracytoplasmic immunoglobulin production when co-cultured with 1.25 x 10^6 B lymphocytes suggesting a ratio of 1:1.25 for maximal augmentation. The viability of the cells in these cultures after six days incubation in the presence of pokeweed mitogen, ranged from 8% to 66%. It is likely that in the well containing 3 x 10^6 Tres cells, the total cell content would be in excess of the optimum culture requirements per Nunclon well. In the wells containing from

INTRACYTOPLASMIC IMMUNOGLOBULIN PRODUCTION IN B CELLS CO-CULTURED WITH Ttot/Tres/Tsens LYMPHOCYTES

TOTAL T	Tsens	Tres	B	PWM 1/400	VIABILITY	LIVE CELL COUNT	No. C_{Ig}+/ 100 CRBS	NO. C_{Ig}+ CELLS/ 10^6 LYMPHOCYTES
1.25×10^6	-	-	1.25×10^6	+	31%	1.1×10^6	89	178×10^3
-	-	1.25×10^6	1.25×10^6	+	36%	1.2×10^6	34	68×10^3
-	1.25×10^6	-	1.25×10^6	+	37%	1.0×10^6	2	4×10^3
1.25×10^6	-	-	1.25×10^6	-	34%	0.7×10^6	0	0
-	-	1.25×10^6	1.25×10^6	-	<5%	0×10^6	0	0
-	1.25×10^6	-	1.25×10^6	-	<5%	0×10^6	0	0

TABLE 7.

CO-CULTURE EXPERIMENT SHOWING AUGMENTATION OF PWM-DRIVEN PFC RESPONSE BY Tres AND SUPPRESSION BY Tsens.

	NO. OF CELLS IN CULTURE*					PFC/10^6 B CELLS		
	TOTAL MNC	Tres	Tsens	B	PWM	IgG	IgA	IgM
1.	-	-	-	10^6	1:400	5810	1690	1780
2.	-	10^6	-	10^6	1:400	7400	7240	16620
3.	-	10^6	10^6	10^6	1:400	4880	3400	4440
4.	-	-	10^6	10^6	1:400	6480	1840	5260
5.	10^6	-	-	-	-	350	210	700

* Adherent cells (final concentration 5%) were added to all combinations with the exception of the last one.

TABLE 8.

DETERMINATION OF OPTIMAL Tres CONCENTRATION FOR MAXIMAL INTRACYTOPLASMIC Ig PRODUCTION

WHEN CO-CULTURED WITH B CELLS IN THE PRESENCE OF PWM

NO. Tres CELLS	B	VIABILITY	LIVE CELL COUNT	NO. C_{Ig}+/ 100 CRBC	NO. C_{Ig}+/10^6 LYMPHOCYTES
3×10^6	1.25×10^6	48%	0.54×10^6	4	8×10^3
10^6	1.25×10^6	53%	0.34×10^6	21	42×10^3
3×10^5	1.25×10^6	8%	0.02×10^6	12	24×10^3
10^5	1.25×10^6	33%	0.10×10^6	17	34×10^3
3×10^4	1.25×10^6	50%	0.20×10^6	2	4×10^3
10^4	1.25×10^6	50%	0.10×10^6	4	8×10^3
3×10^3	1.25×10^6	44%	0.16×10^6	0	0×10^3
10^3	1.25×10^6	56	0.18×10^6	3	6×10^3
0	1.25×10^6	66%	0.20×10^6	0.8	1.6×10^3

TABLE 9.

10^3 to 3×10^4 Tres cells, it is possible that the monocyte contamination could have played a suppressive role in the immunoglobulin production.

(vi) Determination of Optimal Tsens Concentrations for suppression.

From Table 10 it can be seen that the failure to generate C_{Ig}^+ cells in the Tres and B cell co-cultures alone would make it impossible to demonstrate any suppressor effect of the Tsens in the varying concentrations shown. When this experiment was repeated (Table 11), there was some suppressor effect evidenced with increased numbers of Tsens but no clear dose related effect was demonstrated.

B CELLS CO-CULTURED WITH VARYING CONCENTRATIONS OF Tsens IN THE PRESENCE OF POKEWEED MITOGEN

B	Tres	Tsens	VIABILITY	LIVE CELL COUNTS	NO. C_{Ig}+/ 100 CRBC	NO. C_{Ig}+/ 10^6 LYMPOCYTES
1.25×10^6	1.25×10^6	-	65%	5.4×10^6/ml	3	6×10^3
1.25×10^6	1.25×10^6	10^5	80%	4.4×10^6/ml	1	2×10^3
1.25×10^6	1.25×10^6	3×10^4	73%	4.4×10^6/ml	5	10×10^3
1.25×10^6	1.25×10^6	10^4	74%	6.0×10^6/ml.	0	0×10^3
1.25×10^6	1.25×10^6	3×10^3	75%	6.6×10^6/ml.	1	2×10^3
1.25×10^6	1.25×10^6	10^3	65%	0.7×10^6/ml.	0	0×10^3
1.25×10^6	-	-	0%	0	-	-

TABLE 10.

REPEAT EXPERIMENT TO DETERMINE SUPPRESSOR EFFECT OF VARYING CONCENTRATIONS OF Tsens.

B	Tres	Tsens	NO. C_{Ig}+/100 CRBC	NO. C_{Ig}+/10^6 LYMPHOCYTES.
10^6	10^6	1×10^6	0	0
10^6	10^6	3×10^5	16	32×10^3
10^6	10^6	10^5	5	10×10^3
10^6	10^6	3×10^4	2	4×10^3
10^6	10^6	10^4	9	18×10^3
10^6	10^6	-	7	14×10^3

TABLE 11.

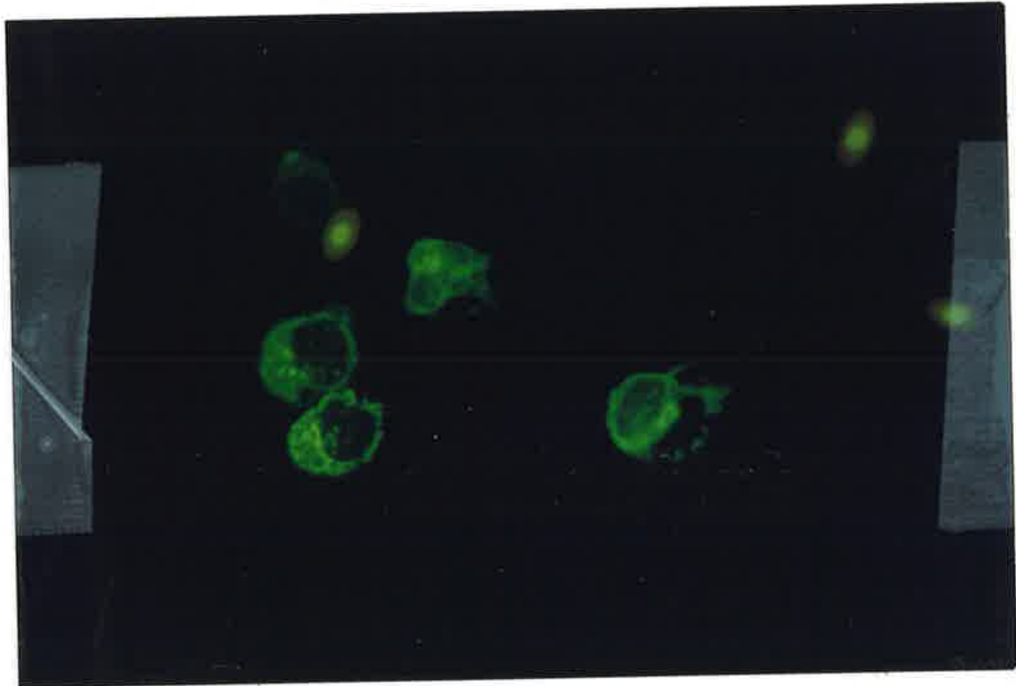

FIGURE 6. Demonstration of intracytoplasmic immunoglobulin production using fluorescein conjugated polyvalent anti-human immunoglobulin and chicken RBC as indicator cells.

