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A functional study of Facb rosette-forming cells in health and rheumatoid disease.

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A FUNCTIONAL STUDY OF FACB ROSETTE-FORMING CELLS IN HEALTH AND RHEUMATOID DISEASE

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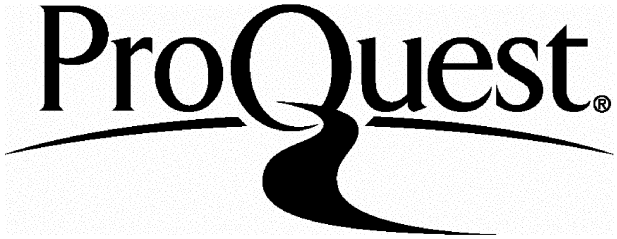

The few talents I possess were given to me by God and nurtured by my parents. Anything I have, or will, achieve is due to them. I hope this work is worthy of the many sacrifices my parents have made on my behalf.

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SUMMARY

A subpopulation of lymphocytes detected by the use of calf erythrocytes sensitized with the Facb fragment of rabbit immunoglobulin G (IgG) were compared in healthy control subjects and in patients with rheumatoid arthritis. These cells (Facb R^+ cells) were found to be less dense in rheumatoid patients than in controls. They were also found to be steroid sensitive in the former group but not in the latter. Electron micrographs indicated that Facb R^+ cells in patients with RA were metabolically more active than in normal controls. It was found that a transient increase in the number of circulating Facb R^+ cells could be induced in control subjects by antigenic challenge.

Studies in mice showed that an increase in %Facb R^+ splenic lymphocytes could be induced by antigenic challenge in sensitized animals. Experiments involving the adoptive transfer of sensitized cells showed that the Facb R^+ cell is involved in suppression of Ig synthesis. This suppression was shown to require interaction between IgG and the Fc receptor on Facb R^+ cells. Similar results were obtained in experiments using human cells. These results are discussed in relation to current concepts of Fc-mediated suppression of humoral immunity.

A longitudinal study of patients with early rheumatoid disease showed that the %Facb R^+ peripheral blood lymphocytes correlated with the clinical assessment of disease activity. This correlation was lost in patients with established RA receiving D-Penicillamine therapy. The implications of these results are discussed in relation to observations made on the in vitro effect of thiol containing compounds on the expression of Fc receptors on Facb R^+ cells.

CONTENTS

CHAPTER 1

INTRODUCTION

SECTION 1

LYMPHOCYTES

The work described in this thesis involves the study of a subpopulation of mononuclear cells first described by Hall, Winrow and Bacon (1980) which bear receptors capable of binding the Facb fragment of IgG (Facb R^+ cells). An attempt has been made to characterise these cells (both phenotypically and functionally) in relation to the known subpopulations of mononuclear cells. Therefore, two major sections of this introduction provide synopses of currently accepted methods of T, B and null cell identification and of the roles of these subpopulations in various immunological reactions

(A) General introduction

Compelling evidence for the involvment of lymphocytes in an immunological response was first presented in the mid 1950's. Medawar (1956) in a discussion on tolerance, cited an experiment in which mice made tolerant to allogeneic grafts could be induced to reject them by the adoptive transfer of lymph node cells from mice actively immunised against the tissue antigens of the grafts.

Although it was well established that graft rejection, delayed-type hypersensitivity (DTH) and antibody production were all immunological phenomena, it was not until the mid 1960's that the cells involved were conclusively identified as lymphocytes. In 1963, M^{c}Gregor and Gowans demonstrated that the removal of small lymphocytes from rats by chronic thoracic duct drainage (TDD) resulted in a severely

depressed primary response to sheep red blood cells (SRBC) and to tetanus toxoid (TT). The same workers later showed that TDD could prolong the survival of allogeneic grafts (M^{C}Gregor and Gowans, 1964). In 1967, Bloom and Chase demonstrated that it was possible to induce specific delayed-type hypersensitivity in guinea-pigs by the adoptive transfer, to naive recipients, of lymphocytes from specifically immunized donors.

Despite this work, there was considerable controversy over the precise involvement of lymphocytes in immunological phenomena. Doubt was cast by the observations of M^{C}Cluskey et al (1963) which showed that very few sensitized cells were found at the site of DTH reactions or in the beds of allografts undergoing rejection. In addition, lymphocytes were (for a long time) considered to be end cells with no potential for further differentiation or proliferation. This was probably due to the fact that at any instant in time, a large proportion of lymphocytes show relatively little metabolic activity. However, the development of in vitro models such as the leukocyte migration inhibition assay (David et al.,1964) and the study of antigen specific leukocyte blastogenesis (Permain, Lycette and Fitzgerald, 1963) eventually confirmed that the lymphocyte played an active role in many immune responses.

Although it had been established that the lymphocyte did perform various functions, it was not obvious whether or not all these activities were mediated by the same cell or by different subpopulations. At this time, lymphocytes were defined by morphological criteria alone, being classified as either small, medium or large. This distribution was found to be continuous and not trimodal, thus precluding the assignment of specific functional activities to the different morphological groups.

Early observations appeared to imply a dichotomy in the immune system. Glick, Chang and Joap (1956) found that neonatal bursectomy in chickens impaired their ability to produce antibody in response to antigenic challenge. Later, Aspinall et al (1963) showed that neonatal thymectomy (either at hatching or 1-3 days later) delayed allograft rejection in Leghorn chickens. Neither steroidal nor surgical bursectomy had this effect. These and other experiments in birds and rodents provided evidence for two separate types of immunological phenomena:- cell-mediated and humoral. Complementary evidence for a similar dichotomy in man was provided by the clinical observations of Good and Varco (1955) on thymoma and agammaglobulinaemia. Abdou and Abdou (1972) suggested that the equivalent environment in man to the Bursa of Fabricius was the bone marrow.

The functional properties of the cells at the basis of the observed dichotomy in the immune system were categorised by Roitt et al (1969) who suggested the terminology ; T cell of thymus derived cell and B cell or bursa-equivalent derived cell. The principle function of the B cell is undoubtedly the synthesis and secretion of antibody. The functions performed by T cells were grouped together under the heading 'cell-mediated immune responses'.

Today, the division of immunological phenomena into cell-mediated and humoral is considered to be largely fallacious since much evidence has accumulated concerning the role of T cells in antibody production. It is quite obvious that immune responses require a complex interaction of a variety of different cells and biochemical mediators. Identification of the cells involved had been greatly facilitated by the development and use of alloantisera (largely in in-bred mouse strains), by the identification of specific surface receptors and more recently by the use of monoclonal antibodies.

(B) Mouse lymphocyte subpopulations

i) Introduction

The mouse is often used as an experimental tool owing to the comparative ease of obtaining large numbers of genetically identical individuals. Elimination of genetic variation allows various functional experiments to be performed which are only possible *in vitro* in humans- a system which has numerous inherent limitations. However, one must not automatically extrapolate from mouse to man, although results observed in the mouse system may be used as an indication of those expected in humans. Since much of our knowledge of immune regulation and cellular interactions stems from work done in the mouse, this section will briefly describe those markers used to identify murine lymphocyte subpopulations, some functions of which are discussed in Section 11 of this chapter.

ii) Surface markers of mouse T cells

Theta/Thy alloantigens

The theta antigen (later renamed Thy 1) was the first serologically detected murine alloantigen with a restricted tissue distribution. Reif (1963) and Reif and Allen (1963;1964) raised isoantisera in C3HeB/Fe mice against AKR thymic lymphocytes which could lyse AKR thymocytes but not non-thymus derived lymphocytes of marrow cells of normal AKR mice. This AKR thymic antigen was found in high concentration in the thymus and adult nervous tissue but only in low concentrations on lymphocytes from the adult spleen and lymph nodes. When tested against other mouse strains, only thymocytes from RF mice showed cytolysis.The reciprocal isoantiserum (i.e. raised in AKR mice against C3HeB/Fe cells) caused the lysis of thymocytes from all the mouse strains tested except those from AKR and RF mice. Reif and Allen (1964) suggested that these two antigens should be termed θ-AKR and θ-C3HeB/Fe. Experiments using

F_1 hybrid mice (AKR x C3HeB/Fe)and isoantisera to θ-AKR and θ-C3HeB/Fe, suggested that these two antigens were the expression of a single locus comprised of two different alleles (Reif and Allen 1966a). These alleles were later termed Thy 1.1 (θ-AKR) and Thy 1.2 (θ-C3HeB/Fe).

The first evidence of a restricted distribution of the Thy 1 antigen was presented by Reif and Allen (1966b). They showed that isoantisera to the theta antigens were ineffective against leukæmic cells of non-thymic origin. Also Raff (1969) using ^{51}Cr release as a measure of cytotoxicity, looked at the effect of chronic antilymphocyte serum (ALS) treatment on the cytolytic activity of anti-θ antiserum towards murine thymus, lymph node and peripheral blood lymphocytes. He showed that 70-80% of lymph node lymphocytes and 30-50% of splenic lymphocytes have θ antigen and that these levels were drastically reduced after ALS treatment (which selectively depletes T cells). Similar results were obtained by Schlesinger and Yron (1969). Raff (1969) also reported the results of preliminary experiments using neonatally and adult thymectomised mice, lethally irradiated and foetal liver reconstituted mice, and congenitally athymic mice. In all cases there was a reduction in the expression of θ antigen in the peripheral lymphoid tissues. This was later substantiated by the work of Raff and Wortis (1970).

All this work indicated that θ antigens were expressed on cells conditioned by the thymus. Direct evidence that 'non-T' cells did not express θ was produced by Raff (1970) and Cerrotini et al (1970). Raff showed that a correlation existed between those cells lacking θ antigen and those bearing surface immunoglobulin (SIg); whilst Cerrotini et al showed that treatment with anti-θ antiserum did not affect the plaque forming ability of sensitised cells. Thus, Thy 1 was accepted as a marker for all 'thymus conditioned murine lymphocytes' (T cells).

Ly (Lymphocyte) alloantigens

In 1968, Boyse et al., were studying two systems of leukaemic antigens in mice. The cytotoxic sera used to identify these systems were found to contain additional lytic antibodies. Experimentation revealed that these antibodies were specific for antigens confined to thymocytes and some lymphocytes. They also showed that the absorptive capacity of bone marrow was very low, suggesting that these specificities were only present on T cells. The genetic loci determining the expression of these antigens were designated as Ly A and Ly B. Backcross experiments showed that each locus consisted of 2 alleles determining the alternative isoantigens Ly A.1 or Ly A.2 and Ly B.1 or Ly B.2.

In 1971, Boyse et al., showed the presence of a third specificity (which they called Ly C) which was always present with Ly B on the cell membrane. The three Ly specificities later became known as Ly 1, Ly 2 and Ly 3. Further specificities have become recognized on both T and B cells which appear to define functionally distinct subpopulations (see Sections 1 C ii and 11 A and B). In order to clarify the nomenclature, it has been suggested that T or B cell distribution of the Ly specificities should be indicated by the prefixes t or b eg. Lyt 1, Lyb 1. This has not yet received widespread acceptance and thus both systems of nomenclature exist in the literature.

The early literature on the Ly alloantigen system appeared to indicate that Ly 1 was only expressed on T cells i.e. Thy 1^+ cells. However, the recent development of monoclonal antibodies has allowed more sensitive work to be performed. Using monoclonal Lyt 1, Ledbetter et al., (1980) showed that approximately 2% of mouse spleen cells expressed easily detectable amounts of Lyt 1 but appeared to lack Thy 1. The authors suggested that Thy 1 and Ly 1 were expressed in inversely proportional amounts on the cell surface. Thus, although these markers are both used

to identify T cells, recent work indicates that Ly 1^+ cells may not automatically be assumed to be Thy 1^+.

Fc receptors

The presence of Fc receptors (FcR) on mouse thymus derived lymphocytes has been demonstrated by several workers, (Yoshida and Andersson, 1972; Andersson and Grey, 1974; Basten et al., 1975; Stout and Hertzenberg, 1975) using various forms of IgG containing immune complexes. The results showed that there was great heterogeneity in the distribution of FcR on a 'per cell' basis (Andersson and Grey, 1974; Stout and Hertzenberg, 1975) and that the amount of complex bound was similar in both T and B cell populations (Stout and Hertzenberg, 1975). The earlier reports suggested that FcR were expressed on 'activated', but not on resting, T cells (Yoshida and Andersson, 1972; Andersson and Grey, 1974). However, using the more sensitive fluorescence activated cell sorter (FACS) system, Stout and Herzenberg (1975) showed that the majority (70%) of FcR^+ T cells in the normal spleen were small, non-activated (i.e. not lymphoblastoid) lymphocytes and that only 50-60% of activated T cells were FcR^+. This indicated that activation did not necessarily lead to an increase in FcR expression.

The distribution of the various classes of FcR and the functions apparently associated with their expression are discussed more fully in Sections 11 A,B,C and D.

iii) Surface markers of mouse B cells

Surface immunoglobulin

Reports of the transforming effect of anti-Ig antisera and of the opsonic adherence of anti-Ig treated lymphocytes to macrophages led Raff, Sternberg and Taylor (1970) to suggest that the antigen- specific

receptors on lymphocytes were immunoglobulins. Using fluorescent labelled rabbit anti-mouse IgM, they demonstrated the presence of IgM on 35% spleen cells and on 19% lymph node cells. They could not demonstrate this immunoglobulin on the surface of thymocytes and hence suggested that this might act as an indicator of B lymphocytes. Using similar methods, Rabellino et al.,(1971) reported the presence of surface immunoglobulin (SIg) on 45% splenocytes. However these workers showed that IgG_1 and IgG_2 were more prevalent than IgM.

Complement receptors

Lay and Nussenzweig (1968) showed the presence of 2 different receptors for complement on various murine leukocytes. One of these receptors was trypsin sensitive and did not require the presence of divalent cations. It was not exhibited on monocytes, polymorphonuclear leukocytes (PMN), or thymocytes. It was present, however, on 10-25% of lymph node cells. These results suggest that the receptor is present on B cells. A second cation dependent complement receptor was found on monocytes and PMN.

Ly (lymphocyte) alloantigens.

The Ly alloantigen series which was originally observed on T cells has been greatly extended in recent years, some of the antigens being observed specifically on B cells. The Ly 4 alloantigen (also termed Lyb 1) was described by Snell et al (1973) and was the first in this series which appeared to be restricted to B cells. M^{c}Kenzie and Snell (1975) showed that the Ly 4 locus governed the appearance of a single reactive specificity on the surface of lymphocytes - Ly 4.2. The distribution of this antigen was shown to be restricted and inversely proportional to that of Thy 1,2. From these results they concluded that Ly 4.2 was present on B cells. Confirmation of this came from experiments

in mice depleted of T cells by various methods. The majority of lymphocytes in these animals were Ly 4.2^+,Thy 1.2^- (Aoki et al.,1974; M^{c}Kenzie and Snell, 1975).Also, M^{c}Kenzie (1975) showed that both direct and indirect antibody forming cells could be inhibited in vitro by anti-Ly 4.2 antiserum. However, Aoki et al (1974) found that additional specificities in the anti-Ly 4.2 antiserum appeared to react with a subpopulation of purified T cells. More recently, Gani and Summerell (1977) showed that there was almost equal distribution of Ly 4.2 on B and T cells.Thus the situation is by no means clear. The reactivity of Ly 4.2 antisera with T cells may be a result of its heterogeneity and investigation with a monoclonal anti-Ly 4.2 may clarify the situation.

Although Ly 4.2 is probably the best categorised of the B cell alloantigens, several other specificities have been defined which are restricted to B lymphocytes - Lyb 2, 3, 4, 5, 6, and 7. The distribution and significance of these antigens were reviewed by M^{c}Kenzie and Potter (1979).

Mouse specific bone marrow-derived lymphocyte antigen (MBLA)

Raff et al (1971) used lymph node cells from thymectomised, irradiated, fœtal liver reconstituted, CBA mice to produce an antiserum which, (when absorbed with red cells, hepatocytes and thymocytes), reacted with approximately 40% of bone marrow cells, and with lymphocytes from the spleen and lymph nodes. This antiserum (anti-MBLA) was found to show reciprocal cytotoxicity to anti-Thy 1 (Raff et al, 1971); to react with direct plaque forming cells (Niederhuber and Moller, 1972); to react with antigen binding B cells (Niederhuber and Moller, 1972). Thus the evidence pointed towards this being an antigen expressed on the surface of mouse B cells.

Fc receptors

Receptors which bind the crystallizable fragment of IgG (Fc) have been demonstrated on mouse B cells (Basten et al.,1972; Paraskevas et al.,1972). Basten et al (1972) showed that FcR bound immune complexes containing IgG but that this binding could be inhibited by pre-incubating the cells in an excess of monomeric IgG. Also, this association was disrupted by washing, which the authors suggested illustrated the weak nature of the FcR-IgG bond on these cells.

The binding specificity of FcR on mouse B cells is discussed more fully in Section 11 D.

iv)Mouse null lymphocytes

Warr et al (1978) isolated a subpopulation of lymphocytes from athymic, nude (T cell deficient) mice which were eluted from nylon wool columns and did not react with the IgG fraction of rabbit anti-mouse IgM covalently bound to sepharose-6B macrobeads. Direct immunofluorescence failed to demonstrate SIg on these cells which were thus considered to be null cells. However, using fluorescent labelled chicken anti-mouse IgM, Warr et al (1978) were able to demonstrate SIg on these null cells in similar amounts to that found on B cells by more convential methods. More recently, Schrader and Nossal (1980) described a class of FcR^+ null cells termed the P cell. These cells are non-phagocytic, Thy 1^-, Lyt 1^-, Lyt 2^-, Ia^+, SIg^-. However, the presence of SIg was investigated using direct fluorescence and thus it is feasible that these cells do possess low levels of SIg. Thus, the available data on the existence of null cells in mice is not conclusive.

v)I-region associated antigens

Immune response gene associated antigens (Ia) have been reported to be present on many different cell types including all classes of

lymphocytes. For this reason these antigens will be discussed separately in this section. The functional relevance of these markers will be discussed in Section 11 of this introduction.