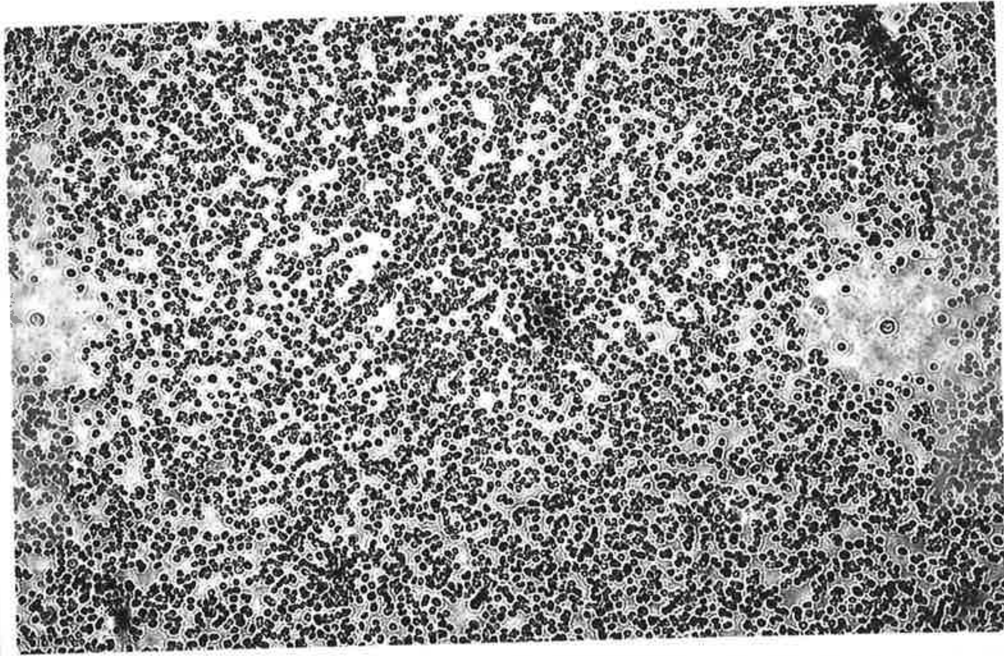

FIGURE 7 Spontaneous plaque forming cells showing circular zone of SRBC lysis around immunoglobulin producing central lymphoid cell.

5C. PERCENTAGE OF THEOPHYLLINE SENSITIVE T LYMPHOCYTES IN VARIOUS CLINICAL CONDITIONS

The percentage of theophylline sensitive T lymphocytes were determined in the following groups:

(i) normal controls

(ii) systemic lupus erythematosus (SLE)

(iii) miscellaneous group

(i) Percentage of Tsens lymphocytes in normal subjects

These estimations were carried out on blood obtained from Red Cross blood donors as well as on some of the laboratory staff. The results of 19 subjects showed a range varying from 2% to 47% with a mean of 17.5% (S.D. $\pm$ 11.5).

(ii) Percentage of Tsens lymphocytes in active/inactive SLE.

The percentage of Tsens lymphocytes was determined on a total of ten patients with SLE. Sixteen determinations were obtained during the active phase of SLE, as determined by clinical parameters; while 19 values were obtained during remission of the disease. Each patient was thus subjected to more than one evaluation, depending on their clinical state.

The results are graphically set out in Fig. 8. In a total of 16 determinations during active SLE, the Tsens values ranged from -31% to +24% of the total lymphocyte population, thus reflecting the presence of theophylline inducible cells in some. The mean value of Tsens here was 7.9% $\pm$ 11.3. During the inactive phase of the disease, 19 estimations showed the Tsens ranging from -15% to 29% with a mean of 14.7% S.D. $\pm$ 12.4.

The values obtained for active SLE (7.9% $\pm$ 11.3) when compared to the normal controls (17.5% $\pm$ 11.5) using the Students' t test, showed a significant difference with a p value <0.02. However, when the mean value of Tsens in inactive SLE (14.7% $\pm$ 12.4) was compared to the normal controls (17.5% $\pm$ 11.5), it was found to be not significant with a p value >0.1.

The Tsens populations in active SLE (7.9% $\pm$ 11.3) was not significantly changed in the inactive phase of the disease (14.7% $\pm$ 12.4) with a $p > 0.1$.

(iii) Percentage of Tsens lymphocytes in other miscellaneous diseases

The Tsens percentages were determined on patients with a variety of other medical conditions. These are listed below and show a range of Tsens from -8% to 25% of the total lymphocytes. The only patient with a value lower than Mean - 2SD was patient No. 6 with -8% Tsens with chronic lymphocytic leukaemia.

	Diagnosis	% Tsens
1.	Alcoholic liver disease	14%
2.	Alcoholic liver disease	- 4%
3.	Alcoholic liver disease	11%
4.	Alcoholic liver disease	25%
5.	Chronic lymphocytic leukaemia	7%
6.	Chronic lymphocytic leukaemia	- 8%
7.	T cell lymphoma	9%
8.	Scleroderma	7%
9.	Active chronic hepatitis	18%
10.	Sarcoidosis	5%
11.	Grave's disease	7%
12.	Myasthenia gravis	25%
13.	Wegener's granulomatosis	9%
14.	Epilepsy (on Dilantin)	18%

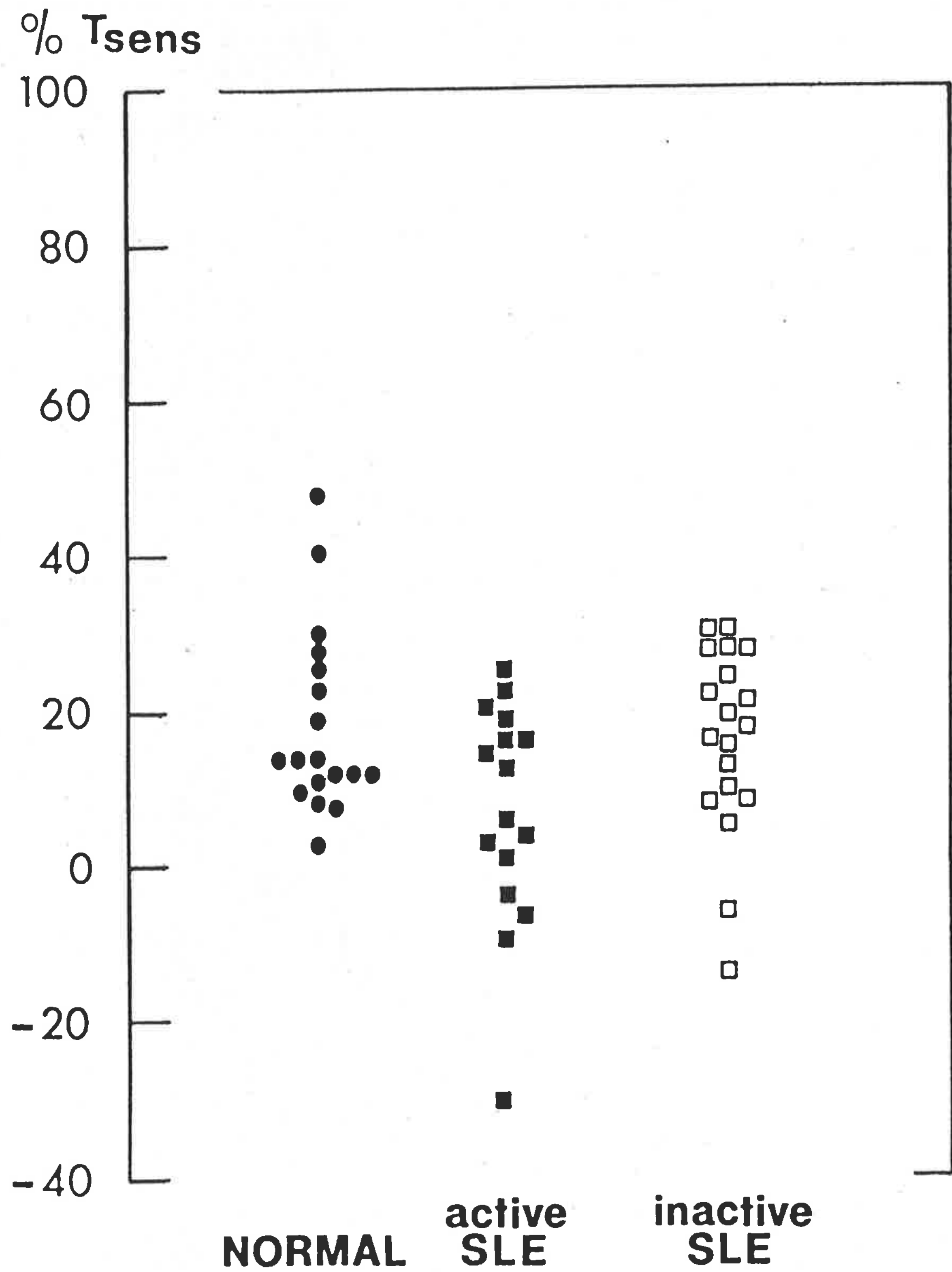

FIGURE 8. Percentage distribution of Tsens lymphocytes in Normal subjects/active/inactive SLE.