The literature concerning the occurrence of Ia antigens is too vast to be described in great detail in this thesis. The subject has been reviewed by Moller (1976) and M^{c}Kenzie and Potter (1979) and the salient features of these reviews are considered below.

The histocompatibility loci code for specificities which appear to be found in all tissues. Many of the loci which code for the membrane alloantigens on lymphocytes already discussed in this section are found within the major histocompatibility complex (MHC) of the mouse (the H-2 complex). At least four loci code for antigens of general distribution - H-2K, -2D, -2G, -2L - and can thus be detected by graft rejection and by their ability to elicit antibody production. Encompassed by the H-2 complex are several loci coding for specificities found largely on lymphocytes. These map in the same region as the immune response genes (Ir genes) and have thus been termed the I-region associated (Ia) antigens. Using recombination studies, it had been shown that the I region is comprised of several sub-regions (designated I-A, I-B, I-J, I-E, and I-C) each having their own marker locus or loci. Four of these loci have been serologically defined- Ia-1, Ia-3, Ia-4, and Ia-5. Other loci determined by functional and serological studies have not yet been designated to particular sub-regions of the Ir genes.

Dickler and Sachs (1974) and Hammerling (1976) have shown by double fluorescent studies that approximately 90% SIg^{+} splenic lymphocytes were Ia^{+}. These workers were unable to detect Ia-related antigens on peripheral blood T cells. However, other workers have demonstrated anti-T cell cytotoxic activity in anti-Ia antisera (Frelinger et al., 1974). Neiderhuber and Frelinger (1976) showed that Con A induced T blasts expressed Ia. Thus, the data imply that both B and T cells express Ia-

related antigens but that the latter are expressed in much lower concentration on T cells than B cells (Hammerling, 1976). More recently, Tada et al., (1978) showed that at least two subpopulations of T helper cells could be identified by their differential expression of Ia determinants. Ia antigens have also been demonstrated on the surface of macrophages and have been shown to participate in antigen-specific stimulation of T helper cells (Erb and Feldmann, 1975). Recently. Asano, Okumura and Tada (1981) have shown that those Ia antigens expressed on helper and suppressor T cells and encoded in the I-J region are not expressed on myeloid antigen presenting cells. The latter express Ia determinants encoded by the I-A and I-E/C subregions. Thus, these cells carry the same specificities as B cells.

The expression of Ia determinants on other cells (eg. epidermal cells and spermatocytes) has been reviewed elsewhere (Hammerling, 1976).

(C) Human lymphocyte subpopulations

The pioneering work demonstrating characteristic cell surface markers in mice lead the way for similar work in man.

i)T lymphocyte markers

Sheep red blood cell rosette formation

'All lymphocytes processed by the thymus bear a receptor capable of binding sheep red blood cells (SRBC)'. This statement is based on evidence which has been accumulating since 1969. Bach et al (1969) reported a chance observation that a few human peripheral blood lymphocytes formed clusters when incubated with SRBC. Later work showed that this phenomenon could be abrogated by anti-lymphocyte antiserum (Bach 1970). There followed a series of reports which, although differing slightly in technique, all demonstrated that a percentage of human lymphocytes was capable of binding SRBC (Coombs er al 1970; Lay et al, 1971; Froland, 1972). This phenomenon proved to be non-immunological and confined to thymus-derived lymphocytes (Wybran, Carr and Fudenberg 1972; Wybran and Fudenberg, 1973; Jondal, 1976). Rosette formation was shown to occur only with living cells and to be cation and temperature dependent; rosettes becoming unstable at temperatures above 37°C (Jondal Holm and Wigzell, 1972).

Thus, the data suggested that all T cells bound SRBC to the same extent. However, it was found that this characteristic could be manipulated to demonstrate heterogeneity amongst T cells. Yu (1975) demonstrated that T cells could be divided into those forming 'active' or early rosettes (immediately after sedimentation) and those forming 'late' rosettes (requiring incubation at 4°C). Recently Semenzato et al (1981) have suggested that 'active' and 'late' rosette formation reflect the maturity of the lymphocytes involved; those involved in late rosette

formation being the more mature.

Other workers have looked at the effect on rosette formation of modifying SRBC either enzymatically (with neuraminidase) or chemically (with 2 aminoethyl-isothiouronium bromide; AET). In general, these techniques have resulted in the formation of a greater percentage of more stable rosettes (Kaplan and Clark, 1974)
However, these techniques may alter the specificity of the assay such that cells other than T cells are being rosetted.

Helix pomatia (HP) receptors

Hammarstrom et al (1973) showed that neuraminidase treatment of lymphocytes (a process which results in the cleavage of sialic acid residues from the glycoproteins of the cell membrane) enabled these cells to bind the A hæmaglutinin of Helix pomatia (the vineyard snail). Utilization of this hæmaglutinin in affinity chromatography resulted in the isolation of enriched T cell populations ≈ 90% E^+ (Hellstrom et al, 1976).

Heteroantisera

The rationale used on the investigation of mouse cell surface antigens was applied to the human system inorder to determine whether or not the equivalent of the murine 'Ly' series could be detected on human cells. Evans et al (1977; 1978) used human lymphocytes as an immunogen in rabbits and rendered the resultant antiserum specific for T cells by absorption. This work resulted in the identification of two subsets termed TH1 and TH2 which appeared to functionally distinct. The activities of these subsets will be discussed in Section 11.

Monoclonal antibodies

When B cells are stimulated to produce antibody, clonal expansion results, each cell of that clone producing an antibody of a single specificity. Stimulation of numerous clones by different antigenic determinants on the immunogen results in the production of a specific heteroantiserum. If however, one took isolated, stimulated B cells and fused them with a rapidly dividing tumor cell, it is theoretically possible to immortalize a clone producing antibody of a single specificity (monoclonal antibody). This technique was first perfected by Köhler and Milstein (1975) and since that time, a wide range of monoclonal antibodies has been produced. The advent of these immunological tools was heralded as a major breakthrough in our understanding of lymphocyte function. Unique surface antigens (identified by specific monoclonal antibodies) appeared to be expressed on the various functional subsets (Reinherz and Schlossman, 1980a). However, problems of apparent cross-reactivity have occurred (reviewed by Lane and Koprowski, 1982) and cellular identification by the use of monoclonal antibodies obviously requires standardization. A list of the more commonly used commercial monoclonal antibodies is given in Table 1.1.

Fc receptors

Receptors which can bind the Fc portion of immunoglobulin molecules have been demonstrated on various human leukocytes. Receptors for IgG (T gamma; $T\gamma$; Farrarini et al., 1975), IgM (T mu; $T\mu$; Moretta et al., 1975), IgD (T delta; $T\delta$; Sjoberg, 1980 a) and IgA (T alpha; $T\alpha$; Lum et al., 1979; Sjoberg, 1980 b), have all been demonstrated on human T lymphocytes. The first of these receptors to be demonstrated were those which bind IgG and IgM ($Fc\gamma$ and $Fc\mu$). Originally, it was thought that they were expressed on different subpopulations of T cells (Moretta et al., 1977; Gupta, Schwartz and Good, 1979). However, subsequent work appeared to contradict this idea. Pichler,Lum and Broder (1978) showed that $Fc\gamma R$

Table 1.1

Monoclonal antibodies used in human leukocyte characterization

Monoclonal Antibody	Specificity
*OKT 1,3 +Leu 1,4,5,	Total peripheral blood T cells
*OKT 4 +Leu 3a,3b	Peripheral blood helper/inducer T cells
*OKT 5,8 +Leu 2a,2b	Peripheral blood cytotoxic/suppressor T cells
*OKT 6,9,10 +All T cell reagents	Thymocytes
*OKI 1 +HLA-DR '@Human DR	Ia-like antigen positive leukocytes
*OKM 1 '@Human monocyte	Monocyte/myeloid leukocytes

*- Ortho Pharmaceutical Company.

'- Bethesda research laboratories

+- Becton Dickinson

@- anti

(after modulation with immune complexes and incubation at 37°C) were endocytosed and/or shed. If these cells were subsequently cultured they expressed $Fc_{\mu}R$ which could not be made to disappear by modulation. In addition, simultaneous expression of $Fc_{\mu}R$ and $Fc_{\gamma}R$ could be demonstrated on these cultured cells. Similar results were obtained by Mingari et al., (1978). Schulof et al., (1980) showed that neuraminidase treatment of T_{μ} cells exposed $Fc_{\gamma}R$ on a small subpopulation of these lymphocytes. Thus, more recent work suggests that the majority of T cells bear receptors for IgM and that a small proportion of these cells simultaneously express $Fc_{\gamma}R$.

Characterization of T_{μ} cells with monoclonal antibodies had shown that they do not form a homogeneous subpopulation since both OKT 4^{+} and OKT $5/8^{+}$ cells are present. The T_{γ} subset did not react with OKT 3 (a universal T cell marker) but possessed an antigen reactive with OKM1, a monocyte marker (Reinherz et al., 1980).

ii)B cell surface markers

Surface immunoglobulin (SIg)

Detection of SIg on murine B lymphocytes initiated an investigation of the same phenomenon in humans. The presence of SIg on human lymphocytes was confirmed by Papamichael, Brown and Holborow (1971) and Wilson and Nossal (1971).The evident lack of these cells in patients with hypogammaglobulinaemia suggested that the SIg^{+} cells were B lymphocytes (Froland and Natvig, 1972 a). Detection of SIg can be achieved by any one of several well documented methods and these have allowed extensive research into the nature of the Ig on the surface of B cells (Froland and Natvig,1972 b). Various workers have demonstrated that 10-20% of peripheral blood lymphocytes have membrane bound Ig as identified by staining with

anti-$F(ab')_2$ or anti-light chain antisera (Froland et al., 1971; Grey et al., 1971; Siegal et al., 1971; Froland and Natvig, 1972a). In most species, including man, it has been shown that IgM (in the monomeric form and not the pentameric form found in serum) is one of the major cell surface immunoglobulins (Vitetta, Baur and Uhr, 1971; Pernis et al., 1971; Preud'homme and Seligman,1972; Vitetta and Uhr, 1972; Andersson et al.,1974). Early evidence suggested that each B lymphocyte bore immunoglobulin from only one class (Grey et al., 1971; Froland and Natvig, 1971;1972a). However, van Boxel et al.,(1972) showed a high percentage of lymphocytes carried IgD on their membrane. Since the presence of this immunoglobulin was not investigated in the earlier studies where IgM and IgG accounted for the total SIg, van Boxel's results suggested that more than one Ig class was present on some B cells. Indeed, subsequent work has shown that approximately 75% of peripheral blood B cells bear both IgM and IgD, the remainder expressing IgG or IgA.

Complement receptors

Whilst it is true that all cells with easily demonstrable SIg also possess receptors for the third component of complement, (C_3R; Bianco Patrick and Nussenzweig, 1970) the reverse is not true (Ross et al., 1973). This is due to the demonstration of a third, non-T, non-B lymphocyte population which may bear complement receptors (Perlmann et al., 1975; 1976). However, more recent evidence appears to contradict this (see Section 1 C iii). Also monocytes have been shown to possess C_3R (Huber et al., 1968).

Two different complement receptors have been identified on lymphocytes - the C_{3b} (or immune adherence receptor) and the C_{3d} receptor (Ross et al., 1973). These are antigenically distinct and express selective specificities for C_4 or C_3 molecules (Ross and Polley, 1975; 1976).

Fc receptors

Receptors capable of binding the Fc region of IgG were first demonstrated on human non-T cells by Hallberg, Gurner and Coombs (1973). Subsequent work suggested that either none, (Gergely et al.,1977; Alexander and Henkart,1978) or only a small fraction (Lobo and Horwitz 1976; Winfield, Lobo and Hamilton, 1977) of these receptors were expressed on B cells. Arbeit, Henkart and Dickler (1977) showed that Fc R could be demonstrated on most B cells by the use of high concentrations of soluble immune complexes.Similarly, Pichler and Broder (1978) showed that IgG sensitized ox erythrocytes could be used to demonstrate $Fc_{\gamma}R$ on most B cells. These authors suggested that the relative instability of the rosettes formed reflected the low avidity of the receptor for IgG and might explain the diverse results reported in the literature. The same authors also investigated the presence of $Fc_{\mu}R$ on B cells. Their results suggested that $Fc_{\mu}R$ were also present on the majority of B cells. This is in contrast to the work of Pichler and Knapp (1978) who suggested that Fc R were expressed only on 2-3% of peripheral blood B cells. Expression of FcR was found not to be restricted to any particular subpopulation of B lymphocytes (as defined by the class of SIg) and hence appeared not to reflect any functional heterogeneity in these cells (Pichler and Broder, 1978).

Mouse erythrocyte rosettes

When human lymphocytes are mixed with mouse red blood cells, a small proportion form rosettes. These M^+ cells have been shown to be B lymphocytes (Forbes and Zalewski, 1976; Gupta,Good and Siegal, 1976). Modification of mouse erythrocytes by pronase has resulted on the identification of four subsets of B cells: $M^+M_{pro}{}^+$; $M^+M_{pro}{}^-$; $M^-M_{pro}{}^+$; $M^-M_{pro}{}^-$, (Forbes et al.,1982). The authors suggested that the binding of normal or pronase treated mouse cells reflected the maturity of the lympho-

cytes involved.

iii)Null cell surface markers

The author has chosen this heading to describe all those lymphocytes which are E rosette negative and lack SIg as detected by direct immunofluorescence. This group of cells has been variously called :"null" lymphoid cells (Kalden et al.,1977; Niaudet, Greaves and Horwitz,1979); non-T,non-B, third population cells (Froland,Wisloff and Michaelsen, 1974); L lymphocytes - cells possessing membrane labile (L) Ig - (Horwitz and Lobo, 1975); unidentified, lymphocyte-like cells(UL cells) (Dickler, 1976b; Arbeit, Henkart and Dickler, 1977). The techniques used to isolate each of these populations suggest that they are not synonymous, but probably all derive from a group of cells expressing neither receptors for SRBC nor SIg.

Fc receptors

In 1974, Froland, Wisloff and Michaelsen showed that human group O erythrocytes sensitized with Ripley anti-CD (EA_{CD}) antiserum were selectively bound by non-T, non-B, lymphocytes. They further showed that the receptors responsible for this binding were trypsin and neuraminidase resistant (pre-incubation with these enzymes even resulted in enhanced rosette formation) but that almost complete inhibition of rosette formation could be achieved by pre-incubation with iodoacetamide (final concentration 10^{-2}M).

Investigation of the specificity of this receptor showed that IgG_1 and IgG_3 were bound with equal affinity whilst IgG_2 and IgG_4 were bound only weakly (Froland et al.,1974). The binding was Fc dependent, being inhibited by the Fc fragment of IgG and even more strongly by the Fch fragment (derived from an IgG_3 myeloma protein). This was thought to suggest involvement of the C 2 region in binding to the Fc receptor. This work was in part confirmed by Horwitz and Lobo (1975)

and by Pang and Wilson (1978) who suggested that the Fc receptor on null cells was structurally different to that on other types of lymphocyte. Lobo and Horwitz (1976) showed that both B cells and null cells bound aggregated guinea pig and human IgG, but only null cells formed EA_{CD} rosettes. They also showed that the former were trypsin sensitive whilst the latter were not. Arbeit, Henkart and Dickler (1977) and Pang and Wilson (1978) showed that only low levels of sensitizing antibody were required to demonstrate null cell Fc R using immune complexes and this has led to the acceptance of the terms "high affinity" or "high avidity" Fc receptors, (Horwitz et al., 1978; Horwitz and Garrett, 1977; Despont et al., 1981).

More recently, Hall, Winrow and Bacon (1980) have described the presence of a receptor on lymphocytes which binds the Facb fragment of IgG - demonstrating the involvement of the C 2 region in Fc receptor -IgG interaction. These receptors preferentially bound IgG_1 and IgG_3 and were expressed on cells which lacked easily demonstrable SIg or receptors for SRBC (Winrow, 1982).

Complement receptors

Receptors for the third component of complement have been demonstrated on the surface of B lymphocytes (Bianco, Patrick and Nussenzweig, 1970) and monocytes (Huber et al., 1968). Evidence exists that these receptors are also present on null cells (Chess et al., 1975; Perlmann et al., 1975; Horwitz et al., 1978). In general, null cell populations prepared by passage of T depleted lymphocytes through nylon wool columns (Horwitz and Lobo, 1975; Froland et al., 1974; Despont et al., 1981) or by EAC rosette depletion (Horwitz and Garrett, 1977). These methods remove all complement receptor positive populations and thus the resulting null cell preparations would be C_3R^-. However, Chess et al., (1975) used a Sephadex G 200 anti - human Fab column to show that

there was a subpopulation of lymphocytes which was E^-,SIg^-, C_3R^+. This was confirmed by Horwitz et al.,(1978) using anti-human Ig immobilized to petri dishes to remove SIg^+ cells.

Problems of null cell classification

The usual definition of null cells depends on the lack of SRBC receptors and SIg on the lymphocytes (Froland, Wisloff and Michaelsen, 1974; Horwitz and Lobo, 1975; Dickler, 1976 b; Kalden et al., 1977; Niaudet, Greaves and Horwitz, 1979). However, recent work has suggested that null cells do in fact bear SIg (Haegert, 1978; Haegert and Coombs, 1979; Haegert, 1980; Pepys, Tennant and Pepys, 1981). The techniques used (Direct Antiglobulin Rosetting Reaction -DARR; Mixed Antiglobulin Rosetting Reaction -MARR; alkaline phosphatase labelled anti-human polyvalent Ig) enhanced the sensitivity of the direct labelling methods usually employed. Results suggested that 99-100% of all peripheral blood non-T cells express SIg, although Haegert (1980) had suggested that 10% of null cells (as detected by direct fluorescence) may truly lack SIg and that this might reflect the heterogeneity expressed amongst conventionally defined null cells.