5D. LONGITUDINAL STUDIES PERFORMED IN FIVE PATIENTS WITH SYSTEMIC LUPUS ERYTHEMATOSUS

Five patients (Table 12) attending The Queen Elizabeth Hospital for the management of systemic lupus erythematosus (SLE) were followed from periods ranging from 5 months to a year in an attempt to correlate changes in theophylline-sensitive T lymphocyte levels with other indices of disease activity.

The following indices were assessed:

1. Erythrocyte sedimentation rate (ESR).
2. Native DNA binding activity.
3. Antinuclear factor titres (ANF)
4. The number of spontaneous plaque forming cells (SPFC) for polyclonal immunoglobulin (IgG, IgA, IgM and total Ig production.)
5. Percentage of theophylline sensitive T cells (T sens).

PATIENT	SEX	AGE	DURATION OF SLE	MANIFESTATION OF SLE DURING PERIOD OF STUDY
1. D.B.	Female	56 yrs	13 yrs	Arthralgia, rash pericardial effusion
2. C.M.T.	Female	23 yrs	2 yrs	Arthritis, haemolytic anaemia
3. M.K.	Male	23 yrs	2½yrs	(R) optic neuritis and lymphadenopathy
4. L.W.	Female	36 yrs	2 yrs	Polyarthritis, encephalopathy, photosensitive dermatitis, intestinal vasculitis.
5. J.W.	Male	72 yrs	1½yrs	Polyarthritis and mesangial glomerulonephritis.

TABLE 12.

CASE NO. 1

D.B., a 56 year old Caucasian female was first seen in October, 1968 for left sided chest pain associated with breathlessness and coughing. She was febrile with signs of basal pneumonia and was anaemic (Hb 8.4g per 100 ml.). A pericardial effusion developed after admission and investigations supported the diagnosis of S.L.E. with a raised ESR 61 mm/hr, hyperglobulinaemia, antinuclear factor positive with homogenous staining at a titre > 1/500 and the presence of LE cells. Prednisolone 60 mg. daily was commenced with good clinical improvement. A renal biopsy in November, 1968 showed no active renal lesions of SLE and she was subsequently maintained on 5 mg prednisolone a day. In November, 1970 she developed deep vein thrombosis of the left leg and required anticoagulant therapy. Subsequently, azathioprine 100 mg/day was added. However, a month later she developed haemarthrosis and suppuration of the right knee and required arthrotomy for relief. Anti-coagulants were ceased and in May, 1971 arthrodesis of the right knee was performed. Meanwhile, diabetes mellitus developed and tolbutamide was instituted at 500 mg bd, in addition to diuretics for congestive cardiac failure. The SLE remained quiescent through the next six years and she was maintained on azathioprine and prednisolone but required insulin to control the diabetes. Steroid induced osteochondritis dissecans of the left knee and a few suicidal attempts with drug overdoses necessitated further admissions over 1979 and 1980 and she remained intermittently depressed and required tricyclic antidepressant therapy. In January, 1981 azathioprine was ceased and in March, 1981 she was re-admitted with right middle lobe pneumonia which progressed to respiratory failure and required management at the Intensive Therapy Unit with parenteral antibiotics and 30 mg prednisolone/day. At this time her DNA binding was > 105 units/ml, the ANF + at 1/640 and ESR was 109 mm/hr. It was felt that she had suffered a relapse of SLE in addition to the pulmonary complication. She improved and was discharged with a return of DNA binding and ESR to normal.

In August, 1981 cervical lymphadenopathy developed and she also complained of persistent low back pain which was thought to be a result of steroid induced lumbar osteoporosis. By December she was feeling well and was on prednisolone 18 mg. maintenance dosage.

CORRELATION OF MEASURED INDICES WITH DISEASE ACTIVITY (Fig.9, Table 13).

SLE was first diagnosed in this patient in October, 1968 when she presented with an auto-immune haemolytic anaemia and subsequent pericarditis. However, Tsens estimations were performed only since July, 1981 when this study was undertaken. At that time she had just recovered from a relapse with complicating right middle lobar pneumonia. The Tsens was 19%, although SPFC estimations still revealed a marked degree of activity of the SLE with 4,700 polyvalent Ig producing cells and 3,260 IgA+ producing lymphocytes per 10^6 lymphocytes. In August, 1981 she had another relapse with cervical lympadenopathy at which time the Tsens fell to -13%, with a rise of DNA binding to 72 units/ml. With treatment she remitted and a concomitant fall in DNA binding in 20 units/ml. in November 1981 was associated with a rise in Tsens to 27%. This subsequently again decreased to 16% on 27.11.81 when the DNA rose yet again to 93.5 units/ml. together with an associated rise in SPFC production. The Tsens returned to 21% in January, 1982 when her other parameters also indicated remission and SPFC production was minimal.

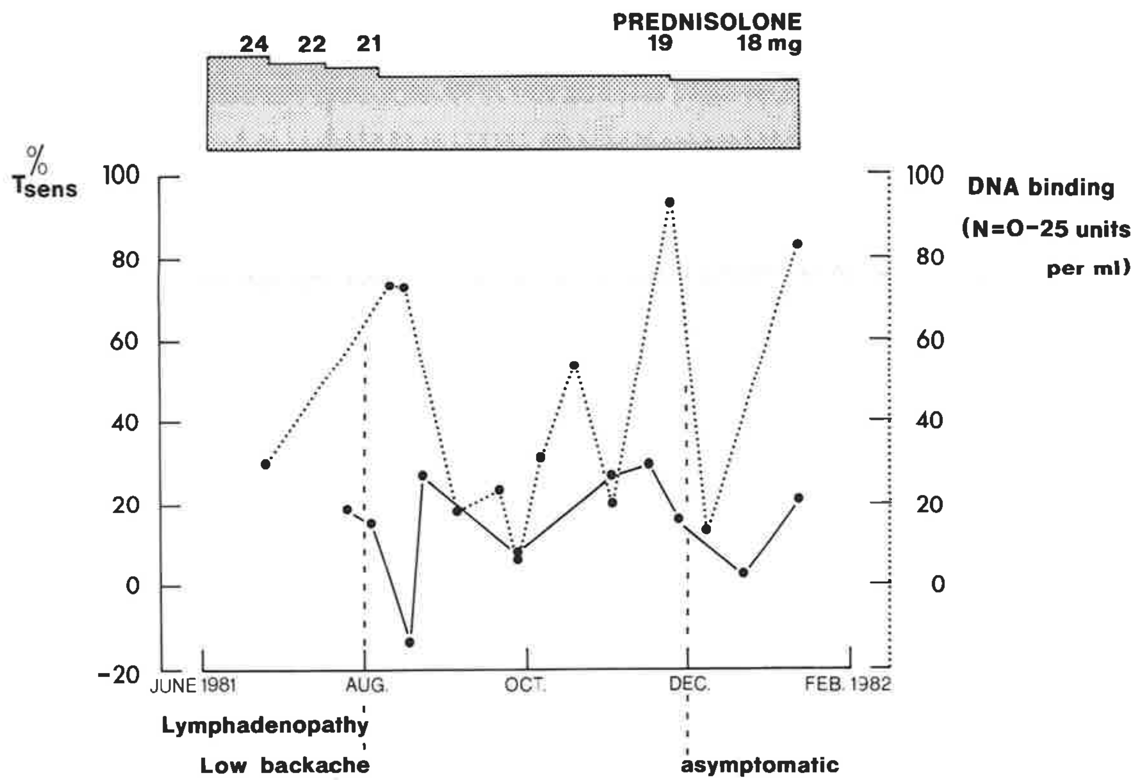

FIGURE 9. Case No. 1 D.B. Female 56 years.

Case No.1 - Addendum

Correlation of Tsens vs SPFC IgG, IgA and n- DNA binding

	SPFC IgG	SPFC IgA	n- DNA
n	10	10	5
Corr. coefficient	0.20	0.08	-0.24
p	$>$ 0.10 (NS)	$>$ 0.10 (NS)	$>$ 0.10 (NS)

CASE NO.1 D.B. FEMALE 56 YEARS

DATE	% ERFC	% T SENS	ESR	n-DNA	ANF	SPFC/10^6 LYMPHOCYTES			
						PV	G	A	M
26.6.81	-	-	19mm	29	+1/320	-	-	-	-
27.7.81	82%	19%	-	-	-	4,700	1,620	3,260	140
3.8.81	89%	16%	-	-	-	2,400	120	2,360	100
17.8.81		-13%	10mm	72	+ +A	1,270	480	2,450	60
24.8.81		27%	-	-	-	170	820	2,670	120
21.9.81	-	-	-	23.5	+1/160	230	10	1,200	50
28.9.81	89%	8%	-	7	-	1,250	110	3,510	80
19.10.81	-	-	-	53	+ 1/80	3,710	320	3,850	320
2.11.81	40%	27%	-	20	+1/160	0	10	20	10
16.11.81	57%	29%	-	-	-	-	25	6,450	220
27.11.81	81%	16%	-	93.5	+1/160	3,340	620	5,440	20
21.12.81	87%	2%	-	61	+ 1/80	310	10	600	0
13.1.82	86%	21%	-	-	+1/160	5	15	920	0

TABLE 13.

CASE NO. 2

C.M.T., a 23 year old Vietnamese female presented in December, 1979 with fever and arthritis of the left ankle together with lymphadenopathy and was found to have a Coomb's positive haemolytic anaemia with Hb 8.3g per 100 ml. Investigations revealed an ESR of 118 mm/hr, numerous LE cells, low serum-C4 level of 0.09G/L (normal 0.2-0.5), hypergammaglobulinaemia and positive antinuclear factor at an end titre of 1:640. The DNA binding was however, normal at 23.5 units/ml. X-rays of the chest and left ankle were normal. The only significant past history was that of malaria while in a refugee camp six months previously and this was excluded as a cause for her present fever. A diagnosis of SLE was made and Prednisolone instituted at a dose of 15 mg/day, with dramatic resolution of the fever, improvement in the arthritis and regression of the lymphadenopathy. She defaulted from follow up after two months of steroid therapy and was next seen in March, 1980 for a persistent cough. Investigations revealed miliary pulmonary tuberculosis and anti-tuberculous chemotherapy was immediately commenced, with Isoniazid 300 mg, Ethambutol 800 mg, Rifampicin 450 mg, Streptomycin 500 mg and Pyridoxine 6 mg. daily. Subsequently resistance to Isoniazid and Streptomycin was discovered and as radiological resolution of the miliary lesions was slow, chemotherapy was changed to Capreomycin 75g, Pyrazinamide 1.25g, para-aminosalicylic acid (PAS) 12g, Ethionamide 750µg and Ethambutol 800 mg, with a maintenance of Prednisolone at 5 mg/day. She had a stormy recovery but eventually improved with radiological clearing and good clinical improvement. The SLE appeared to be in remission then and DNA binding remained within normal limits. However, in September, 1981 just as anti-tuberculous chemotherapy was due to cease, laboratory tests suggested a reactivation of the SLE although she remained clinically well. There was increased SPFC activity, an increased DNA binding of 107 units/ml, and a raised ESR of 45 mm/hr.

In view of these findings prednisolone was increased to 15 mg/day, with resulting improvement in these parameters.

CORRELATION OF MEASURED INDICES WITH DISEASE ACTIVITY (Fig.10, Table 14).

This patient initially presented in December, 1979 with SLE but investigations on the Tsens population were only obtained later in September, 1981 when she was found to have a relapse. DNA binding at that time was 107 units/ml, with an ESR of 45 mm/hr. The Tsens at this time was 24% of her total lymphocyte population. The patient had just completed a course of anti-tuberculous chemotherapy for miliary pulmonary TB, but did not have any specific complaints relating to relapse of her SLE. The Tsens percentage further decreased to -5% a week later and her SPFC also showed a concomitant increase to 1,100 of polyvalent Ig producing lymphocytes. Prednisolone therapy was consequently increased to 15 mg daily on the assumption that these parameters might herald a clinical relapse. There appeared to be some improvement with a fall in ESR to 27 mm/hr, decrease in SPFC production and a return of Tsens to 17%. The patient subsequently defaulted from follow up and her subsequent clinical progress is thus unknown.

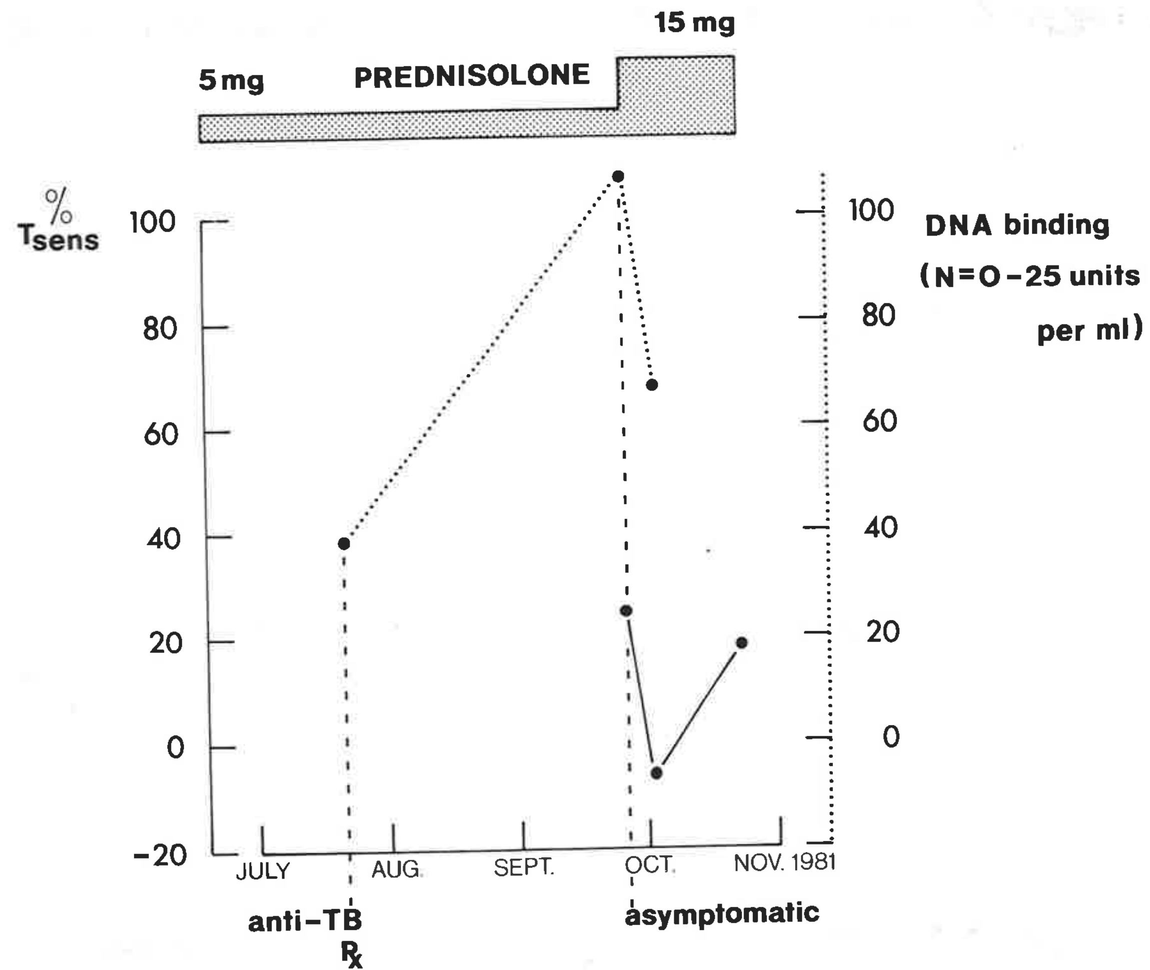

FIGURE 10. Case No. 2. CMT. Female 23 years.