It has also been suggested that null cells may be T cells. Horwitz et al., (1978) reported that 11% of their L cell population consisted of 'contaminating T cells which reacted with anti-human T lymphocyte antiserum and with sheep erythrocytes. Also, electron micrographs have indicated that null cells and T cells are identical morphologically (Ferrarini et al., 1980). The same workers showed that both these cells have paranuclear localization of acid hydrolases.

Similarities between null cells and monocytes have also been observed. Niaudet, Greaves and Horwitz, (1979) and Horwitz et al., (1978) have shown that null cells react with anti-Ia and anti-myeloid antisera. More

recently, Kay and Horwitz (1980) showed that null cells were $OKM1^+$ but were non-phagocytic and lacked α-naphthyl esterase. Thus, the true ontogeny of null cells remains unclear and obviously more work is required in this field.

iv)HLA-DR related (human Ia-like) antigens

Human Ia-like antigens were first recognised as a series of HLA-linked alloantigens, principally on B lymphocytes, using sera from multiparous women (Winchester et al., 1975). The subsequent preparation of hetero-antisera from rebbits made hyperimmune to isolated cell membrane proteins from B lymphoblastoid cell lines, allowed the identification of these anti-gens on other cells. Using such an antiserum, Fu et al., (1978) and Yu et al., (1980) demonstrated the presence of Ia-like antigens on a small proportion of normal circulating T cells. However, Evans et al., (1978) were unable to detect Ia-like antigens on normal cells using heteroantisera and a fluorescence activated cell sorter (FACS) system. In vitro stimulation of lymphocytes (by mitogens, soluble antigens and allogeneic MLC) has been shown to induce Ia-like antigens on T blasts which appear in culture after 4.6 days (Evans et al., 1978; Yu et al., 1980). In contrast, in vivo immunization induces an increase in small, DR^+ lymphocytes after 2-3 days. When cultured, these cells do not incorporate thymidine. Despite these conflicting results, the data clearly indicate that HLA-DR antigens are expressed on T cells. The third major lymphocyte subpopulation has also been shown to express Ia-like antigens. Horwitz et al., (1978) showed that 42% of the cells in an L cell enriched population expressed Ia-like antigens. Thus, all the major subpopulations of lymphocytes have been shown to express HLA-DR related antigens. Accessory cells also express Ia-like antigens. Engleman et al., (1980) showed that approximately two thirds of the peripheral blood monocyte population was DR^+. Expression of Ia-like antigens by other accessory cells has been reviewed by Lipsky and Kettman

(1982). The functional relevance of these antigens will be discussed in Chapter 1 Section 11 of this thesis.

SECTION 11

(A) Introduction

In section A 1 of this thesis the early experimental work which lead to the identification of the major lymphocyte subpopulations was described. Most of this work involved in vivo functional studies and it is the functional activities of lymphocytes which will be considered in this section. Two early, important observations showed that neonatal bursectomy in chickens reduced serum antibody levels and that neonatal thymectomy impaired delayed-type hypersensitivity (DTH) reactions (Jankowic,1963) and delayed homograft rejection (GVH) (Aspinall et al., 1963). This lead Warner and Szenberg (1964) to suggest a functional dissociation between cell mediated (DTH, GVH) and humoral (Ab production) responses - the former being mediated by T cells, the latter by B cells. Although it is no longer accepted that this strict dichotomy really exists it is helpful to use these terms when describing various immune responses.

(B) Humoral immunity

Humoral immunity (ie. the production of antibody) was originally thought to be mediated solely by B cells (Warner and Szenberg, 1964). However, Claman et al., (1966) suggested that the humoral response to some antigens depended on a synergistic action between T and B cells. This early work stimulated an investigation into the regulation of the humoral response by cellular interaction. It is now known that both positive (help) and negative (suppression) signals control the production of antibody. These effects and the cells which mediate them are discussed below.

i) Positive regulation of the humoral response (helper effects)

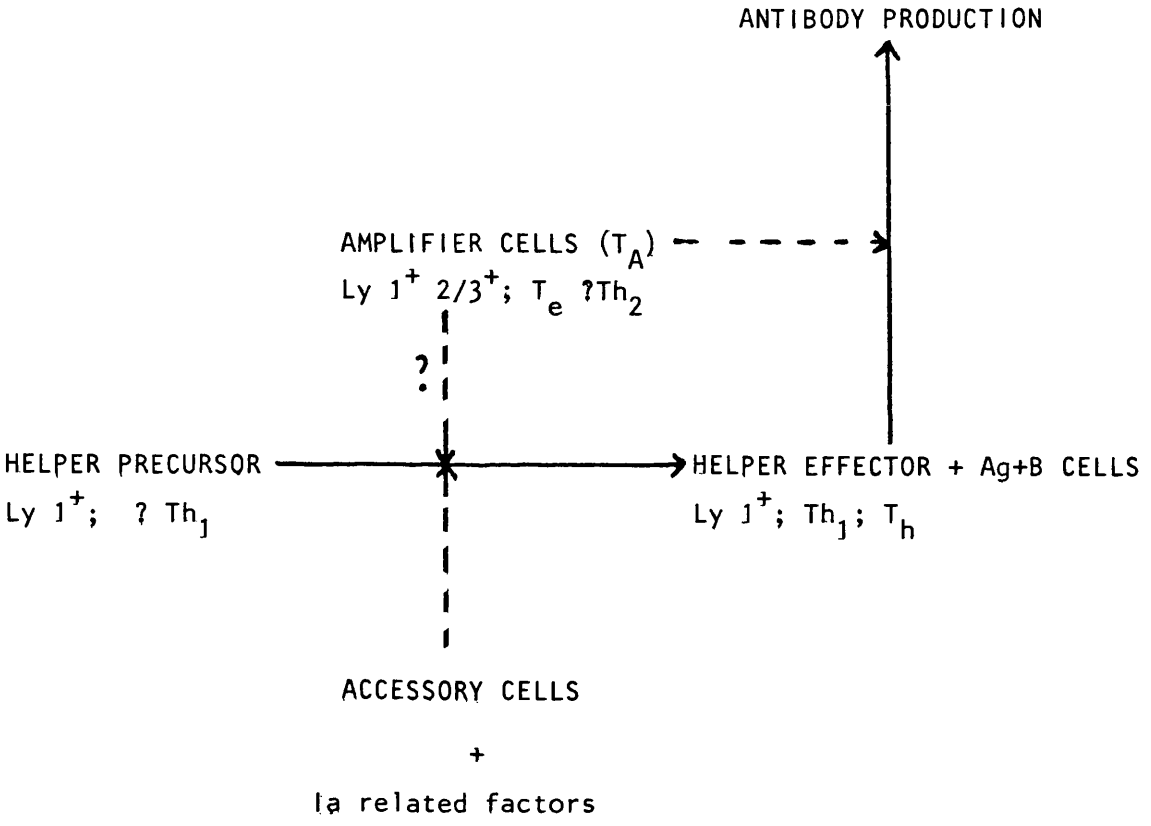
The synergy suggested by Claman et al.,(1966) was later examined using

the 'hapten-carrier' effect (Mitchison, 1971). The hapten (dinitrophenol-DNP) conjugated to the carrier-chicken gamma globulin (CGG) was injected into mice. The resulting primed splenocytes were transferred to virgin recipients which were challenged with DNP-BSA (DNP-bovine serum albumin). A good secondary anti-DNP response was only observed when DNP-CGG primed cells were transferred with the BSA primed T cells. These cells were termed helper cells (T_h). With the development of anti-sera against T cell specific antigens, it became possible to identify those subpopulations which provided this help. It also lead to the realization that T cell mediated help was a complex process requiring the interaction of several different cell types (see Fig 1.1).

Amongst the first alloantigens found to be present on T cells were those of the Ly series (Sec 1 B ii). Their presence on T_h cells was investigated by Feldmann, Beverley and Dunkley (1975) who showed that the in vitro production of anti-keyhole limper haemocyanin (KLH) antibody (determined by a plaque forming cell (PFC) assay) could be abrogated by treating the cells with complement and anti-Ly 1 antiserum. Anti-Ly 2 antiserum had no effect, thus suggesting that T_h cells are Ly 1^+2^-. These results were confirmed by Cantor, Shen and Boyse (1976) using sheep red blood cells (SRBC) as antigen. In addition, they showed that after antigenic challenge, the development of T cell help was favoured by the presence of 'high responder' Ir genes . They also reported the presence of a further subgroup of T cells with the phenotype Ly $1^+2^+3^+$ which they termed early appearing or immature T cells (T_e cells).

The Ly 1^+ T_h effector cells were shown to be derived from precursor cells which expressed the same phenotype as the effector cells (Feldmann et al.,1977). These workers found that the differentiation of precursors to effectors was mediated by amplifier cells which expressed the phenotype Ly$1^+2^+3^+$. These cells are probably identical to the T_e cells

Figure 1.1 A possible mechanism for T cell help in antibody production in mice



described by Cantor, Shen and Boyse (1976). Further evidence for the requirement of at least two T cell subpopulations for the expression of T cell help came from the work of Muirhead and Cudkowicz (1978). They examined T cell help in response to SRBC in (C57BL/6 x C3H/He) F1 mice and described two subpopulations involved in the reaction; T_H (which were sensitive to anti-thymocyte serum-ATS- and persisted after adult thymectomy-ATx) and T_A (resistant to ATS; short lived after ATx). T_H cells were required to initiate the anti-SRBC response whilst addition of T_A cells resulted in its exponential amplification. T_A cells exhibited the Ly $1^+2^+3^+$ phenotype. Studies using vinblastine lead the authors to suggest that T_H cells triggered unprimed B cells in conjunction with antigen whilst T_A cells increased the number of PFC produced by the triggered B cells. Tada et al.,(1978) using BALB/c and C3H/He mice also described two helper T cell populations called Th_1 and Th_2. Th_1 cells were shown to initiate antibody production by B cells and to express the phenotype Ly 1^+ Ia^-. Th_2 cells acted synergistically with Th_1, very small numbers augmenting the antibody response. Th_2 were also shown to be Ly 1^+ but Ia^+.

Thus, the data suggests that T cell help in mice is mediated by the interaction of several different T cell subsets. The effector cell appears to be Ly 1^+. However, the phenotypes of the amplifier and effector precursor cells are uncertain. Amplifier cells (T_A) with the phenotype Ly $1^+2^+3^+$ probably act upon effector precursors (Th_1) causing their differentiation into helper effector cells (Th_2).

Helper T cells in humans were found to have specific cell surface markers by Moretta et al.,(1977). These workers showed that T cells expressing Fc R helped PWM induced proliferation of B cells and suggested that T_μ and T_γ cells were responsible for help and suppression respectively. However, subsequent work (discussed in Sec.1 C i) indicated that both Fc_μ and Fc_γ could be expressed on the same cell and hence these markers

did not define functionally distinct subpopulations. Recently Endoh et al.,(1981) have shown that Tα cells help B cells to produce IgA. This suggests that T cells expressing different FcR may provide help for the production of different classes of Ig.

The recent development of monoclonal antibodies has enabled a more precise identification to be made of the T cells which provide help in a humoral response. The best documented of these antibodies are those of the OK (Ortho Pharmaceutical Comp.) series. T_h cells are contained in the OKT 4^+ subset of T cells (Reinherz and Schlossman, 1980a,b; reviews). The OKT 4^+ cells (which correspond to the TH2 subset described by Evans et al., 1978)(Reinherz et al., 1980)are present in the peripheral blood in similar proportions to the Ly 1^+ subset in mice (approximately 60% of T cells). These authors also reported that Ly 1^+ and OKT 4^+ cells responded similarly to stimulation by Con A and PHA. More recently, Reinherz et al., (1982) developed a monoclonal antibody (T Q1) which reacted with 70-85% OKT 4^+ cells, 50% OKT 4^-/OKT 8^+ cells and a fraction of B and null cells. Those T cells which provided help for PWM stimulated Ig production belonged to the small OKT 4^+ TQ 1^- subpopulation. Cortesi et al., (1982) produced a monoclonal antibody to human T cells activated in a secondary mixed lymphocyte culture (MLC). The antibody 5/9 reacted with only 15-20% peripheral T cells and about 33% null cells. These workers found that the helper activity required for PWM stimulated B cell differentiation was restricted to and highly enriched in the $5/9^+$ subpopulation of peripheral blood T cells. They also examined FcR expression in the $5/9^+$ population and found that 25% were $Fc\gamma R^+$ and 50% were $Fc\mu R^+$, again indicating that T helper cells may express $Fc\mu R$ and $Fc\gamma R$. Virtually all $5/9^+$ cells were also OKT 4^+/OKT 8^-. These results suggest that the subpopulation defined by the monoclonal antibody 5/9 is the same as the OKT 4^+ $TQ1^-$ subpopulation. A monoclonal antibody to the human T cell line HSB-2 (3A1) was found to react with 85% E^+ peripheral blood lymphocytes.

The $3A1^+$ subset of T cells helped autologous B cells to proliferate and differentiate in response to PWM stimulation (Haynes et al.,1980). However, these cells could also be stimulated by Con A to suppress *in vitro* Ig synthesis. These results suggest that the 3A1 monoclonal antibody defines a functionally heterogeneous subpopulation of T cells. Hoffman et al.,(1981) produced heteroantisera to the HSB-2 and to the Sezary cell lines. These workers found that the helper cells for PWM dependent Ig synthesis were included in the anti-Sezary $cell^+$ subpopulation (50-70%) whilst the anti-$HSB\text{-}2^+$ population (30-50%) contained the cells responsible for suppression in this system. The conflicting results produced by Haynes et al.,(1980) concerning the relationship between the expression of the HSB-2 derived antigen and T cell help may reflect the drawbacks of the use of monoclonal antibodies proposed by Lane and Koprowski (1982).

Thus T_h cells may be defined in the human system by the use of numerous monoclonal antibodies. Whether or not both helper inducer and helper effector cells exist in humans has not yet been clearly established.

Positive regulation by non-T cells

Several workers have shown that cellular proliferation and antibody production in response to antigenic or mitogenic stimulation have been greatly reduced or totally abolished by rigorous depletion of monocytes from the cultures (Pierce,1969; Shortman et al.,1970; Gehrz and Knorr, 1979; Innes et al., 1979; Eardley et al., 1979; Scott Boswell Sharrow and Singer, 1980; Abramson, Puck and Rich, 1981; Neefe, Curl and Woody, 1981; Ruscetti and Gallo, 1981; Alpert et al.,1981).Recognition of monocyte presented antigen by T cells requires the expression of Ia/DR related determinants on the monocyte (Schwartz, 1978; Cowing et al., 1978; Hodes et al.,1978; Alpert et al., 1981). A variety of other cells have been shown to perform the same functions as monocytes (reviewed by Lipsky and Kettman, 1982). These accessory cells lack specificity and are non-lymphoid (Lipsky and Kettman, 1982). However, null cells have

also been reported to perform accessory cell functions. Horwitz and Garret (1977) and Lobo(1981) showed that L cells enhanced T cell blastogenesis in response to mitogens and antigens but only in the presence of monocytes. Caraux et al.,(1982) also observed this effect but suggested that there was no requirement for monocytes. However, contaminating monocytes in the null cell population (<0.5%) may have been sufficient to mediate T cell stimulation.

Carvalho, Davis and Horwitz (1980) examined the effect (on Con A induced T cell blastogenesis) of mitomycin treatment of T cells or L cells. The results showed that proliferation of L cells is not required for them to enhance T cell blastogenesis.Null cells have been reported also to affect mitogen induced Ig production by peripheral blood lymphocytes Lobo,1981). When L cells were reacted with antibody sensitized erythrocytes, Lobo (1981) found that although blastogenesis was enhanced, Ig production was significantly reduced.In contrast, Carvalho, Davis and Horwitz (1980) found that L cells enhanced Ig synthesis when stimulated with KLH-antiKLH complexes.However, these opposing results may be due to the different stimuli used in the two reports. Thus, the data suggests that null cells are capable of performing a type of accessory role in immune responses. Indeed, the recent observation by Stavitsky (1980) of a non-T, non-B, FcR^+ cell in mice which is capable of presenting Ag to reactive lymphocytes would agree with this idea.

Soluble helper factors

Whilst it has been known for many years that T cells participate in humoral immune responses, the precise nature of the helper factors involved is unclear. Feldmann and Basten (1972) and Feldmann (1972) showed that soluble factors were produced by T cells which in the presence of monocytes, enabled B cells to produce an antigen specific response.Two types of helper factors (HF) have been defined: i) non-specific HF (produced by allogeneic stimulation of T cells; Schimpl and

Wecker, 1972; Amerding and Katz, 1974); ii) antigen specific HF (Feldmann 1972; Taussig et al ., 1975; Zvaifler et al., 1979).

Accessory cells also produce soluble factors which enhance both T and B cell proliferation. Lymphocyte activating factor (LAF) or interleukin 1 (IL1) is produced by monocytes. It is generally assayed by its affect on T cell activation (Gery et al., 1972) although it has not been demonstrated that it acts directly on T cells. A molecule similar to LAF, BAF (B cell activating factor) has been shown to play a role in the activation of B cells to IgM production (Schrader, 1980). It also influences B cells to respond to an amplifying signal (T cell replacing factor - TRF) produced by activated T cells (Schimpl and Wecker, 1975; Schrader et al, 1980). The functions of both LAF and BAF are probably fulfilled by IL1. The latter is vital for the production of IL2 (T cell growth factor - TCGF) a soluble factor released by a subpopulation of T cells (on stimulation by IL1) which initiates the proliferation of other T cell subsets (Ruscetti and Gallo, 1981).