CASE NO.2   C.M.T., Female, 23 years.

DATE	% ERFC	% TSENS	ESR	n-DNA	ANF	PV	SPFC/10^6 LYMPHOCYTES G	A	M
23.7.81	-	-	55mm	36.5	-	1,620	640	1,680	70
24.9.81	56%	24%	45mm	107	+ 1/160	650	70	955	35
1.10.81	55%	-5%	26mm	66	-	1,100	20	460	80
22.10.81	40%	17%	27mm	-	+ 1/160	24	0	285	18

TABLE 14.

CASE NO. 3

M.K. a 23 year old Polish male presented in August, 1979 with a maculopapular rash over the hands and face for five weeks, and blurred vision in the right eye for one week. This was preceded by increasing fatigue and poor night vision. Clinical examination revealed an erythematous telangiectatic rash over the face and hands, marked generalised lymphadenopathy, and loss of vision in the right eye. The fundi appeared to be within normal limits. Investigations supported the diagnosis of an autoimmune disorder with vasculitis causing right optic nerve ischaemia and included a raised DNA binding activity of 55 units/ml, positive antinuclear factor, and marked hypocomplementaemia with total serum haemolytic complement <20% (normal 90-96%) and S-C_3 level of 0.28G/L (N 0.5-1.5) and S-C_4 of 0.01 G/L (N 0.2-0.5). The ESR, however, did not rise beyond 9 mm/hr, during the acute illness. Prednisolone was begun at a dose of 60 mg/day with some improvement of visual acuity and dramatic improvement of the rash. Because of persistent hypocomplementaemia, Prednisolone was increased to 100 mg daily and Cyclophosphamide 200 mg/day was added. Visual acuity improved slightly in the right eye and he remained otherwise well and returned to work. He was maintained on Cyclophosphamide 100 mg daily and Prednisolone 25 mg a day, with a return of the serum complement levels to normal.

During follow up he remained well and visual acuity in the right eye continued to improve. DNA binding, serum complement levels and antinuclear factor titres reverted to normal levels and Cyclophosphamide was gradually withdrawn and Prednisolone reduced very gradually and ceased in February, 1981. The patient then defaulted from follow up for some months but when reviewed in July, 1981 investigations suggested reactivation with rising ANF titres and raised DNA binding of 50 units/ml, increased SPFC activity with 900 IgA producing SPFC/10^6 lymphocytes and borderline serum complement levels. Axillary lymphadenopathy developed later though

the patient remained asymptomatic and Prednisolone was recommenced at a dose of 20 mg/day. There was gradual improvement once steroids were instituted with regression of the lymphadenopathy, a reduction of SPFC activity, and a fall in anti-DNA antibody titres. He is currently maintained on 13 mg Prednisolone daily.

CORRELATION OF MEASURED INDICES WITH DISEASE ACTIVITY (Fig. 11, Table 15).

This patient was investigated for Tsens levels only at the time of his relapse in September, 1981. At this time indices of SLE activity suggested a reactivation of the disease with an ANF titre of 1:160, a raised DNA binding to 100 units/ml, and 1560 IgA producing SPFC 10^6 lymphocytes. The ESR remained normal at 8 mm/hr, but the percentage of Tsens lymphocytes decreased to -10% suggesting the presence of theophylline induced rosetting of T lymphocytes. With the reintroduction of Prednisolone there was a reduction in SPFC activity and a fall in DNA binding, and the Tsens level rose to 15%. This was accompanied by clinical regression of the lymphadenopathy.

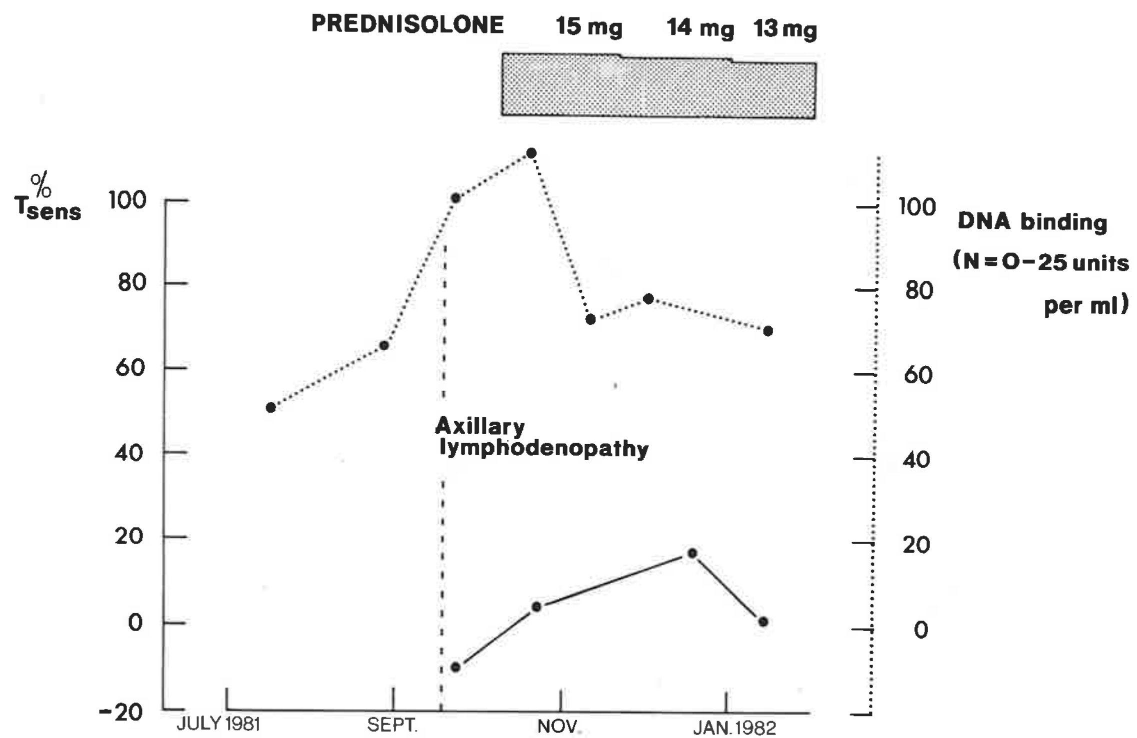

FIGURE 11. Case No. 3. M.K. Male 23 years.

CASE NO.3 M.K. MALE 23 YEARS

DATE	% ERFC	% TSENS	ESR	n-DNA	ANF	SPFC/10^6 LYMPHOCYTES PV	G	A	M
16.7.81	-	22%	13mm	50	+ 1/40	1,405	825	900	110
27.8.81	-	-	6mm	65.5	+1/160	440	140	300	0
25.9.81	-	-10%	8mm	100	+ 1/80	-	-	-	-
22.10.81	30%	3%	5mm	≥107	+ 1/80	1,160	120	1,560	170
12.11.81	-	-	-	71	+ 1/80	530	30	260	40
3.12.81	-	-	4mm	76	+ 1/40	10	0	0	0
17.12.81	66%	15%	-	-	+ 1/80	90	0	35	0
14.1.82	49%	0%	-	-	+ 1/80		- lysed -		

TABLE 15.

CASE NO. 4

L.W., a 36 year old female first presented in 1979 with a rash over the face and upper body and polyarthritis involving the proximal interphalangeal joints, wrists and knee joints. DNA binding was markedly raised at >105 units/ml and the antinuclear factor test was positive with homogeneous staining to a titre of 1 in 160 rising to 1 in 640. The ESR was 15 mm/hr. and there was evidence of microscopic haematuria. She subsequently became thrombocytopenic with a platelet count of 34,000/cmm and was shown to have platelet antibodies to a titre of 1/16. Prednisolone 100 mg daily was instituted and gradually reduced to a maintenance dose of 10 mg over four months.

During follow up she developed an episode of right sided pleurisy in August, 1980 and Prednisolone was increased to 40 mg. daily. During this time she also developed a depressive illness requiring tricyclic antidepressants. Late in 1980 she developed left sided trigeminal nerve involvement resulting in sensory loss involving the second and third divisions of the trigeminal nerve. In June, 1981 she had another exacerbation of SLE with alopecia and a flare up of polyarthritis, and at this stage an antimalarial, hydroxychloroquine sulphate 200 mg. daily, was added. She then developed a grand mal seizure in August, 1981 and an electroencephalogram showed evidence of cerebral dysrhythmia. Sodium phenytoin 400 mg daily was started and Prednisolone was increased to 30 mg. daily. Late in August she had a pyrexial illness with a generalised rash and lymphadenopathy which resolved on its own. In December, 1981 she developed lower abdominal pain with marked peritoneal irritation and diarrhoea which was attributed to vasculitis of the small bowel. She has been maintained on 20 mg. Prednisolone daily since then, and has remained asymptomatic.

CORRELATION OF MEASURED INDICES WITH DISEASE ACTIVITY.

(See Table 16 and Figure 12).

During the period of study from April to December, 1981, L.W. experienced several episodes of increased disease activity with alopecia and polyarthritis in June, a grand mal seizure in early August and later pyrexia and lymphadenopathy, and small intestinal vasculitis in December. It can be seen that during all these episodes, the ESR remained low, not exceeding 20 mm/hr, and as such did not appear to be a useful parameter in this case to predict relapse.

However, the spontaneous plaque forming cell numbers were high in July and the IgA producing SPFC peaked at 1,420 (Control = 124±97) in early August. At this time the Tsens level appeared to be within the normal range (18%) but then fell to a low 4% with DNA binding at 60 units. This preceded the onset of the pyrexia, rash and lympadenopathy in August. The antinuclear factor titre at this time was also raised at 1:640. With clinical resolution, it can be seen from Table 16 that the number of SPFC in October returned to normal but unfortunately a Tsens measurement is not available at this time for correlation. The DNA binding, SPFC production and ANF titre were returning to normal in late November and at this time she was clinically stable.

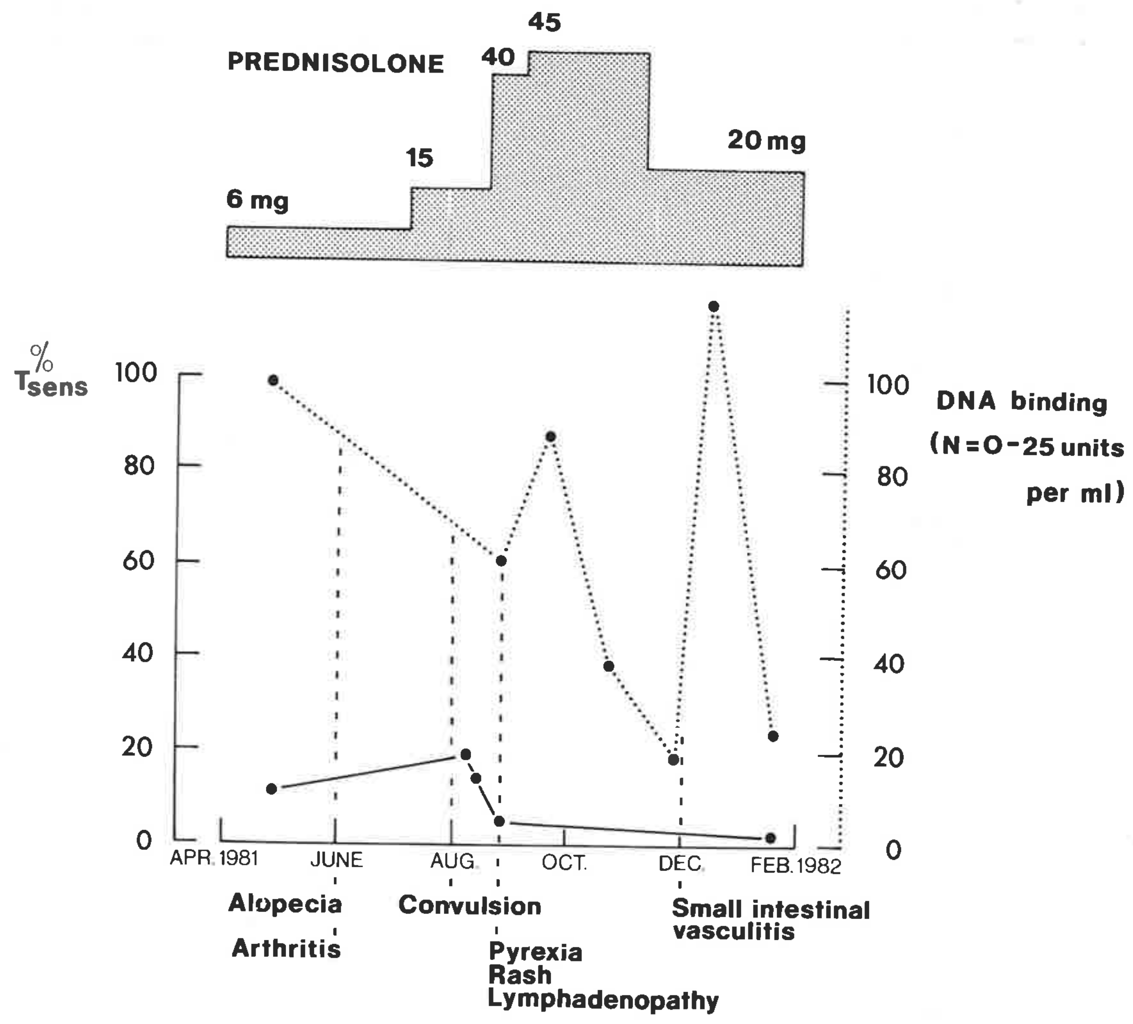

FIGURE 12. Case No. 4. L.W. Female 36 years.

CASE NO. 4. L.W. FEMALE 36 YEARS

DATE	% ERFC	% TSENS	ESR	n-DNA	ANF	SPFC/10^6 LYMPHOCYTES			
						PV	G	A	M
29.4.81	-	10%	-	98	+ 1/1,280	-	-	-	-
10.8.81	47%	18%	11mm	-	-	260	40	1,420	20
12.8.81		14%	-	-	-	120	20	120	0
24.8.81	-	4%	20mm	60	+ 1/640	1,040	520	180	60
21.9.81	-	-	7mm	86.5	+ 1/640	-	-	-	-
19.10.81	-	-	3mm	36.5	+ 1/640	40	0	70	10
24.11.81	-	-	-	19.5	+ 1/160	35	5	15	5
9.12.81	-	-	2mm	116.5	+ 1/320	0	0	0	0
15.1.82	70%	1%	-	-	+ 1/320		- Nil -		

TABLE 16.

CASE NO. 5.

J.W., a 72 year old male with a six month history of arthritis, first presented to Casualty in January, 1981 with a two day history of severe epigastric pain. He was taking codeine and salicylates as well as Indomethacin suppositories for pain relief at the time of admission, but had also taken Ibuprofen and Naproxen tablets in the preceding months.

Significant findings on admission included consolidation/ collapse of both lower lung lobes with small bilateral pleural effusions, a raised ESR of 96 mm/hr, hypergammaglobulinaemia and an elevated serum creatinine of 220mmol/1. Upper gastrointestinal endoscopy did not reveal any mucosal lesion to account for the epigastric pain. Further tests revealed a markedly elevated DNA binding >105 units/ml, antinuclear factor positive at a 1:1280 titre with homogeneous and rim patterns and microscopic haematuria with hyaline and granular casts in the urinary sediment. A renal biopsy showed diffuse lupus glomerulonephritis with mesangial swelling and proliferation with occasional areas of segmental necrosis and some crescent formation. Immunofluorescence studies showed variable mesangial and capillary wall reactions for IgG, IgA, IgM, C1 and C3. Prednisolone 20 mg was instituted with significant improvement in symptoms and a return of the serum creatinine to normal levels within a week. Glucose intolerance however, developed, and as dietary control was ineffective, the introduction of insulin was necessitated and he was satisfactorily controlled on 16 units of insulin daily. The Prednisolone was gradually reduced to 10 mg/day and an antimalarial, hydroxychloroquine sulphate 400 mg daily was added. He has remained asymptomatic.