Null cell mediated help is also thought to occur through the agency of soluble factors. Caraux et al., (1972) showed (using an agar layer technique) that Con A stimulated cells produced soluble factors which enhanced T cell blastogenesis.

Thus, enhancement of the humoral response appears to depend upon a complex interaction of T cell subsets, B cells, null cells, accessory cells and the soluble factors produced by them.

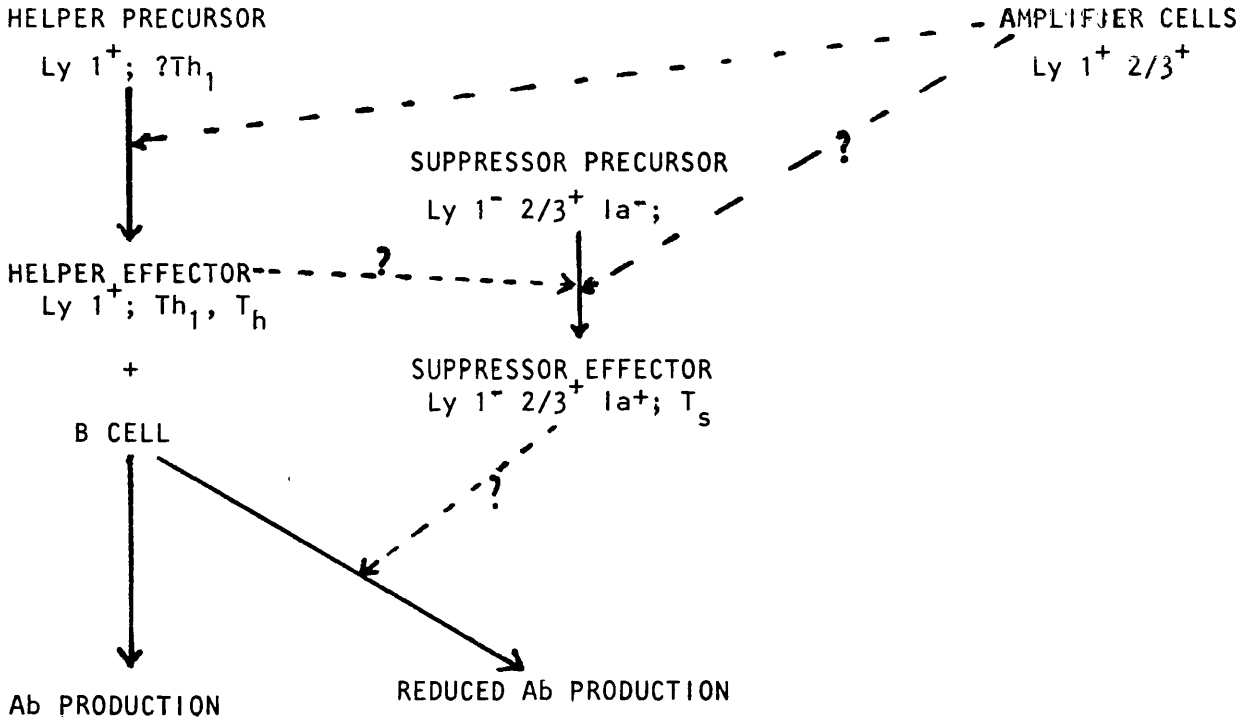
ii) Negative regulation of the immune response (suppression)

The complex interaction of different cell populations required to augment Ab production has also been shown to be required for its suppression. Using an antigen (KLH) stimulated system, Feldmann, Beverley and Dunkeley (1975) showed that suppression of Ab production was mediated by a lymphocyte expressing the Ly 2^+ phenotype. This was confirmed and extended by

Beverley et al., (1976) who showed that antigen stimulated T suppressor (Ts) cells have the phenotype Ly $1^-2^+3^+Ia^+$. Mitogen activated Ts cells were also shown to express the Ly 2/3 antigenic complex (Jandinski et al., 1976). Herzenberg et al., (1976) found that the development of T cell mediated suppression was dependent on a Ly 1^+ T cell. Eardley et al., (1978;1979) using an SRBC stimulated system, later showed that $Ly1^+$ cells induced suppressor activity in a very small proportion of the resting Ly $1^+2/3^+$ subpopulation of T cells. In contrast, Feldmann et al., (1977) using a KLH stimulated system, found that the cells effecting suppression were Ly $2/3^+Ia^+$ and differentiated from Ly $2/3^+Ia^-$ precursor cells after interaction with Ly $1^+2/3^+Ia^-$ amplifier cells. A recent review of T cell mediated suppression has suggested that the phenotype of T suppressor cells depends upon the stimuli used to induce them (Germain and Benacerraf, 1981). This may explain the contrasting results quoted above. A possible mechanism of Ag specific suppression of Ab production is outlined in Figure 1.2.

Suppressor cells have also been identified in the human system. Shou, Schwartz and Good (1976) showed that preincubation of cells with Con A, generated suppressor cells which inhibited mitogen or antigen stimulated blastogenesis in cultures of fresh cells. The degree of suppression observed in such cultures was found to partially correlate with an observed increase in T_γ cells in the Con A stimulated population (Gupta, Schwartz and Good, 1979). Moretta et al., (1977) reported that T_γ cells could suppress PWM induced blastogenesis after preincubation with immune complexes. Thus, Ts cells were thought to invariantly express $Fc_\gamma R$. However, as discussed previously (Section 1 C ii), this was found to be untrue and other methods of cell subset identification were sought. Reinherz et al., (1980 a) immunized CAF_1/J mice with human thymocytes and fused the spleen cells with P3 x 63 Ag 8UI myeloma cells. The resulting monoclonal antibody -OKT 5- reacted with those peripheral blood T cells

Figure 1.2 A possible mechanism for T cell suppression in antibody production in mice



(20%) which formed the $TH2^+$ subset described by Evans et al., (1978;1979). The OKT 5^+ cells were found to exhibit suppressor activity after stimulation by Con A. Also Morimoto, Reinherz and Schlossman (1981) found that the monoclonal antibody OKT 8 reacted with Ts cells induced in cultures stimulated with KLH. More recently, Thomas et al., (1982) reported the generation in a PWM stimulated system of suppressor cells with the phenotype OKT $4^+/17^+$. The monoclonal antibody OKT17 reacted with the majority of resting normal peripheral blood T cells. Upon stimulation with PWM, radiosensitive helper cells were found to lose their reactivity with OKT 17 whilst suppressor cells and radio-resistant helper cells did not. Morimoto et al., (1982) showed that suppression during the induction of a primary in vitro anti-DNP response required both OKT 4^+ and OKT $5/8^+$ cells. After induction of the response, only OKT $5/8^+$ cells were required. This may explain the contrasting results of Thomas et al.,(1982) and Morimoto, Reinherz and Schlossman (1981). T cells expressing an antigen reactive with the monoclonal antibody 3A1 shown by Haynes et al., (1980) to enhance Ig production in a PWM stimulated system, were also found to suppress Ig synthesis when stimulated with Con A. In contrast, a hetero-antiserum to HSB-2 (3A1 is a monoclonal antibody produced to HSB-2) reacted with only suppressor cells (Hoffman et al., 1981).

Thus, T cell mediated suppression of Ig synthesis involves the interaction of two or more T cell subsets. However, other cells have been implicated in this phenomenon and these will be discussed, briefly, below.

Other cells involved in the suppression of Ig synthesis.

The role of monocytes/macrophages in the development of T cell mediated suppression is contentious. Feldmann et al., (1977) using a KLH stimulated system suggested that monocytes were not important. However, Innes et al., (1979) using a Con A stimulated system showed that extensive depletion of monocytes inhibited the development of Ts cells. This anomaly

may result from the problems of total moncyte depletion or from the different methods of stimulation used. Tsokos and Balow (1981) showed that suppression induced in PWM stimulated cultured was mediated by a soluble factor, the production of which was abolished by monocyte depletion.

Null cells have also been implicated as mediators of suppression. Workers have shown that L cells could be stimulated by mitogens to produce soluble factors which could enhance the proliferation of T cells in the culture (Carvalho, Davis and Horwitz, 1980; Caraux et al., 1982). Also, Lobo (1981) showed that production of Ig was significantly reduced when L cells were reacted with antibody sensitized erythrocytes prior to mitogen stimulation.

Thus, monocytes and null cells both play a part in suppression of Ab production. B cells have also been implicated in this phenomenon. Shimamura, Hashimoto and Sasaki, (1982) showed that the adoptive transfer of SRBC primed splenic B lymphocytes resulted in the suppression of Ag specific Ab synthesis in the recipient mice. Cell division and protein synthesis were not required for expression of this effect, neither were soluble factors. The authors suggested that Ts cells were activated by immune B cells in the presence of Ag in the recipients. However, the methods used for the isolation of the transferred B cells did not exclude the possibility that the effect may have been mediated by null cells.

Soluble factors mediating suppression

Many workers have reported the existence of soluble immune response suppressor factors (SIRS; Rich and Pierce, 1974; Tadakuma and Pierce, 1976; 1978; Kontiainen, Todd and Feldmann, 1982). Tadakuma and Pierce (1978) showed that SIRS were produced by Con A activated Ly $2/3^+$ T cells and mediated suppression by their effect on monocytes which limited B cell proliferation to both T dependent and T independent antigens. Kontiainen, Todd and Feldmann (1982) showed that SIRS produced *in vitro* could mediate suppression *in vivo*. Suppression was not the result of altered kinetics

but was due to a decrease in peak Ig production.

Thus, the work presented in this section shows that regulation of the humoral immune response is a complex phenomenon involving the actions of T, B and null lymphocytes and various accessory cells.

(C) Cell mediated immune responses.

The cell mediated branch of the immune response may be divided into delayed-type hypersensitivity reactions (DTH; Type 1 hypersensitivity reaction) and the responses involving cytotoxic cells (Type 11 hypersensitivity reaction). The cellular events involved in these reactions are complex and have been extensively reviewed. It is not intended to discuss this topic at any length in this section. However, since null cells have been shown to be involved in cytotoxic reactions, their role in cell mediated immune responses will be discussed, briefly, below.

i) The role of null cells in cytotoxic reactions

The best documented function of null cells is their activity in natural killer (NK; Heberman et al., 1979) and antibody dependent cellular cytotoxicity (ADCC; Perlmann, Perlmann and Wigzell, 1972) reactions, although there is some controversy over the true nature of the effector cells in these reactions. Brier, Chess and Schlossman (1975) showed that the effector cells in ADCC activity against Ab coated autologous lymphocytes were SIg^-, lacked receptors for SRBC but did possess receptors for complement. They also showed that ADCC depends on the presence on the effector cells (K cells) of Fc R. These results were confirmed by Kiessling et al., (1976) and Perlmann et al., (1976). However, other workers have reported null cell populations depleted of C_3R^+ cells which could still exhibit ADCC activity (Horwitz and Garrett, 1977; Despont et al., 1981). HLA-DR related antigens (Ia-like) have not been demonstrated on K cells. Thus, there does not appear to be any MHC restriction governing K cell activity (in contrast to cytotoxic T cells; Ozer et al., 1979; Ng et al., 1980; Despont et al., 1981).

NK activity (cytolysis which is not dependent on the presence of sensitizing antibody on the target cell) has also been demonstrated to be concentrated in the null cell fraction (Kiessling et al., 1976; Ozer et al., 1979; Ng et al., 1980; Despont et al., 1981). Kiessling et al.,(1976) showed that these cells do not possess C_3R whilst Pape, Troye and Perlmann (1979) demonstrated that they are FcR^+. These results, plus the suggestion by Ozer et al.,(1979) that K and NK activities are mediated by the same cell would also imply that K cells are C_3R^-. Although the evidence for NK and K cell activity in null cells is convincing, occasional reports have suggested that these functions are also mediated by T cells. (Pape, Troye and Perlmann, 1979; Johnsen and Madsen, 1979). Pape, Troye and Perlmann (1979) measured NK activity in cell populations which bound to (HP^+;T cells) or were eluted from (HP^-;non-T cells) Helix pomatia lectin columns. Both populations lysed K562 target cells and the effectors were Fc R^+. Johnsen and Madsen (1979) showed that both T and null cells could mediate ADCC. The two effector cells showed different dose response curves and the authors suggested that the target cell may determine which population exhibits cytotoxic activity. Thus, these results indicate that cytotoxicity is a complex phenomenon mediated by a variety of lymphocyte subpopulations.

(D) The role of the Fc receptor in immune regulation

Receptors which bind the Fc portion of all classes of Ig have been demonstrated on a variety of cell types, in several different species. Comprehensive reviews have been published concerning the specificity and distribution of these receptors (Dickler, 1976; Unkeless, Fleit and Mellman, 1981) and this subject will be discussed below.

i) Receptors for IgM, IgA, IgD and IgE

Receptors for IgE,A and M have not been demonstrated on human monocytes/macrophages (Hellstrom and Spiegelberg, 1979; Lawrence, Wiegle and Spiegelberg, 1975). However, macrophage binding of IgA,E and M has been

demonstrated in other species (Dorrington, 1977;Boltz-Nitulescu et al., 1981).

Neutrophils have been shown to bind IgA but not IgM,D orE (Henson, 1977), whilst basophils and mast cells have been shown to express receptors for IgE (Dickler, 1976 b; Dorrington, 1977).

Receptors for IgM on T cells have been discussed previously (Chap. 1 Sec. 1.B i; C i). Fc R have been demonstrated on both T and B cells (Lum et al., 1979; Sjoberg, 1980a). Lum et al. (1979) showed that receptors for IgA were expressed on 5-6% peripheral blood T cells and this percentage increased after overnight incubation at 37°C. Sjoberg (1980 b) confirmed these findings and reported that 10-15% peripheral blood non-T cells also expressed Fc R.The same author also showed that receptors for IgD were expressed on both peripheral blood T and non-T cells (Sjoberg, 1980 a). Helbstrom and Spiegelberg (1979) showed that a very small percentage of peripheral blood lymphocytes (1.2±0.4%) expressed receptors for IgE. T cells did not express this receptor and of those Fc R^+ B cells, 50-80% expressed SIgM and only 0-28% expressed SIgD.

Null cells, isolated using anti-rhesus antibodies, did not express receptors for IgM,IgA,IgD or IgE (Froland et al., 1974).

ii)Receptors for IgG

Work demonstrating the presence of $Fc_\gamma R$ on T, B and null cells has been discussed in the relevant preceding sections. Other cells including monocytes/macrophages (Huber and Fudenberg, 1968;Alexander et al., 1978; Barnett Foster,Dorrington and Painter, 1980;Lane et al,1980),neutrophils (Messner and Jelinek, 1970; Lawrence, Wiegle and Spiegelberg, 1975), platelets (Hawiger et al., 1979) and epithelial cells (van der Muelen et al., 1980) have all been shown to possess receptors for IgG.

Many workers have studied the specificity of the binding between IgG and the $Fc_\gamma R$. The Fc region of IgG is composed of 2 domains, the second and third constant regions on the heavy chains comprising IgG -

CH_2 and CH_3 (see Figure 2.1).Workers have shown that IgG binding by $Fc_\gamma R$ depends on regions in both these segments (Spiegelberg, 1975; Klein et al., 1977; Alexander et al., 1978; Barnett Foster, Dorrington and Painter 1980). Klein et al., (1977) showed that the region in IgG responsible for binding to FcR on activated, murine T cells was located in the CH_3 domain. However, the observation that purified CH_2 fragments (but not $F(ab')_2$ were also bound, lead the authors to suggest that stable Ig-FcR interaction was due to co-operative binding of sites in both the CH_2 and CH_3 regions.Similar results were found with guinea-pig peritoneal macrophages Alexander et al., 1978). Spiegelberg (1975) using an IgG_1 myeloma with a deletion in the CH_3 region, suggested that the binding of IgG_1 by FcR on human lymphocytes involved sites in both the CH_2 and the CH_3 domains. Thus, although it was obvious that binding in both regions was required for a stable interaction between IgG and $Fc_\gamma R$, it was not clear whether or not one site dominated the other. Ciccimara, Rosen and Merler (1975) showed that the primary site of attachment of IgG_1 and IgG_3 to the FcR on human monocytes was in the CH_3 domain of the Fc region. In contrast, the FcR on third population lymphocytes exhibited principal binding in the CH_2 region (Froland et al., 1974). However, K cell activity (which is thought to be mediated by these lymphocytes) was not inhibited by the Facb fragment of IgG (which contains the CH_2 but not the CH_3 region; MacLennan, Connell and Gotch, 1974). This discrepancy may reflect the different techniques used to detect CH_2 region binding (Fch fragments from IgG_3 in contrast to Facb) or alternatively may be evidence for FcR heterogeneity on null cells.

Several other methods have been used to examine the nature of Fc R including competition experiments employing various subclasses of IgG, heat aggregated IgG(Hagg) or immune complexes; enzymatic stripping of FcR and heat induced shedding of FcR.Huber et al., (1971), Froland et al., (1974), Alexander et al., (1978) and Hall and Bacon (1981) amongst others have shown that IgG_1 and IgG_3 are more avidly bound by mononuclear cells

than IgG_2 or IgG_4. Treatment of human peripheral blood lymphocytes with pronase or papain indicated that Fc R which bind immune complexes (either human ORh^+ erythrocytes and anti-Rh (Ripley) serum (EA_{Ri}), or chicken erythrocytes(ChRBC) and anti-ChRBC, are different to those which bind Hagg (Gormus and Woodson and Kaplan, 1978). Pronase treatment enhanced Fc R expression for EA_{Ri} whilst removing or inactivating those receptors which bound Hagg. Similar results were observed by Winchester et al., (1979) using keyhole limpet haemocyanin (KLH) anti-KLH and IgG anti-IgG complexes. Further evidence of Fc R heterogeneity was produced by Lobo and Horwitz (1976). They showed that Fc R expressed on B cells were trypsin sensitive, did not bind EA_{Ri} and binding to Hagg was variable. In contrast, L cells bound Hagg and formed EA_{Ri} and the FcR was trypsin resistant. This work was confirmed by Winfield, Lobo and Hamilton (1977) who showed that 10-15% of peripheral blood lymphocytes formed EA_{Ri} rosettes, their receptors being pronase sensitive and trypsin resistant. The FcR expressed on T and B cells were all pronase and trypsin sensitive. In contrast to this, Winchester et al., (1979) suggested that the FcR on T and null cells are similar and differ from those on B cells. Sandor et al.,(1978;1979) also demonstrated the existence of two types of Fc R based on their susceptibility to temperature shock. Thus, when cells incubated at 4^oC were transferred to 37^oC for 45 minutes, type 1 FcR (FcR 1) were shed whilst type 11 (FcR 11) were not. The FcR 1 correspond to the low avidity Fc R demonstrated on 50% $SIgM^+$ cells and approximately 10% T cells (Winfield, Lobo and Hamilton 1977).

From this brief out-line it can be seen that Fc R expressed on various cell populations are heterogeneous. Whether or not this non-uniformity reflects different functional capacities in the Fc R^+ populations is unclear. The following section will briefly discuss the possible roles of Fc R^+ cells in the regulation of humoral immunity.

iii) Fc R mediated suppression of Ig production

A role for FcR in immune regulation was implied by experiments

which demonstrated that antibody to an immunogen, when administered concurrently with, or shortly after, the immunogen, abrogated the humoral response observed with immunogen alone (Sinclair, 1969; Chan and Sinclair, 1971; Hoffman et al., 1974, Kappler, Hoffman and Dutton, 1978; Oberbarnscheidt and Kolsch, 1978). Kappler, Hoffman and Dutton (1971) showed that passively administered IgG anti-SRBC blocked the development of anti-SRBC IgM PFC in the spleens of naive mice by an Fc dependent mechanism. Since this effect may have been due to inhibition of T cell help, Hoffman and Kappler (1973) developed a system to investigate this possibility. They showed that SRBC and Burro RBC (BRBC) cross react at the T cell help level but not at the B cell level. Thus, if the effect was due to inhibition of T_H cell activity, antibody to either SRBC or BRBC would inhibit the PFC response to both immunogens (when administered concurrently). This was found not to occur (Kappler et al., 1973) implying that antibody mediated suppression was not effected by inhibition of T cell help. The effect was also shown to be antigen specific,but not determinant specific, since the anti-trinitrophemol(TNP) and the anti-SRBC response to TNP-substituted SRBC were abrogated by anti-TNP (Kappler et al., 1973; Hoffman, 1980). However, SRBC administered at the same time as TNP-SRBC could elicit an anti-SRBC response. This and other work by these authors, reviewed by Hoffman, (1980), demonstrated that this was not a 'central effect', rendering the cells unresponsive to subsequent challenge. Much of this work has been confirmed by Kolsch et al.,(1980).Hoffman and Kappler (1978) examined the effect of soluble, T cell replacing factors on immune complex mediated suppression. Using nu/nu mice (which lacked T cells), these workers showed that tumor necrosis serum (TNS) could replace T cells in an immune response. This serum contains T cell replacing factor (TRF) and a factor also produced by Con A activated macrophages (TRF-M). These factors are thought to act synergistically in recruitment of B cells (Hoffman, 1980). Immune complex mediated suppression could be reversed by

TNS but not by TRF (Oberbarnscheidt and Kolsch, 1978; Hoffman, 1980). Kolsch et al., (1980) suggested that these results indicate that binding of immune complexes to FcR inhibits the binding of TRF (since TRF and Fc appear to bind to different sites on the same molecule; the binding of one resulting in allosteric inhibition of the other) and hence results in inhibition of B cell differentiation. Alternatively, Hoffman (1980) suggested that they indicate physical impairment of T-B-accessory cell co-operation which is necessary for the release of T cell helper factors. It is feasible that this Fc dependent inhibition of B cell differentiation could be mediated by a third type of FcR^+ lymphocyte - the null cell. Indeed, Lobo (1981) has shown that L cells pretreated with EA inhibited Ig production in a PWM stimulated system by up to 50%. However, other workers have suggested that immune complex dependent suppression is mediated by FcR^+ T cells (Setcavage and Kim, 1980) or B cells (Miyama-Inaba et al., 1982). However, isolation of subpopulations in both these reports is by negative selection and therefore does not remove null cells. These studies show that low concentrations of antibody to the immunogen may mediate suppression of specific antibody production via interaction with an FcR^+ cell. However, the identity of this cell is unclear. This may, ofcourse, only be of academic interest if the observed phenomenon merely occurs in vitro or as a result of passive immunization in vivo . Thus, the results of Kolsch et al., (1980) which demonstrate that adoptively transferred, primed murine splenocytes can, when challenged, suppress the naive host cell primary response, are particularly important. A similar conclusion was drawn by Grantham and Fitch (1975) in their study with high and low dose priming in mice.

iv) FcR mediated enhancement of Ig production

Administration of immune complexes rather than antigen alone had been reported to have an enhancing effect on blastogenesis and IgG

production (Carvalho, Davis and Horwitz, 1979; Klaus, 1979; Farkas et al., 1982). Taylor (1982) has suggested that this enhancement may result from either enhanced Ag uptake by antigen presenting cells, or by possibly cross-linking Ag and Fc receptors on cells thereby leading to an enhancing effect. The stimulatory effect of immune complexes observed by Carvalho, Davis and Horwitz (1979) only occurred at sub-optimal Ag concentrations. Although the authors suggest that immune complexes containing small amounts of Ag stimulate T_H cells, they did not study the effect of immune complexes on antibody production and hence the results may be misleading. However, Klaus (1978) has shown that immune complexes are much more effective than soluble antigen in priming B cells. This effect is Fc dependent and probably demonstrates enhanced antigen presentation.

Thus, it appears that antibody to the immunogen may either enhance or suppress Ig production. Taylor (1982) has suggested that either the ratio of Ab to Ag or the isotype of the Ab may govern which effect predominates. This latter explanation has been partially confirmed by the work of Farkas et al., (1982). These authors showed that immune complexes containing IgG_{2a} enhanced antibody production whilst those containing IgG_1 or IgG_{2b} were only as effective as antigen alone.

SECTION 111

THE IMMUNOPATHOGENIC ROLE OF LYMPHOCYTES IN RHEUMATIC DISEASE

Rheumatoid arthritis (RA) is a chronic, recurrent connective tissue disease of unknown aetiology and is characterized chiefly by an inflammatory erosive polyarthritis which classically begins in the small joints of the hands and feet and progresses symmetrically in a centripetal fashion. Extra-articular manifestations include vasculitis, atrophy of the skin and muscle, subcutaneous nodules, lymphadenopathy, splenomegaly and leukopaenia. Constitutional symptoms include malaise, fever and weight loss (Fye, Moutsopoulos and Talal, 1978). In this section, the pathological changes observed in RA and a possible aetiology for this disease will be discussed briefly. The majority of this section will be concerned with a comparative description of the distribution and function of lymphocytes in RA and in health.

(A) Anatomy, Pathology and Pathogenesis

i) Structural changes in the rheumatoid joint

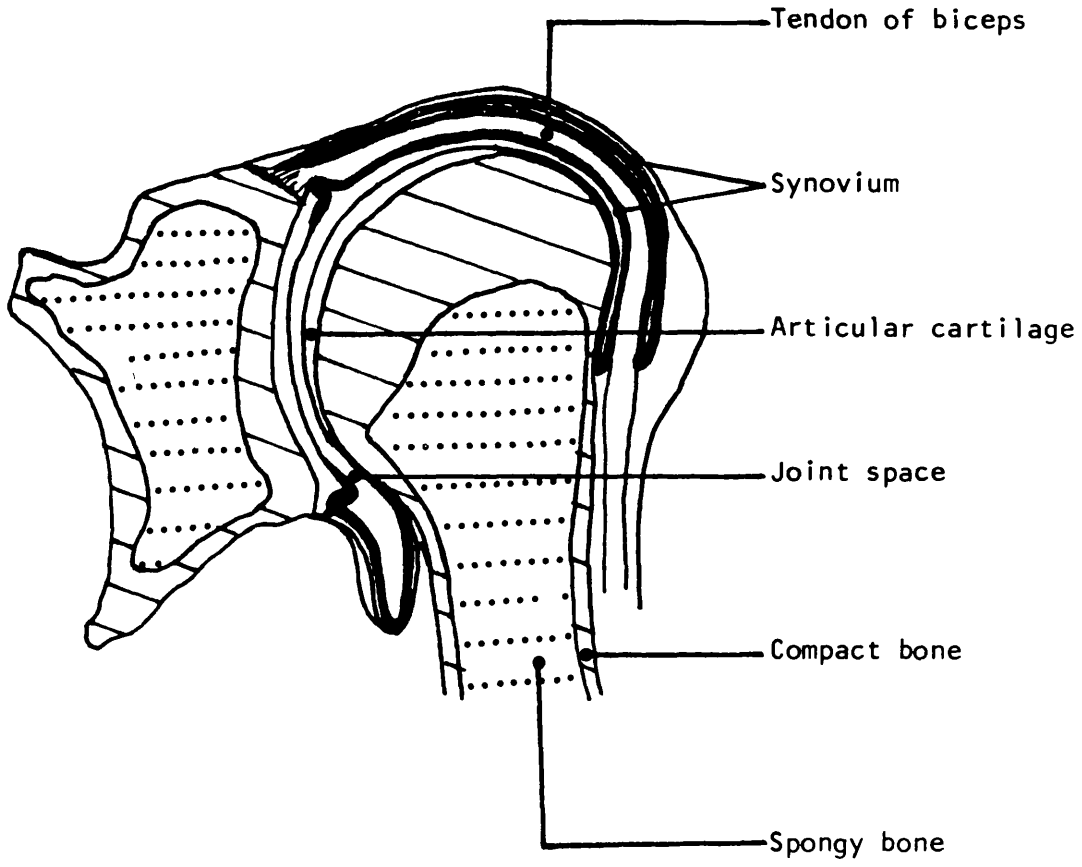
Joint deformity resulting from an inflammatory response has been described as one of the most outstanding features of RA (Glynn, 1972). The structure of a typical synovial joint is shown in Figure 1.3. The inner surface of the joint capsule and all intra-articular surfaces, except the articular cartilage, are lined by synovial tissue. The latter is comprised of an intimal layer of synoviocytes (1-4 cells thick) and a layer of subsynovial tissue which is highly vascularised and may be either adipose, fibrous or areolar in nature (Castor, 1960).

The synoviocytes are of two types: Type A cells which are the more abundant, are phagocytic and probably of myelo-monocytic lineage; Type B cells which are fibroblastic and characterised by highly conspicuous rough endoplasmic reticulum (Chayen and Bitensky, 1971; Zvaifler, 1973). The functions of these cells include secretion of hyaluronic acid, removal

Fig. 1.3 Diagrammatic representation of a normal synovial joint.

The figure shows a diagram of a normal shoulder joint.

DIAGRAMMATIC REPRESENTATION OF A NORMAL SYNOVIAL JOINT



of particulate matter from the joint cavity and mediation in the activation of the fibrinolytic system - an activity which is thought to prevent fibrin accumulation in the normal joint (Zvaifler,1973; Fell,1978).

The articular manifestations of RA are characterised by a proliferative lesion of the synovium which is associated with (and probably caused by) localised inflammation (Harris, 1974 b). The synovial cells reproduce rapidly becoming organised into a polarised, invasive front - the pannus.The latter is comprised of an excess of connective tissue, small blood vessels, mesenchymal cells (with both synthetic and phagocytic functions) and monocytes (Harris, 1976; Harvey, 1978). It is the pannus which is thought to be responsible for the cartilage and bone destrcution observed in severe cases of RA.

In the healthy joint, the cartilage appears smooth and shiny. Articular cartilage is hypocellular. Those cells which are present (chondrocytes) are embedded in a matrix consisting of water electrostatically bound by proteoglycans enmeshed in a network of collagen fibrils. It is the hydrostatic pressure of this water which gives the cartilage its load bearing properties (Harris, 1972; Ghadially, 1981).In rheumatoids, cartilage has been shown to be subject to proteoglycan depletion shortly after the onset of synovitis as a result of several processes including i) the release of proteases and glycosidases from Type A synovial cells (Hamerman,et al,1967).ii) the release of similar enzymes by chondrocytes activated by the inflammatory process (Millroy, 1974); iii) the release of proteolytic enzymes from polymorphonuclear leukocytes and subsequent intracellular digestion of solubilised proteoglycans within phagolysosomes of polymorphonuclear phagocytes and monocytes (Weissman, 1972). Depletion of proteoglycan from cartilage impairs its ability to rebound after removal of a deforming load (Harris, 1972). However, permanent joint destruction only occurs as a result of collagen loss (Harris, 1970; 1974 a,b,c).

Cartilage is not bounded by a limiting membrane and therefore there is no physical barrier to delineate where cartilage ends and synovial fluid begins. Thus, the latter is defined chemically by the absence of collagen (Hadler, 1981). The normal joint contains approximately 2ml of fluid which does not clot due to the absence of fibrinogen, although all the elements of the fibrinolytic system are present (Zvaifler, 1973; Swinson and Swinburn, 1980). The fluid is rendered viscous by the presence of macromolecules such as hyaluronate (a glycosaminoglycan) which is thought to be continuous with that of the cartilage (Balazs et al., 1966; Hadler, 1981). Striking changes occur in the synovial fluid of the rheumatoid joint. The volume may be greatly increased, reaching levels as high as 400ml. The composition of rheumatoid synovial fluid also differs from that of the healthy joint. The most obvious anomalies are its ability to clot (Zvaifler, 1973) and the presence of vast amounts of fibrin within the joint space (Jasani, 1979). This protein may comprise as much as 34% of the total synovial fluid volume and may be responsible for the development of the classic articular manifestations of RA (Jasani, 1979). Other biochemical changes include grossly elevated protein and lipid levels and depressed haemolytic complement activity. This latter observation is thought to be the result of *in situ* binding of complement to immune complexes (Britton and Schur, 1971) and subsequent activation of other complement components.

The cellular composition of the synovial fluid is quite diverse. The majority of the cells are polymorphonuclear granulocytes (75-90%) the remainder being comprised of 5-10% lymphocytes, some monocytes, macrophages and synovial lining cells (Zvaifler, 1973). All of these cells may be involved in the pathogenesis of RA. Paulus et al.,(1977) showed that thoracic duct drainage of patients with RA produced clinical remission whilst reinfusion of these cells resulted in exacerbation of disease activity. This provided direct evidence of a role for lymphocytes

in the pathogenesis of the disease.

ii)Humoral immunity in RA

The most striking serological feature of RA is the presence of anti-immunoglobulin G antibody (rheumatoid factor, RF). The existence of RF has long been known (Waaler, 1940; Rose et al., 1948) and its nature and immunopathogenic significance has been extensively reviewed (Johnson and Falk, 1976; Panayi, 1977; Ziff, 1980). It has been shown that anti-IgG activity may be present in the IgM, IgG or IgA classes of serum antibody (Franklin, 1957; Kunkel, 1961; Johnson and Falk, 1976). Although the presence of immune complexes containing RF has been demonstrated in numerous tissues in the rheumatoid patient, this phenomenon is not restricted to RA since such immune complexes have been found in other inflammatory conditions (Britton and Schur, 1971; Zvaifler, 1973; Hallgren and Wilde, 1976; Lurhama et al., 1976; Zubler et al., 1976). Lambert and Casali (1978) suggested that the presence of immune complexes in the tissues or blood does not imply that they have a pathogenic role in the disease. However, the vascular lesions associated with RA have been shown to be closely linked with the deposition of immune complexes in the vessel walls, (Zvaifler, 1973).

The synovium in rheumatoid patients contains cells which actively secrete immunoglobulin. Natvig and Munthe (1975) showed that approximately 50% of the plasma cells in the rheumatoid joint were secreting IgG. Also Smiley et al., (1968) showed that the rheumatoid synovial membrane mainly synthesized IgG; RF-IgG complexes being formed intracellularly and subsequently secreted. Such complexes are able to fix complement (Bianco et al., 1974; Tanimoto et al., 1975; Taylor-Upsahl et al., 1976) and this ability appears to explain the extensive activation of the complement system observed in the rheumatoid joint (Hurd et al., 1977).

A variety of other autoantibodies have been reported in patients

with RA. Alspaugh and Tan (1976) found that 67% of seropositive rheumatoid patients possessed an antibody (RA precipitin;RAP) which reacted with an antigen expressed on a human B cell line (WIL-2) transformed by EBV. This antigen was termed rheumatoid arthritis associated nuclear antigen (RANA). However, other workers have reported the presence of this antibody in healthy controls previously infected by EBV (Catalano et al., 1980). Thus the presence of anti-RANA antibody is not specific to RA. Anti-nuclear antibody (ANA), originally demonstrated in patients with systemic lupus erythematosus, is also present in the serum of patients with RA. Grennan et al., (1977) showed that rheumatoid patients with ANAs to double stranded DNA were more likely to have severe disease and extra-articular complications than those patients without these antibodies. Other autoantibodies have been described which are directed against tissue components such as collagen (Menzel et al.,1978; Clague et al., 1981). Anti-collagen antibodies have been detected in both rheumatoid serum and synovial fluid and collagenase activity has been shown to be present in the synovial membrane and fluid of patients with RA (Steffan.,1980). The relevance of the presence of these autoantibodies is unclear. Their role in the initiation and/or perpetuation of the inflammatory process in RA is a contentious issue and is clearly a long way from being resolved.

iii)Cell mediated immunity in RA

The leukocyte migration inhibition test (LMT; originally developed by David et al., 1964) has been extensively used to assay cellular responses to antigens. Using such a test, Bacon et al., (1973) and Robinson and Muirden (1980) have shown that rheumatoid cells respond specifically to synovial fluid eluted antigen. However, Morgan et al., (1980) using a larger population found this effect to be non-specific.