CORRELATION OF MEASURED INDICES WITH DISEASE ACTIVITY (Table 17, Fig.13).

Measurement of the percentage of theophylline sensitive T lymphocytes and SPFC was only done two weeks after the patient's admission as he was being managed at another unit of the Hospital. At this time

he had been on 20 mg Prednisolone and the symptoms had regressed and ESR reduced to 50 mm from 96 mm/hr. SPFC activity still appeared to be marked with 1,330 IgA producing SPFC/10^6 lymphocytes, but the Tsens level was 12% increasing to 14% the following week and was within normal at 22% after four weeks of Prednisolone therapy. The ESR (47 mm/hr.) and DNA binding 115 units/ml however, remained elevated at this time despite clinical improvement. This raises the possibility that the Tsens % may reflect more accurately the day to day fluctuations in immunological response to therapy preceding the return of other parameters to normal. The subsequent fall in Tsens levels shown in October to -15% indicates the presence of theophylline inducible cells which rosette only after exposure to theophylline. This does not appear to have been followed by a clinical relapse in this patient and is difficult to account for here.

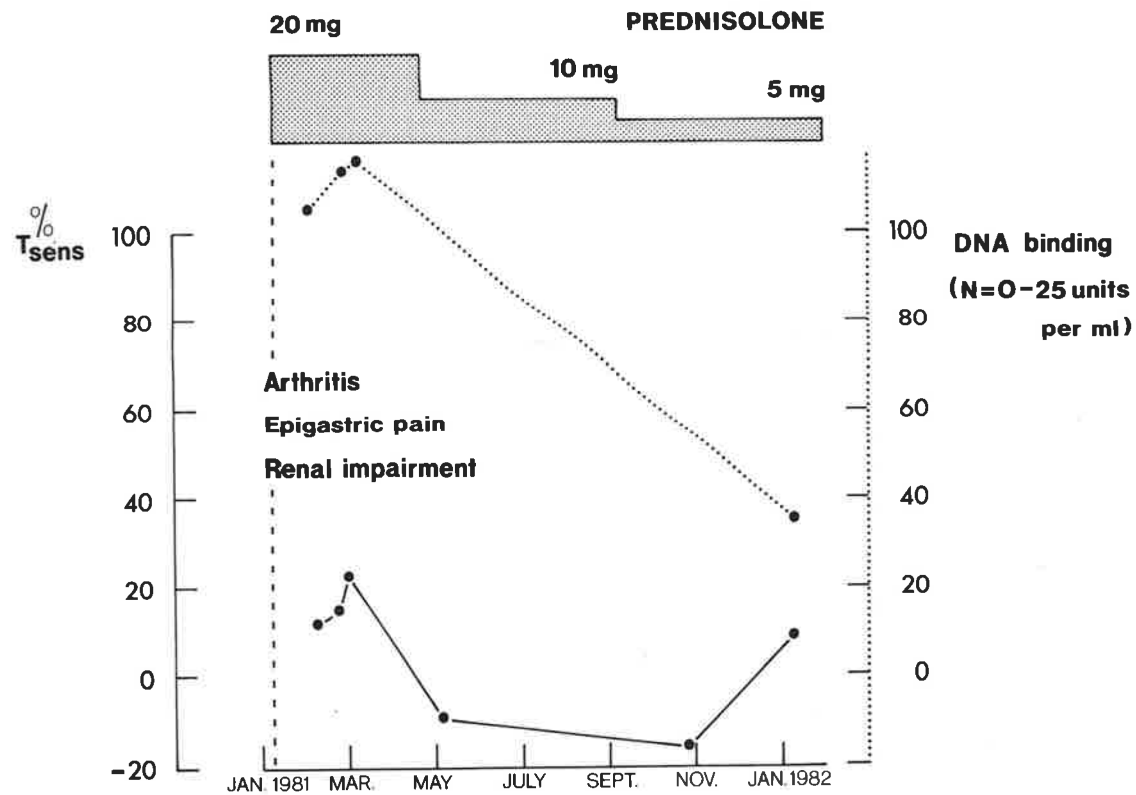

FIGURE 13. Case No. 5. J.W. Male, 72 years.

Case No.5 - Addendum

Correlation of Tsens vs SPFC IgG, IgA and n- DNA binding

	SPFC IgG	SPFC IgA	n- DNA
n	5	5	5
Corr. coefficient	0.94	0.86	0.76
p	<0.02	<0.10 (NS)	<0.10 (NS)

CASE NO.5   J.W. MALE   72 YEARS

DATE	% ERFC	% TSENS	ESR	n-DNA	ANF	SPFC/10^6 LYMPHOCYTES			
						PV	G	A	M
10.2.81	-	12%	50mm	>105	+1/1,280	400	185	1,330	670
17.2.81	-	14%	34mm	-	-	-	160	960	210
25.2.81	-	22%	47mm	115	-	550	380	1,090	260
11.3.81	-	-	43mm	>114	-	-	280	780	10
4.5.81	-	-8%	-	-	-	-	80	75	10
29.10.81	46%	-15%	-	-	-	160	10	180	10
7.1.82	81%	9%	-	34	-	-	-	-	-

TABLE 17.

CHAPTER 6 DISCUSSION

CHAPTER 6.

DISCUSSION

This study was undertaken to assess whether theophylline sensivitivity was a constant and reliable marker for separating T cells with suppressor or helper function, suitable for use in a clinical setting. Limatibul (1978) first demonstrated the ability of theophylline to differentially alter the rosetting ability of T lymphocytes with sheep red blood cells. In the present study T lymphocytes isolated from the blood of Red Cross donors were further separated into theophylline sensitive and theophylline resistant cells depending on their ability to rosette with sheep red blood cells in the presence of theophylline. The fractions were then analysed to determine other established markers claimed to correlate with helper or suppressor activity viz. reactivity with monoclonal antibodies, Fc receptor status, the presence or absence of Gall bodies and the acid esterase content as determined by ANAE staining. The functional behaviour of the fractions in co-cultures with B cells was also studied. Following initial studies by Limatibul and Shore (1978), subsequent studies (Birch, 1981, 1982) have not sought to establish the usefulness of this property in assessing the helper or suppressor status in clinical conditions. In this study, an attempt was also made to correlate the clinical course of five patients with systemic lupus erythematosus with the variations in their theophylline sensitive cell populations and to compare these with other established parameters of disease activity.

Theophylline treatment of total T lympocytes did not significantly alter their reactivity with monoclonal antibodies of OKT3, OKT4, OKT8 and OKM1 specificity, although their SRBC rosetting ability was significantly altered. Cell viability, assessed by Trypan Blue dye exclusion was maintained at >95% following incubation with 3mM theophylline at $37^{o}C$ for 60 mins, showing that significant cell damage did not account for this altered rosetting ability.

The theophylline resistant fraction contained 93% OKT3$^+$ cells and was found to be reasonably homogeneous with regards to putative markers of helper activity. Previous studies have shown (Kung 1979) that in general, T cells with helper function are found in the OKT4$^+$ or Leu 3A$^+$ fraction while suppressor cells predominate in the OKT8$^+$ and Leu 2A$^+$ fractions although none of these markers is specific for helper or suppressor cells. About 60% of the Tres cells in this study were OKT4 positive and 63% were Leu 3A positive, whereas only 27% were OKT8$^+$ and 31% Leu 2A$^+$. However, the proportion of OKT4$^+$ and OKT8$^+$ cells in the Tres fraction was not significantly different from the proportions in total T lymphocytes. The number of cells with positive ANAE staining was significantly greater (81%) in the Tres population than in the total T lymphocytes, as was the number of cells showing Gall bodies on phase microscopy; Gall bodies were found in about half (49%) of the theophylline resistant cells. Marcus and Hurtubise (1981) demonstrated that 68% of the prospectively identified Gall granules had spatially coincident focal esterase staining when stained with α-naphthylacetate esterase. They proceeded to demonstrate that almost no B cells had Gall granules, in contrast to T-helper cells defined with monoclonal antibodies, which were shown to contain Gall bodies in 48% of the cells and in which 88% of all Gall bodies resided. They concluded that the Gall body largely correlated with focal lymphocyte acid esterase activity, and that it was a simple and rather good marker of T-helper lymphocytes. We have demonstrated focal lymphocyte acid esterase activity in 81% of theophylline resistant cells which have other markers indicating that they are of the T-helper subset. The reason that only 49% of these cells actually showed Gall bodies under phase microscopy of a fresh cell suspension may be attributed to the fact that these refractile bodies move with apparent freedom through the cytoplasm in the perinuclear zone and may not have been visible at one pole at the time of examination. One reason why the

Gall body has remained virtually unknown even to haematologists may be due to its solubility in methanol and acetone which are widely used as blood fixatives. This combined with its Neutral Red and Sudan Black staining and apparent birefringence suggests that it has a micellar lipoid nature (Hempelman 1953).