Patients with RA have also been reported to respond abnormally to autologous IgG (Weisbart, Bluestone and Goldberg, 1975). This aberration

was greater if the IgG was altered in some way (eg., by heat aggregation Froland and Gaarder, 1971; Hall, 1978).

Other measures of cell mediated immunity such as cytotoxicity and mitogen responsiveness are discussed in subsequent sections which describe the functions of discrete lymphocyte subpopulations in RA.

(B) Aetiology

Workers have tried for many years to produce convincing evidence that bacteria, mycoplasmas and viruses play an important role in the development of rheumatoid arthritis (RA). Bacteria are known to be the causative agents in reactive arthritis - an explosive, monoarthritis occuring as a consequence of a distal infection, which may be resolved by chemotherapy designed to eliminate the precipitating infectious agent. This subject has been extensively reviewed by Munoz (1978), Wolski (1978) and Goldberg and Cohen (1978 a,b) amongst others.

A more attractive candidate for the causative agent of RA is a virus since the reactive arthritis caused by viruses mimics the symptoms of RA. Onset is characterized by fever and malaise, the arthritis is usually symmetrical and affects the same spectrum of joints as RA. The major difference between the two disease states is that reactive arthritis is transitory and self-limiting (van Sauter and Utsinger, 1978). One of the chief viruses implicated as an aetiological agent for RA is the Epstein-Barr virus (EBV). As discussed previously, patients with RA have been shown to produce anti-RANA antibody. Alspaugh et al.,(1978) showed that RANA and EBV associated nuclear antigen (EBNA) were always expressed together on the same cell and suggested that the presence of anti-RANA Ab resulted from an immune response to EBV infected lymphocytes. Thus indicating that patients with anti-RANA antibody are immune to EBV. Rickinson, Wallace and Epstein (1980) have shown that EBV infects B cells and remains latent within these cells due to the controlling effect of specific anti-EBV T lymphocytes. Tosato, Steinberg and Blaese (1981) showed

that Ig production by EBV immune B cells stimulated in vitro by EBV was inhibited after 12 days by T cells present in the culture. This effect was not observed when lymphocytes from from non-immune individuals were used. When cells from patients with RA were used, the suppression did not occur despite evidence of sensitivity to EBV.These results suggest that the production of autoantibodies in RA may be due to a defect in T cell suppression of EBV stimulated Ig production. However, if this were true, one might expect this to result in the generation of whole, infective EBV virions and thus to increased titres of antibody to structural antigens of the virion such as viral capsid antigen (VCA). No such increase has been observed (Philips et al.,1976). Thus, the role of EBV in the pathogenesis of RA remains a highly contentious issue, with supporters both for (Vaughan, 1979; Fudenberg, 1980) and against (Venables et al.,1981; Silverman and Schumacher, 1981) the hypothesis that EBV is an important aetiological factor in the development of RA.

Genetic factors

The importance of the influence of genetics on the aetiology and pathogenesis of RA has recently been realised. The impetus for the current interest in this topic stemmed, initially, from the work of Brewerton et al (1973) who demonstrated a strong association between ankylosing spondylitis and the presence of HLA-B27. Improvements in the techniques available for tissue and serological typing have facilitated the accumulation of data concerning the genetically determined phenotype of rheumatoid patients. Two recent reviews cover this subject quite succinctly (Miller and Glass, 1981; Svedgaard et al., 1981). The more widely documented associations of RA and the products of the MHC will be discussed, briefly, below.

Several groups have observed a high relative risk of predisposition to RA in HLA-DRw 4^+ compared to DRw 4^- individuals (Roitt et al., 1978; Doubloug, Forre and Thorsby, 1979; Karr et al., 1980; Dallest and Roux,

1980; Scherak, Smolen and Mayr, 1980; Llorca et al., 1981). This association has been reported to be more significant in men than in women (Mercier, Dallest and Roux, 1980). The expression of DRw 4 has been shown by some workers to relate to the severity of the disease (Roitt et al.,1978) although not by others (Scherak, Smollen and Mayr, 1980; Llorca et al., 1981). Llorca et al., (1981) have shown an association between the presence of DRw 4 and rheumatoid factor. Similar observations were made by Dobloug, Forre and Thorsby (1979) who suggested that DRw 4, or a closely associated gene product, played a role in the pathogenesis of sero positive RA.

Other DR antigens have been reported as slightly increased (DRw 1, 7; Mercier, Dallest and Roux, 1980) or slightly decreased (DRw 3,2; Mercier, Dallest and Roux, 1980; Karr et al., 1980; Scherak, Smollen and Mayr, 1980) in RA. However, none of these variations is statistically significant. Thus, although the pathological changes occuring in RA are well documented, the aetiology and genetics of the disease are unclear.

(C) Lymphocyte subpopulations in RA

Any study of lymphocyte activity is fraught with problems owing to the genetic diversity of the human population. Such a study in patients with rheumatoid disease is even more complex because of the spectrum of overlapping syndroms characteristic of the disease, which impedes a strict categorization of the patients. These problems are highlighted by the conflicting experimental data concerning lymphocyte activity in rheumatoid patients.Despite these drawbacks, a wealth of data has accumulated on lymphocyte subpopulations in RA and this section will attempt to summarise the currently accepted views on this subject.

i)T lymphocytes

The level of T lymphocytes in the peripheral blood had been shown to

be the same in both control subjects and patients with RA (Micheli and Bron, 1974; Winchester et al., 1974; Horwitz and Juul-Nielsen, 1977; Slavin and Strober, 1981). However, there are reports of both reduced (Burmester et al., 1978) and increased (Froland, Natvig and Husby, 1973; Froland, Natvig and Wisloff, 1975) numbers. This discrepancy probably arises because Burmester et al., (1978) considered total cell numbers rather than relative percentages, and all his patients exhibited significant lymphocytopaenia.

In rheumatoid synovial fluid, the lymphocytes are predominantly T cells and these are present in significantly higher numbers than in the peripheral blood of either patients with RA or of controls (Froland, Natvig and Husby, 1973; Winchester et al., 1974; Sheldon, Papamichail and Holborow, 1974; Froland, Natvig and Wisloff, 1975; van de Putte et al., 1976). Similarly, T cells are thought to be the predominant type of lymphocyte in rheumatoid synovial tissue (Abrahamsen et al., 1975; Bankhurst, Husby and Williams, 1976).

T cells expressing a receptor for the Fc portion of IgG (T_γ; putative suppressor cells) have been reported to be present in normal numbers in the peripheral blood of patients with RA (Meijer et al., 1980 a; Pfreundschuh et al., 1980; Dobloug et al., 1981; Mathieu, Mereu and Pisano, 1981). T cells with Fc receptors for IgM (T_μ; putative helper cells) have been reported to be present in normal (Dobloug et al., 1981) increased (Pfreundschuh et al., 1980) or decreased (Meijer et al., 1980 a) numbers. Meijer et al., (1980 a) showed a correlation between low T_μ numbers and disease activity in rheumatoid patients. In the synovial fluid the percentage of T_γ cells in RA is significantly lower than that in control subjects (Mathieu, Mereu and Pisano, 1981).

T cells expressing HLA-DR associated antigens have been reported to be significantly raised in the synovial fluid and tissues when compared with the peripheral blood of patients with RA (Burmester et al., 1981). It

is not known whether this is due to increased expression of HLA-DR *in situ* or whether it reflects a specific accumulation of $HLA\text{-}DR^+$ cells.

ii)B lymphocytes

The peripheral blood of patients with RA has been shown to contain a similar proportion of surface Ig positive (SIg^+) lymphocytes to that of healthy control subjects (Mellbye et al., 1972; Sheldon, Papamichael and Holborow, 1974; Froland, Natvig and Wisloff, 1975; Horwitz and Juul-Nielsen, 1977; Burmester et al., 1978; Meijer et al., 1980 a; Slavin and Strober, 1981) However, patients in remission have been reported to have significantly raised numbers of SIg^+ lymphocytes in the peripheral blood (Froebel et al., 1979). In contrast, SIg^+ lymphocytes have been found to be reduced in number in rheumatoid synovial fluid compared with the peripheral blood (Froland, Natvig and Husby, 1973; Sheldon, Papamichael and Holborow, 1974; Winchester et al., 1974; Froland, Natvig and Wisloff 1975; van de Putte et al., 1976). Occasional groups have reported no significant differences (Vernon-Roberts, Currey and Perrin, 1974; Burmester et al., 1978). Low numbers of SIg^+ lymphocytes have also been reported in synovial tissue (Wangel and Klockars, 1977; Abrahamsen, Froland, Natvig and Pahle, 1976; Meijer et al., 1980 b).

A few groups have looked at complement receptors as a marker for B lymphocytes. Brenner, Scheinberg and Cathcart (1975) and Burmester et al., (1978) have reported normal levels of complement receptor positive cells in the peripheral blood and reduced levels in the synovial fluid of patients with RA. However, Mellbye et al., (1972) reported the reverse findings.

iii)Null cells

The problems concerning the terminology used to describe those cells which lack the conventional markers for either T or B cells have already been discussed (Sec.1 C iii). Thus, it is proposed that the term null cell

be used when considering this population. Null cells have been found in normal proportions in the peripheral blood of patients with RA (Froland, Natvig and Wisloff, 1975; Horwitz and Juul-Nielsen, 1977). However, since null cells are thought to be a heterogeneous group, other workers have looked at those cells with 'high avidity' receptors for IgG, assuming this to be a more reliable marker for null cells.

iv) Fc receptor bearing lymphocytes

Initial reports of differences between Fc receptor bearing lymphocytes in patients with RA and control subjects were based on results obtained using antibody sensitized human (O Rh^+) erythrocytes. These rheumatoid rosettes were found to be increased in the peripheral blood of patients with RA, especially in early disease (Bach, Delrieu, and Delbarre, 1970). The lymphocytes forming these rosettes were found to be neither T nor B cells since SRBC-rosette depletion and filtration through nylon fibre columns increased the number of rosetting cells (Sany et al., 1975). This increase in Fc R^+ lymphocytes in RA was confirmed by Scherak et al (1976) and Sharpin and Wilson (1977 a). However, several groups have been unable to detect any difference between the incidence of Fc R^+ lymphocytes in RA and control peripheral blood (Durance, Micheli and Fallet, 1974; Burmester et al., 1978; Froebel et al., 1979). Using heat aggregated, radiolabelled IgG, Wooley and Panayi (1978) demonstrated increased binding by cells from patients with RA compared with control cells. These workers suggested this might be due to either increased avidity or an increased number of Fc R per cell in rheumatoid patients.

Froland, Natvig and Wisloff (1975) and Dobloug et al., (1981) have used erythrocytes sensitised with Ripley antibody to detect Fc R on third population (null) cells. They were unable to show any differences in the binding capacity of rheumatoid and control lymphocytes. Similarly, Horwitz and Juul-Nielsen (1977) showed that L cells (lymphocytes with

Fc R capable of passively absorbing IgG) were present in normal percentages in rheumatoid peripheral blood. However, absolute numbers of L cells were depressed.

A novel assay for FcγR (the capacity to bind calf erythrocytes sensitized with the Facb fragment of IgG) was described by Hall, Winrow and Bacon (1980). These workers showed that Facb R^+ lymphocytes were present in increased percentages in the peripheral blood of patients with RA (but not in patients with other arthritides) when compared with healthy controls. These cells have been identified as null cells (Winrow 1982).

Reports of Fc R^+ lymphocytes in the synovial fluid in RA, suggest that they are present at a level comparable to that in the peripheral blood (Burmester et al., 1978). Similar observations were made for third population (null) cells (Froland, Natvig and Wisloff, 1975; Abrahamsen, 1981). Vernon-Roberts, Currey and Perrin (1974) reported significantly reduced numbers of null cells in rheumatoid synovial fluid. In contrast, using binding of aggregated IgG as a measure of Fc R, Winchester et al., (1974) showed the presence of high levels of these cells.

v)Problems associated with cell number determination

The preceding data highlight the problems involved in determining cell numbers. The main problem arises from trying to compare data obtained using different experimental techniques. Thus, T cell estimates may be greatly enhanced using neuraminidase treated or aminoethylisothiouronium bromide (AET) treated sheep erythrocytes. It has been suggested that T cell estimates using untreated sheep cells fail to include 'late' rosetting cells (Sharpin and Wilson, 1977 a; Chow and Kaplan, 1976).

Detection of B cells by the presence of SIg presents even more problems. Many estimates have failed to allow for Fc receptor bound Ig and immune complexes, cell bound lymphocytotoxic antibodies (often

present in rheumatoid sera; Meijer et al., 1982) and the problem of cell bound rheumatoid factor.

The paucity of B cells detected by SIg in synovial tissue may be due to blast transformation and concomitant loss of SIg. This seems likely since there is evidence of B cell activity in synovial tissue, for example the production of rheumatoid factor (Kobayashi and Ziff, 1973). Also, there are reports of high null cell levels in the synovium which may be plasma cells (Tannenbaum et al., 1975; Meijer et al., 1980 b). The low levels of B cells in the synovial fluid perhaps lend weight to the argument that there are relatively few B cells in the synovium. However, this has been opposed by the suggestion that migration of B cells to the synovial fluid is not favoured by the local physiological conditions and that the environment encourages the selective localization of T cells in the synovial fluid (Froland and Abrahamsen,1979).Credence is given to this idea by the observations of Fryden (1977) and Manconi et al.,(1978) who have shown that T cells are the predominant type of lymphocyte in other extravascular fluids.

Thus, trying to draw conclusions about the relevance of altered lymphocyte subpopulations in RA is fraught with problems. Not only is it difficult to strictly identify the cells, but at the classic sites of active inflammation (the joints), it is very difficult to obtain comparative values from control (uninflammed) joints. The problems have been partly overcome by examining *in vitro* correlates of lymphocyte activity *in vivo*.

vi)Mitogen responsiveness

Most workers have shown that RA peripheral blood lymphocytes respond normally to mitogenic stimulation, although several groups have shown slightly (non-significantly) reduced responses when compared with control values (Panayi, 1973; Sheldon, Papamichael and Holborow, 1974; Burmester

et al., 1978; Meijer et al., 1980 b). Only rarely did the observed decrease reach significance (Lockshin et al., 1975; Froebel et al., 1979).

Synovial fluid lymphocytes have been shown to be less susceptible to transformation by Phytohaemagglutinin (PHA), Concanavalin A (Con A), and Pokeweed mitogen (PWM) than autologous peripheral blood lymphocytes (Stratton and Peter, 1978). However, this may be because synovial fluid lymphocytes show a higher degree of spontaneous DNA synthesis and are thus thought to be a precommitted activated population. Despite these problems, it has been shown that synovial fluid lymphocytes generally respond poorly to PHA (Panayi, 1973; Sheldon, Papamichael and Holborow, 1974; Abrahamsen, Froland and Natvig, 1978; Burmester et al., 1978; Corrigall, Panayi and Laurent, 1979). There is some controversy over the response of synovial fluid lymphocytes to Con A and PWM stimulation; both having been described as normal (Con A and PWM: Corrigall, Panayi and Laurent, 1979; PWM: Burmester et al., 1978) or reduced (Con A: Burmester et al., 1978; PWM: Abrahamsen, Froland and Natvig, 1978). Lymphocytes eluted from synovial tissue show poor blastogenic responses to PHA, Con A and PWM when compared to autologous peripheral blood lymphocytes (Abrahamsen, Froland, Natvig and Pahle, 1976; Meijer et al., 1980 b). Whether or not this reduced activity was due to the methods employed to obtain a lymphocyte suspension from synovial tissue is unclear.

Cells which have been cultured with Con A may, under the correct culture conditions, suppress mitogen induced blastogenesis in fresh cultures (Shou, Schwartz and Good, 1976). Such Con A-induced suppressor cell activity has been reported to be reduced in both the peripheral blood (Sany et al., 1979) and in the synovial tissue (Chattopadhyay et al., 1979 a) in patients with RA. Chattopadhyay et al (1979 b) also reported that lymphocytes from RA synovial tissue were unable to suppress Ig production even at high T:B cell ratios.

vii) Responses to specific antigens

Abrahamsen, Froland and Natvig (1978) compared the responses to specific antigens of lymphocytes from the synovial fluid and peripheral blood of patients with RA. The response to Candida was similar in both groups of lymphocytes. However, purified protein derivative (PPD) induced a significantly higher degree of blastogenesis in synovial fluid lymphocytes than in those from the peripheral blood. This effect of PPD has not been substantiated (Burmester et al., 1978). However, Abrahamsen, Froland Natvig and Pahle (1976) reported identical results using lymphocytes extracted from synovial tissue stimulated with Candida or PPD. Since the lymphocytes from the synovium migrate to the synovial fluid, these results need further investigation to determine whether this apparent, localized, selective enrichment of antigen sensitive cells is typical of extravascular fluids or is a feature peculiar to RA. Other reports of antigen induced lymphocyte blastogenesis in the synovial tissue have suggested that responses to varidase (streptokinase/streptodornase; SK/SD) and PPD are low in comparison to that in autologous peripheral blood (Meijer et al., 1980 b).

viii) Mitogen induced cellular cytotoxicity

No significant differences in the cytotoxic potential of PHA stimulated synovial fluid or peripheral blood lymphocytes from patients with RA were found when compared to patients with other inflammatory diseases (Corrigall and Panayi, 1978) or when compared with peripheral blood lymphocytes from healthy control subjects (Rosenberg and Currey, 1979).

ix) Spontaneous cell mediated cytotoxicity (SCMC)

SCMS appears to be similar in both normal and RA peripheral blood lymphocyte populations (Penschow and MacKay, 1980; Highton and Panayi,

1980). Burmester et al., (1978) demonstrated a reduced activity in RA peripheral blood but only at high effector:target cell ratios (100:1). In synovial fluid, SCMC is raised in comparison to the activity observed in RA and normal peripheral blood (Panayi and Corrigall, 1977; Burmester et al., 1978).

x)Antibody-dependent cellular cytotoxicity (ADCC)

A number of workers have investigated the activity of K cells (ie those responsible for ADCC) using a variety of target cells (Froland, Natvig and Wisloff, 1975; Panayi and Corrigall, 1977; Burmester et al., 1978; Cooke, Hay and Perumel, 1979; Rosenberg and Currey, 1979; Penschow and MacKay, 1980). The general conclusion to be drawn from all these results is that ADCC is unaltered in RA compared with healthy controls.