Analysis of the theophylline sensitive population showed it to be a markedly heterogeneous group of cells displaying both helper and suppressor cell markers in addition to significant monocyte contamination. Gall bodies were found in only 7% of those cells, with ANAE staining demonstrable in 11%. Only 46% of these theophylline sensitive cells were $OKT3^+$, suggesting that they were a heterogeneous population of cells. Thirty nine percent were OKM1 positive compared to 11% in the total T lymphocyte pool. The OKM1 marker is said to define monocytes and null cells. This degree of monocytic contamination may be the result of only single adherent cell removal on plastic petri dishes incubated for one hour at 37°C, and also the magnitude of the total cells per dish which could reach as high as 50×10^6 per petri dish when incubated.

Techniques described for removal of adherent cells involve incubation of mononuclear cells on plastic dishes at 37°C for 45 minutes in a 5% CO_2 enriched atmosphere in some cases (Pichler 1978), whereas other workers (Evans 1977) plate unfractionated mononuclear cells on glass Petri dishes and incubate at 37°C overnight, and yet others (Keightley 1976) suggest incubation at 37°C for one hour. Birch and Polmar (1982) however, claimed effective adherent cell removal following incubation in plastic flasks at 37°C for 35 mins. with less than 3% monocyte contamination in the resulting non-adherent cell suspension. More recently Fauci and co-workers (1982) employed fractionation of Hypaque-Ficoll separated mono-nuclear cells over Percoll gradients and elutriation of this produced monocyte-depleted populations that contained between 1-3% monocytes.

Theophylline sensitive cells did show a significant proportion of cells bearing Fcγ receptors (55%) compared to 20% in the total T lymphocyte pool and only 11% in the Tres group; however, other markers for suppressor activity did not appear to substantiate a significant suppressor cell population within the Tsens group. Thus only 44% were OKT8$^+$ and 29% Leu 2A$^+$, both being predominant monoclonal antibody markers for suppressor cells. These results bear some similarity to those of Reinherz et al (1980d) who found the Tγ enriched population to be a heterogeneous population of cells with only 15% reactivity to OKT3 antisera and a larger percentage (50-88%) OKMI$^+$ compared with unfractionated T cells. Thus they showed little correlation between T subsets defined by monoclonal antibodies and those defined by Fc receptor status and it was suggested that Tγ cells were of a monocyte-myeloid lineage, possibly natural killer cells. In the present study the Tsens population had only 46% OKT3$^+$ cells but 39% OKM1$^+$ cells. Since they contained a significant porportion (55%) of cells bearing Fcγ receptors, the possibility of natural killer cells being theophylline sensitive needs to be borne in mind.

Experiments to assess the functional characteristics of the two subsets proved difficult to reproduce. The Tres lymphocyte subset was expected to enhance immunoglobulin production when co-cultured with B cells. This was demonstrated in certain experiments (Table 7), where Tres provided help for Tsens suppression of PWM driven generation of plaque forming cells. However in other identical experiments (Tables 5, 6.) such help and suppression were not clearly demonstrable. In the initial functional assays (Tables 5 & 6.) poor intracytoplasmic immunoglobulin production was obtained when Tres were incubated with B cells in the presence of PWM. Certain experimental factors may have contributed to this. The carbon dioxide levels in the humidified 5% CO_2 chamber had been known to be unstable, although the extent to which this affected the culture conditions could not be determined. The foetal calf serum may

not have sustained immunoglobulin synthesis optimally. Monoctye depletion had not been carried out in the earlier experiments, but in subsequent assays monocytes were removed by adherence on plastic petri dishes at 37°C for 60 mins. and then added back to 5% of the total cell content in a culture well. However T cell depleted B cells isolated by centrifugation on a Ficoll-Hypaque gradient could still contain some monocytes which in turn could exert a suppressive effect on immunoglobulin production.

Monocytes have been shown to play a central role in modulating lymphocyte responsiveness. This function may involve transmission of information via cell-to-cell contact with lymphocytes as during antigen presentation or augmentation of mitogen induced proliferation. The intact monocyte as well as a secreted monocyte factor, Interleukin I are required for pokeweed mitogen stimulated B cell differentiation (Rosenberg 1982). Fauci et al (1982) showed a marked decrease in plaque forming cells when monocytes were excluded from cell cultures. However, in reconstitution experiments it was shown that following initial response with Ig production, subsequent increases in monocyte concentrations led to marked suppression. This suppression of B cell function by excess monocytes has also been shown in disease states such as multiple myeloma, sarcoidosis, SLE and active tuberculosis (Fauci 1982). The mechanism of this monocytic suppression on B cell function is unclear. However it has been suggested that one of the mechanisms of the monocyte-mediated suppression is an interaction with an irradiation-sensitive T-cell. The preferential binding of pokeweed mitogen to the monocyte was thought to be one of the main functions it exerted in culture and this was shown to be associated with the presence of the Ia antigen on its surface. It was also found that the proportion of monocytes to non-monocytes for a given individual on a given day could be either too high or too low for maximal Ig production. In our study, the production of intra-cytoplasmic immunoglobulin in B cells did not appear to be predictable when either Tsens or Tres were used in the co-cultures.

The high percentage of OKMI$^+$ cells in the Tsens population should theoretically cause a significant suppression in immunoglobulin production, but this was not substantiated in all the experiments. O'Malley and co-workers (1982) showed that the Tγ OKMI$^+$ lymphocyte subset was responsible for elaborating human γ interferon (or "type II" interferon) in response to phytohaemagglutinin stimulation and attributed the suppressor function of this subset to the anti-proliferative activity of γ-interferon.

The clinical component of this study involved estimating the percentage of Tsens cells in normal healthy donors as well as in known clinical conditions. Systemic lupus erythematosus is a not uncommon auto-immune disease encountered in daily clinical practice which has been claimed to result from impaired suppressor activity. (Kaufman 1979 - Bresnihan, 1977). The study of patients with SLE in both the active phase of the disease as well as in remission was undertaken to determine the variations of their Tsens during these phases. The normal range determined for Tsens was 17% ($\pm$ 11) compared to 8% ($\pm$ 11) in active SLE, showing a significant decrease. However during the inactive phase of the disease 19 estimations showed the Tsens to be 14% ($\pm$ 12) which was not significantly different from the normal range. Sakane et al (1980) showed that during periods of clinical inactivity the abnormalities of suppressor cell generation returned to normal in whole T cell populations in SLE. Wangel et al (1982) using a reverse haemolytic plaque assay similarly demonstrated a markedly increased number of lymphocytes secreting IgG, IgA and IgM during clinically active SLE with a return to control values during the inactive phase of the disease. Anti-T cell antibodies have been demonstrated during the active phase of the disease and a defect in suppressor cell generation can be induced in the lymphocytes of normal individuals when these antibodies are used to treat the lymphocytes in vitro. (Sakane 1979).

Five patients with SLE were followed over varying periods of up to a year in an attempt to correlate changes in Tsens numbers with other indices of activity. Tsens percentages generally showed a decrease during clinical relapses and in some cases they appeared to herald a relapse in a manner similar to other parameters such as D.N.A. binding and SPFC formation. The ESR was non-discriminatory in this respect and was normal in certain instances even during active disease. In a few patients theophylline inducible T-cells appeared to predominate during the acute phase of the disease. The significance of this is not known, but one might speculate that it indicated a population of immature T-cells or pre-suppressors that do not achieve full suppressor activity during the active phase of the disease.

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