(D) Chemotherapy in rheumatoid arthritis

Most of the rheumatoid patients studied in this thesis were on non-steroidal anti-inflammatory drugs (NSAIDs) in order to avoid the problems of drug induced alterations in lymphocyte activity produced with 'second-line' therapy (eg: gold, D-penicillamine, levamisole) or immunosuppressive and steroidal drugs. NSAIDs have been shown to affect lymphocyte functions in vitro . Panayi and Corrigall (1979) showed that indomethacin enhanced PHA induced blastogenesis in peripheral blood lymphocytes but not in those from the synovial fluid. Thus, patients on indomethacin were not used for the functional studies described in this thesis. Although NSAIDs may reduce inflammation, they cannot halt the progression of the disease in those patients with aggressive, erosive RA. In such patients, the clinician must resort to second-line, immunosuppressive or steroidal drugs. The choice of second therapy is largely arbitrary, successive drugs being tried until one is found which suits the patient. Amongst the chief drugs used at the Royal National Hospital for Rheumatic Diseases in patients with aggressive disease are

D-penicillamine and hydrocortisone (a potent anti-inflammatory drug). Since these drugs have been shown to affect disease progression and inflammation, their in vivo effects on Facb R^+ cells was studied in order to determine whether or not these cells were involved in the reduction of disease activity which occurred as a result of chemotherapy. In this section, the effect of these drugs on leukocyte populations and lymphocyte function will be discussed.

i) D-Penicillamine (D-Pen)

The name 'Penicillamine' was first coined by Abraham et al., (1943) to describe an amino acid characteristically found as a degradation product of penicillin. Ritzman, Coleman and Levin (1959) demonstrated that both Penicillamine and cysteamine were capable of cleaving macroglobulins. As a result of this work, Jaffe (1962) studied the effect of Penicillamine in rheumatoid disease and showed that although intra-articular injection of D-Pen resulted in a reduction of IgM rheumatoid factor titres in the synovial fluid, it did not relieve the symptoms. Also, systemic administration reduced the serum RF titre only after several months of treatment by which time clinical improvement had already become apparent (Jaffe, 1965)

Despite pharmacological and immunological techniques developed in the recent past, the way in which D-Pen mediates anti-inflammatory activity is still obscure. However, one hypothesis currently being examined is that D-Pen acts as a scavenger of free-radicals.

Free radical scavenging

Attention has recently become focussed on the damage to connective tissue mediated by oxygen-derived free radicals such as the superoxide anion ($O_2^{\cdot-}$), the hydroxyl radical ($OH^{\cdot}$) and hydrogen peroxide (H_2O_2). Data suggest that free radicals may play a part in the development of the characteristic lesions of the rheumatoid

joint and other sites of inflammation. The superoxide radical has been shown to impede soluble collagen gelation and to cause a decrease in hyaluronic acid viscosity (reviewed by Greenwald, 1981). It has also been implicated in the suppression of activity of serum protease inhibitors (Carp, 1979). Specific in situ generation of free radicals has not been demonstrated in inflammatory foci (Greenwald, 1981) but this may be due to their high reactivity (and hence instability). Indeed, products of free radical activity have been demonstrated in vivo (Lunec et al., 1979). Thus, the evidence suggests that free radicals may be responsible for some of the inflammatory lesions observed in RA. They may be inactivated by reduction; in the case of superoxide to oxygen and hydrogen peroxide. It is this reaction (which is normally catalysed by the enzyme superoxide dismutase) which D-Pen is thought to enhance.

Cellular effects of D-Penicillamine

The effect of D-Pen on human leukocyte populations is unclear. Kendall and Hutchings (1976) have shown that D-Pen may have either an enhancing , or an inhibitory effect on tritiated thymidine incorporation in Con A stimulated cells, according to the time at which the drug is added to the culture. These workers suggested that this effect is partially due to an interaction between D-Pen and cystine which starves the cells of essential L cystine. However, Froland et al., (1976) showed that T and B lymphocytes from patients with RA treated with D-Pen were unaffected (either functionally or numerically) by this drug.

Animal models of RA have produced some contradictory results about the effect of D-Pen on inflammatory reactions. Liyanage and Currey (1972) found that the drug had no effect in adjuvant arthritis. Hunneyball, Stewart and Stanworth (1977) examined the effect of D-Pen in rabbit experimental arthritis. The animals showed significant

reduction in joint swelling after 4 - 8 weeks of therapy; post mortem examination showing less joint damage in the treated animals than the controls. These authors have suggested that the theraputic effect of D-Pen in RA results from a reduction of the delayed-type hypersensitivity response by an action on T cells. This consequently affects immunoglobulin production. However, Zilko, Dawkins and Cohen (1977) were unable to show such an effect of D-Pen on the DTH response to intradermal tuberculin in patients with RA.

Thus, although many clinical reports exist which support the theory that D-Pen halts the progression of RA (reviewed by Lyle, 1979), the precise effects of this drug on biochemical and immunological parameters of disease activity remain unclear.

ii) Hydrocortisone

Hydrocortisone (HCO) is a glucocorticoid which is produced in vivo. One of the chief clinical uses of this drug is as a potent anti-inflammatory agent. HCO has profound effects on lymphocytes and monocytes which contribute to the overall usefulness of steroid therapy. The effects have been extensively reviewed elsewhere (Fauci 1978-1979; Parrillo and Fauci, 1979; Pearson, Clements and Yu, 1979); the principal ones will be summarized (briefly) below.

Monocytes

HCO induces a monocytopaenia which peaks 4 - 6 hours after oral administration (Fauci, 1978 - 1979; Parrillo and Fauci, 1979). The mechanism involved is unclear but is thought to reflect relocation of the monocytes to the non-recirculating leukocyte pool.

Lymphocytes

HCO also induces a lymphocytopaenia which shows similar kinetics

to the observed monocytopaenia. Again, the mechanism involved is thought to be relocation. However, not all the lymphocytes show the same sensitivity to HCO. T cells are selectively depleted such that at the point of maximum effect, only 25% of the pre-HCO level remains in the circulation. In contrast, 67% of the B cells remain, (Pearson, Clements and Yu, 1979). Examination of the presence of Fc R on T cells showed that HCO treatment selectively depletes T_{μ} and Fc R^{-} T cells, both relatively and absolutely (Haynes and Fauci, 1978). The relative percentage of T_{γ} increased from 22 to 66 although no absolute increase was observed.

In vitro studies have shown that pharmacologic levels of HCO inhibit the generation of Con A induced suppressor cells (Knapp and Posch, 1980). This effect may be explained by the observation that glucocorticosteroids inhibit the production of T cell growth factor (TCGF; Gillis, Crabtree and Smith, 1979; 1980). Knapp and Posch (1980) also showed that when Con A activated cells are added to virgin cells in the presence of HCO, suppression of blastogenesis of the responder cells is markedly increased. The authors suggest that HCO inhibits proliferation of the responder cells making them more sensitive to suppression. In contrast to this, Ifeld and Krakauer (1982) have shown that HCO blocks the suppression of Ig synthesis by Con A induced soluble suppressor factors. This agrees with the in vivo observations which show that HCO administration after immunization results in higher circulating specific antibody levels (Pearson, Clements and Yu, 1979).

It is difficult to compare in vivo and in vitro responses to HCO since the cellular populations are significantly altered in vivo due to relocation. However, it is thought that HCO does increase suppressive activity in the circulating lymphocyte pool; although the importance of this effect is unclear since suppression of blastogenesis (in vitro) appears not to have a profound effect on Ig production. The alteration

in T, B and accessory cell ratios may in itself give erroneous results rather than the effect being due to the activity of HCO on cellular function.

CHAPTER 11

MATERIALS AND METHODS

SECTION 1

MATERIALS

All chemicals were obtained from the British Drug Houses Ltd., Poole, unless otherwise stated.

All media reagents were obtained from Gibco Bio-Cult Ltd., Scotland, except those listed below.

All antisera use were obtained from Sigma Chemical Co. Ltd., Poole, unless otherwise stated.

MATERIALS	SUPPLIER
Monoclonal mouse IgG anti-human monocyte antigen; ascites fluid.	Bethesda Research Labs. Inc., Gaithersburg.
Monoclonal mouse IgG anti-human HLA-DR; ascites fluid.	
Carbonyl Iron Powder (type SF)	GAF (G.B) Ltd., Wythenshawe, Manchester.
Pokeweed Mitogen (lyophilised; crude preparation from Phytolacca americana)	Gibco Bio-Cult Ltd., Paisley, Renfrewshire, Scotland.
Plasmin (human from Cohn fraction 1; lyophilised)	Kabi Vitrum Ltd., Ealing, London.
Rabbit antiserum to bovine serum albumin.	Miles Biochemicals, Stoke Poges, Slough.
Concanavalin A (Lyophilised; from the jack bean Canavalia ensiformis)	Pharmacia, (G.B) Ltd., Hounslow, Middlesex.
Ficoll 400	
Ficoll-Paque (density 1.077 +/- 0.001 g/ml)	
Percoll	
Protein A - Sepharose 4B	
DEAE Sephadex A50	
Sephadex G100 (fractionation range 4000 - 150 000 daltons	

(Methyl - ^{3}H) Thymidine Specific activity 5 Ci/mol;21mCi/mg Radioactive concentration 1μmCi/ml	Radiochemical Centre Ltd., Amersham, Bucks.
Mouse T cell function test kit (comprising anti-Thy 1.1, anti-Ly 1.2 and guinea pig complement)	Sera Lab. Ltd., Morden, Surrey.
Aminoethylisothiouronium bromide Hydrobromide (AET)	Sigma Chemical Company Ltd., Fancy Road, Poole, Dorset.
Crystal violet	
Fluorescein isthiocyanate	
Lipopolysaccharide (from Escherichia coli serotype 055;B5 and Salmonella typhosa	
Neuraminidase Type V from Clostridium perfringens	
Pepsin (hog stomach mucosa;lyophilised)	
Erythrocytes in Alsevers preservative: Adult chicken cells Calf blood (Animal Nos. 21, 24, 18) Goat blood Sheep blood	Tissue Culture Services, Slough, Berks.
Guinea pig complement in 20% Richardson's preservative	
Purified Phytohaemagglutinin (purified from Phaeseolus spp; lyophilised)	Wellcome Reagents Ltd., Beckenham, Kent.

ROUTINE BUFFERS AND MEDIA

Phosphate buffered saline (PBS)

x10 concentrate

	g/litre	
Sodium chloride	80.00	
Potassium chloride	2.00	
Disodium hydrogen phosphate	11.50	
Potassium dihydrogen phophate	2.00	pH 7.3

The above were dissolved in one litre of distilled water, filter sterilised (Millipore filter 0.45 m pore size) and stored at $4^{o}C$ until required.

Before use, the solution was diluted to ten times its volume with distilled water. To this was added the equivalent of:-

0.1 g/l calcium chloride

0.1 g/l magnesium chloride

The pH was corrected to 7.3 with 1M sodium hydroxide solution.

Calcium magnesium free salt solution (CMFSS)

x10 concentrate

	g/litre	
Sodium chloride	80.00	
Potassium chloride	4.00	
Glucose	16.00	
Potassium dihydrogen phosphate	0.60	
Disodium hydrogen phosphate	0.48	pH 7.3

The above were dissolved in one litre of distilled water, filter sterilised, and stored at $4^{o}C$. Before use, a 10 fold dilution was performed with distilled water and the pH corrected to 7.3 with 1M NaOH.

10/150mM buffer

This buffer was used in the preparation of IgG, Facb and $F(ab')_2$,

and as a washing buffer in the enzyme-linked immunosorbent assay (ELISA) technique.

0.1M Disodium hydrogen phosphate (A)

0.1M Potassium dihydrogen phosphate (B)

Approximately 50ml solution A were added to 200ml solution B to give a pH of 7.4. The resulting solution was diluted to ten times its volume with distilled water. To each litre of the diluted solution was added 8.77g sodium chloride to give a final concentration of 10mM phosphate and 150mM sodium chloride.

To obtain 10mM phosphate buffer (pH 6.5) used in the preparation of IgG (using DEAE sephadex) approximately 50ml of each of the above constituents were mixed to give pH 6.5. The resulting solution was diluted 10 fold to give the required phosphate concentration.

Hanks balanced salt solution (HBSS)

HBSS (x10 concentrate) was diluted with sterile distilled water and brought to pH 7.4 with sterile 0.15M sodium bicarbonate solution.

Ammonium chloride solution

This solution was used for the lysis of extraneous red cells when it was deemed that the treatment would not affect the subsequent results.

	g/litre
Ammonium chloride	8.290
Potassium hydrogen carbonate	1.000
Ethelenediaminetetracetic acid	0.004

The above constituents were dissolved in one litre of distilled water and the pH corrected to 7.4 with 1M NaOH.

Lymphocyte culture medium- RPMI 1640

	Volume (ml)	
RPMI 1640 (x10 strength)	10.00	
Penicillin/Streptomycin solution (5000 IU/ml of each; P/S)	2.00	
200mM glutamine (GLN)	2.00	
7.5% sodium bicarbonate	2.70	pH 7.4

The above mixture was diluted to 100ml with sterile, distilled water. The pH was corrected to 7.4 with sterile 1M NaOH. Immediately before use, the medium was supplemented with foetal calf serum to give a final concentration of 10%

K562 culture medium

K562 cells were cultured in RPMI 1640 supplemented with 15% foetal calf serum, instead of the 10% used in the lymphocyte culture medium above.

Absorbed,heat inactivated newborn calf serum

Sheep erythrocytes were washed three times in PBS. The washed, packed cells were mixed at a ratio of 1:9 with heat inactivated new born calf serum (Gibco Bio-Cult Ltd., Scotland; HINBCS).

The suspension was kept at $4^{o}C$ overnight and subsequently centrifuged at 400g for 3 minutes. The supernatant (SRBC absorbed HINBCS) was stored at $-20^{o}C$ in 0.5ml aliquots.

Buffers used in the ELISA technique

Coating buffer

	g/litre	
Sodium carbonate	1.59	
Sodium bicarbonate	2.93	
Sodium azide	0.20	pH 9.6

The constituents were dissolved in sterile, distilled water and the solution stored at room temperature for not more than two weeks.

PBS/Tween

Tween 20 (Polyoxyethylene sorbitan monolaurate)	0.5 ml
Sodium azide	0.2 g

The above constituents were added to one litre of PBS and the buffer stored at room temperature.

Diethanolamine buffer (10%)

Diethanolamine	97.0 ml
Distilled water	800.0 ml
Sodium azide	0.2 g
Magnesium chloride hexahydrate	100.0 mg

The above constituents were mixed and adjusted to pH 9.8 with 1M hydrochloric acid. The total volume was made up to 1 litre with distilled water and the buffer stored in an amber bottle at room temperature.

Scintillation fluid

To 2.5 litres of toluene was added:-

12.50 g 2,5 - Diphenyloxazole (PPO)

0.75 g 1,4 - Di-2-(4-methyl-5-phenyloxazolyl) benzene (POPOP)

SECTION II

METHODS

(A) Isolation of Immunoglobulin G (IgG)

i) Preparation of hyperimmune rabbit serum

Calf red blood cells (CRBC) were washed three times in sterile PBS. Enough packed cells were then added to a suspension of Bacille Calmette-Guerin (BCG;2 mg/ml) in PBS to give a final concentration of 10^9 CRBC/ml. Each rabbit (New Zealand White; male) was injected intradermally in four of five sites with a total of 1ml of this suspension. This was repeated at weekly intervals for three weeks, a test bleed being performed in week four.

ii) Affinity chromatography

Protein A is a constituent of the cell wall of Staphylococcus aureus. The commercial preparation used for the isolation of IgG is obtained from a mutant strain (Cowan strain I) and purified by affinity chromatography. Its importance as a biological tool lies in its ability to interact with subclasses of IgG from a number of different animals (Hjelm, Hjelm and Sjoquist,1972;Chalon,Milne and Vaerman,1979).

The reaction between IgG and protein A is specific for the Fc portion of the molecule (Forsgren and Sjoquist,1966; Kronvall and Fromell 1970). More recently it has been shown that neither Facb nor pFc' fragments of IgG bind to protein A (Stewart, Varro and Stanworth,1978). This observation has allowed the use of protein A in the preparation of Facb .

Protein A column chromatography is achieved by covalently coupling protein A to Sepharose CL-4B by the cyanogen bromide method. The commercial preparation is supplied as a freeze dried powder which when swollen, has a binding capacity of approximately 20mg IgG/ml gel. The method used for IgG preparation was that described by Hjelm,Hjelm

and Sjoquist (1972) with some modifications. The separation was carried out at 4°C. A column (0.9x15cm; Pharmacia K9) was filled with approximately 5ml of swollen gel and washed through with several volumes of 10/150 buffer. 2-5ml of hyperimmune rabbit serum was applied to the column and after washing out the other serum proteins with 10/150 buffer, the IgG was eluted with 1M acetic acid and concentrated over an Amicon Diaflo XM50 membrane (nominal exclusion limit 50 000 Daltons).The acid concentrate was then loaded on to a Sepharose CL6B column (90 x 1.5 cm) and eluted with 10/150 at a flow rate of 8ml buffer/ hour, collecting 4ml fractions. Monomeric IgG fractions (assessed from an OD_{280} trace) were pooled, concentrated as above and total protein measured using the Folin-Ciocalteau method (Lowry et al, 1951).

iii)Ion exchange chromatography-Diethyl aminoethyl (DEAE) Sephadex

Since protein A only has a binding capacity of approximately 20 mg IgG/ml gel, IgG separation from myeloma sera was performed using DEAE sephadex. A modification of the method of Ishizaka (1965) was used. The freeze dried commercial preparation was swollen in 10mM phosphate buffer (pH 6.5) according to the supplier's directions. After filtration through a Buchner funnel, the 'dried' Sephadex was mixed with enough myeloma serum to form a thick paste. This was stirred for 1 hour at 4°C, filtered again, and then thoroughly washed with 10mM phosphate buffer. The filtrate was concentrated over an Amicon Diaflo XM50 membrane and dialysed overnight against 10/150 buffer. The IgG concentration of the resulting solution was determined using the Folin-Ciocalteau method of protein estimation (Lowry, 1951). A small sample was applied to a Sepharose CL-6B column to check for aggregation.

iv)Heat aggregation of IgG

A solution of IgG (approximately 4mg/ml) was maintained at 63°C

for 20-30 minutes. Insoluble aggregates were removed by centrifugation at 400g for 10 minutes. The degree of aggregation was assessed by analysing a small volume of the solution by gel filtration on Sepharose CL-6B as described in Section II A ii.

(B) Enzymic digestion of IgG

i)Plasmin - Preparation of rabbit Facb anti-calf red blood cell

The action of plasmin was first reported by Porter (1959). Plasmin is a biologically active protease which under the correct conditions will cleave IgG between residues 326/327 (see Figure 2.1) resulting in the formation of Facb and pFc'. Facb was prepared by a modification of the method of Stewart, Smith and Stanworth (1973). IgG was separated from hyperimmune rabbit serum using protein A affinity chromatography (Section II A ii). The concentrated acid eluate was maintained at 37°C for 1 hour in a shaking water bath. After this period, the pH was adjusted to 7.0 with 1M NaOH and human plasmin added (Kabi Vitrum Ltd.,London; 3CU/100mg IgG). The solution was then returned to the water bath for a further 4 hours, after which time the digest was applied to a Sephadex G100 column (90 x 1.5 cm) and eluted with 6M urea in 0.1M acetate buffer (pH 4.5) at a flow rate of 8ml/hour. The fractions containing the Facb and unreacted IgG (see Figure 2.2) were concentrated, dialysed against 10/150 for 4 hours at 4°C and then applied to the protein A-Sepharose 4B column (Section II A ii). The Facb was eluted with the 10/150 buffer whilst the residual IgG was retained on the column. The Facb was concentrated and the protein content estimated by the Folin-Ciocalteau method (Lowry et al, 1951).

ii)Pepsin - Preparation of rabbit $F(ab')_2$ anti-calf red blood cell

The method of Nisonoff, Markus and Wissler (1961) was used to prepare the $F(ab')_2$ fragment of IgG.IgG was isolated on protein A Sepharose 4B (Section II A ii) dialysed against 0.01M acetate buffer (pH 4.5) for 4 hours at 4°C and adjusted to 10-30 mg/ml. Pepsin in acetate buffer (1 mg/ml) was added to the IgG solution to give an

Figure 2.1 Diagrammatic representation of the structure of IgG

The figure shows a typical IgG molecule consisting of two heavy chains and two light chains.

KEY

CH - constant regions of the heavy chains

VH - variable regions of the heavy chains

CL - constant regions of the light chains

VL - variable regions of the light chains

S-S- inter- or intra- chain disulphide bonds

---- point of cleavage by plasmin to yield Facb and pFc' fragments

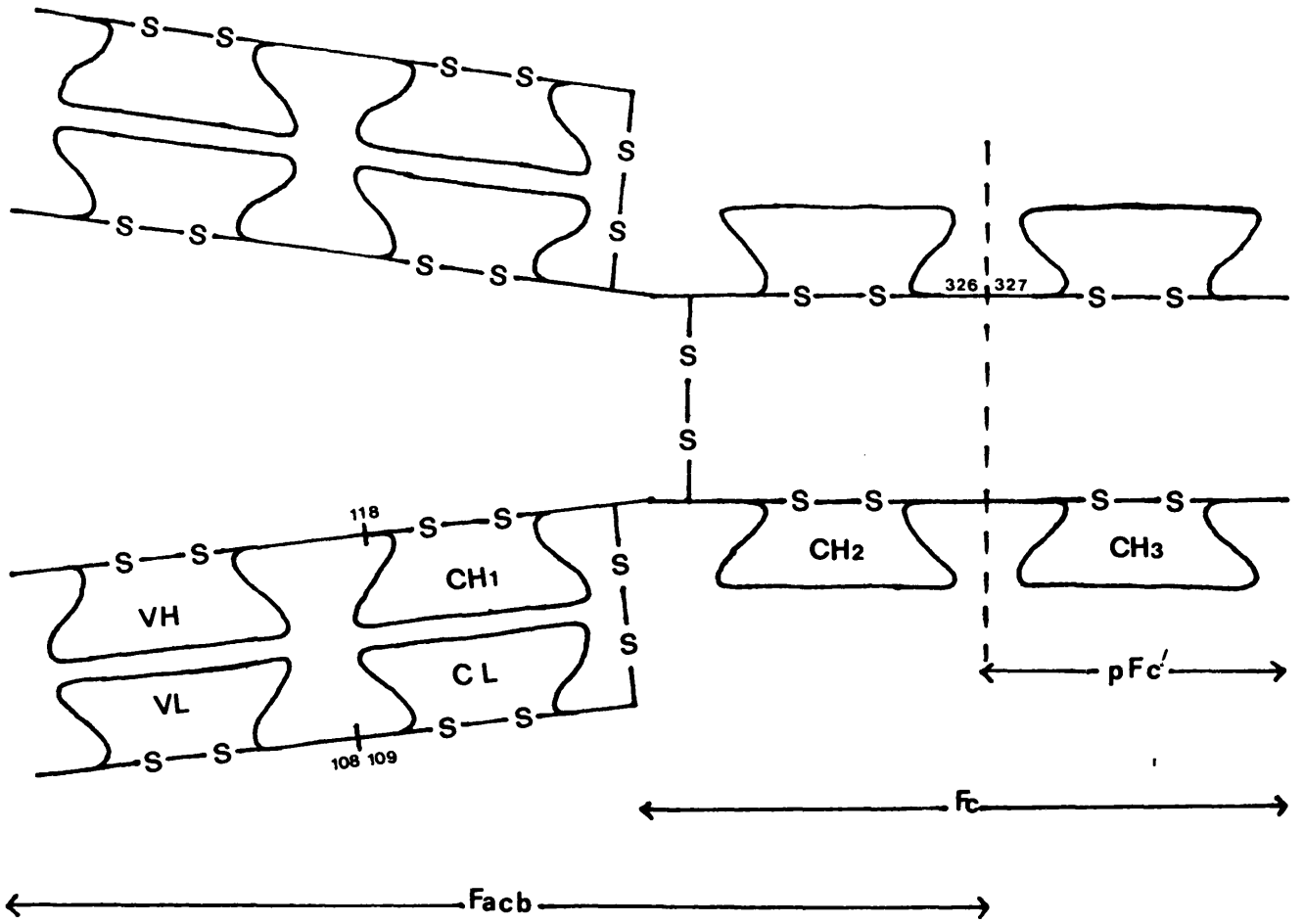
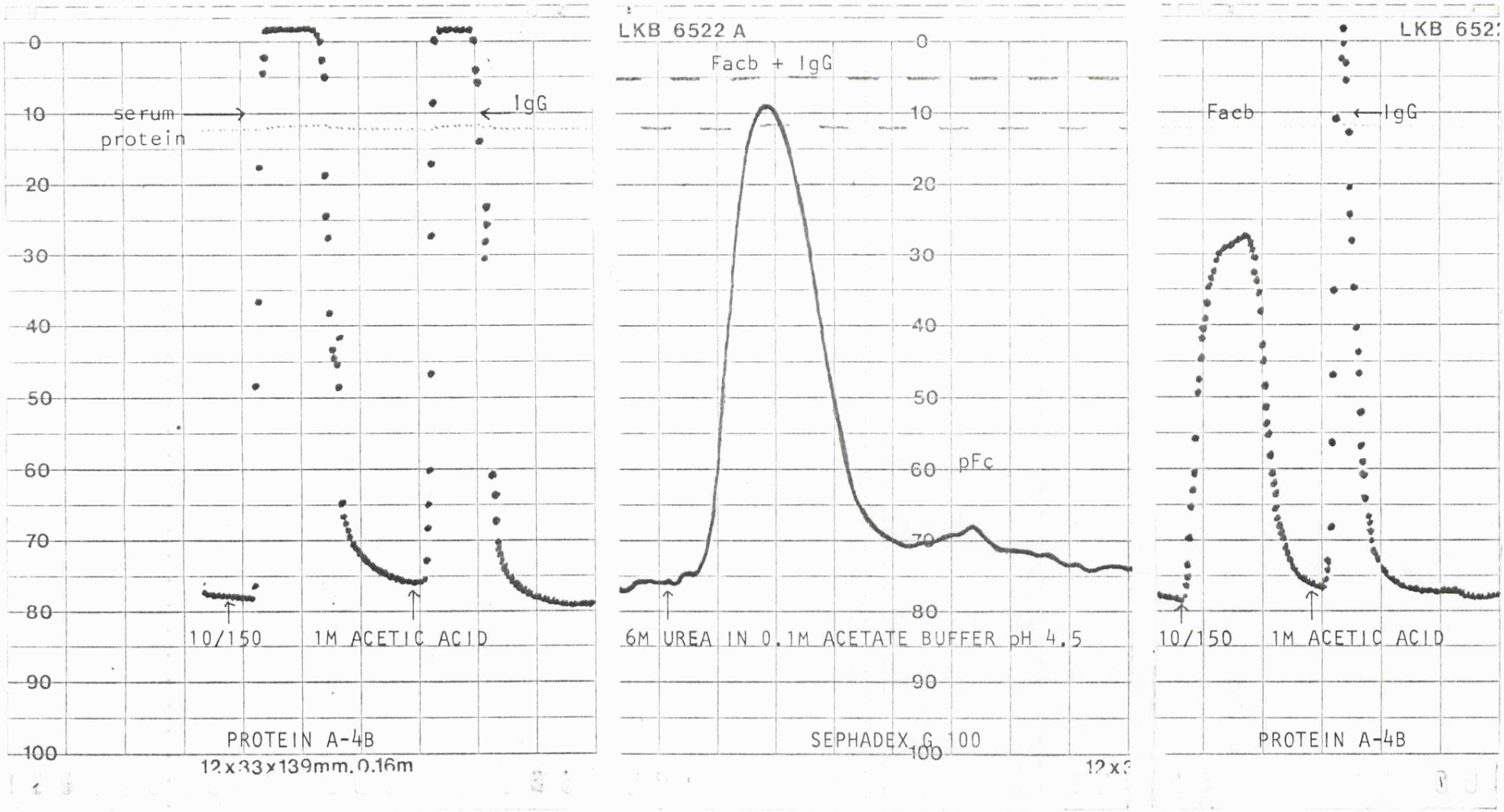

S
326
327
118
CH1
CH2
CH3
VH
VL
C L
108 109
pFc'
Fc
Facb

Figure 2.2 Optical density trace for the preparation of Facb

Ig G was prepared from hyperimmune rabbit serum by affinity chromatography on protein A - Sepharose 4B (Chap.11 Sec. 11 A ii) and incubated with plasmin (Chap 11 Sec. 11 B i). The digest was fractionate on Sephadex G100 and the protein eluted in the void volume (comprising Facb and unreacted IgG) was concentrated and applied to a protein A - Sepharose 4B column to purify the Facb. This figure shows a typical elution profile (absorbance at 280nm) for this procedure.


0
10
20
30
40
50
60
70
80
90
100
serum protein
IgG
10/150
1M ACETIC ACID
PROTEIN A-4B
12x33x139mm. 0.16m
LKB 6522 A
Facb + IgG
pFc
6M UREA IN 0.1M ACETATE BUFFER pH 4.5
SEPHADEX G 100
12x3
LKB 652
Facb
IgG
10/150
1M ACETIC ACID
PROTEIN A-4B

enzyme substrate ratio of 1:100. After incubation for 22 hours at 37°C pepsin was inactivated by the addition of solid TRIS (hydroxymethyl) methylamine; TRIS) to pH 8.0. This solution was eluted from a G100 Sephadex column (90 x 1.5 cm) with 10/150 buffer at a flow rate of 8ml/hour. The protein emerging in the void volume was pooled, concentrated and applied to the protein A Sepharose 4B column. The protein eluted with 10/150 -$F(ab')_2$- was concentrated and its concentration determined using the Folin-Ciocalteau method (Lowry et al, 1951). The $F(ab')_2$ was stored in aliquots at -90°C until required.

(C) Lymphocyte separation

i)Human peripheral blood - Introduction

Lymphocytes were separated by the density flotation method described by Boyum (1968). The principle of the technique is based on Stokes Law which determines the rate at which a body sediments through a fluid phase and is dependent on the size and density of the body concerned.Boyum (1968) demonstrated that by inducing aggregation of erythrocytes (this may be achieved by the use of such agents as ficoll, dextran, methylcellulose or polyvinylpyrrolidone (PVP) -coated silica-Percoll) their sedimentation rate increased,thus enabling their separation from leukocytes. He also demonstrated that 'isopaque' (diatrizoate sodium) ethelenediamine tetracetic acid (EDTA) and various salts enhanced this aggregation thus necessitating the dilution of blood with an equal volume of PBS before it is layered onto Ficoll-Paque.Owing to the sensitivity of granulocytes to osmotic variation and the low density of lymphocytes, the slight density gradient at the blood/Ficoll interface (formed by diffusion) enhances granulocyte sedimentation leaving an almost pure mononuclear cell population.

ii)Method

Freshly drawn, heparinised venous blood (30IU preservative free heparin/ml blood) was diluted to twice its initial volume with

CMFSS. Each 10ml of this mixture was carefully layered onto 3ml Ficoll-Paque (density 1.077±0.001g/ml) in a centrifuge tube and spun at 400g for 30 minutes at 20°C. After centrifugation, the lymphocytes, monocytes and platelets which formed a discrete band at the Ficoll-plasma interface, were harvested and subsequently washed three times with CMFSS by gentle resuspension and pelleting at 400g for 10 minutes.

iii)Murine splenic lymphocytes

Mice were killed by severance of the cervical spine and their spleens immediately removed and placed in ice cold HBSS.Each spleen was gently teased apart in 2ml of fresh HBSS contained in a sterile petri dish. This was then gently drawn into and expelled from a 19 gauge hypodermic needle and strained through a fine mesh wire gauze filter to give a single cell suspension.

iv)Cell counts and viabilities

Lymphocytes were enumerated using an improved Neubauer haemocytometer. The cell suspension was diluted in counting fluid (3% acetic acid/0.1%methylene blue) the latter causing the lysis of any contaminating erythrocytes. Cell viabilities were assessed by the trypan blue exclusion method. Non viable cells appear dark blue by this method. However, this is not a sensitive measure of cytotoxicity since cells which appear to be in an advanced stage of degeneration by phase contrast microscopy may exclude the dye (Tennant, 1964).

(D) Cell identification by rosette techniques

i)Rosette techniques for the enumeration of cells bearing receptors which bind IgG and Facb - Introduction

In 1973, Hallberg, Gurner and Coombs described the opsonic adherence of sensitised ox red cells to human lymphocytes measured by rosette formation. This was the first description of the phenomenon and a modification of their method was used as described by Hall, Winrow and Bacon (1980).

Preparation of sensitised cells

Calf red blood cells (CRBC) were washed 3 times in PBS (400g; 10 minutes; 20°C). After the final wash, the supernatant was carefully removed leaving the packed cells undisturbed. Appropriate dilutions of anti-CRBC IgG and Facb were made in PBS (predetermined to give an optimal, sub-agglutinating titre) and enough packed CRBC added to give a final red cell concentration of 1% v/v. The suspensions were mixed, incubated at 37°C for thirty minutes and then washed 3 times in PBS. The cells were then diluted to give a 1%v/v suspension of sensitised CRBC.

Rosette formation

Lymphocytes were obtained by separation on Ficoll-Paque (Sec. 11 C ii) washed 3 times in CMFSS and resuspended at a concentration of 2×10^6 cells/ml. A 200µl aliquot of this suspension was mixed with an equal volume of a 1% suspension of sensitised CRBC in a round bottomed tube (LP3; Luckham Ltd.,) sedimented at 400g for 3 minutes and maintained at 4°C for 1 hour. Two drops of a 2% solution of crystal violet in PBS were added to each tube, the cells being resuspended for 1 minute using a rotary turntable inclined at 45° (20 rpm; Baird and Tatlock Ltd.) and subsequently placed in a counting chamber (Neubauer improved haemocytometer). Lymphocytes (stained violet) surrounded by 4 or more erythrocytes were scored as rosettes. A total of 200 lymphocytes was counted and the number of rosettes observed was expressed as a percentage of this total.

ii) Sheep erythrocyte rosettes

Sheep red blood cells (SRBC) were washed 3 times in PBS (400g; 10 minutes). After the third wash, the supernatant was carefully discarded leaving the cell pellet undisturbed. Twenty-five microlitres of the packed cells were added to 500µl of SRBC- absorbed heat inactivated

new born calf serum ($SRBC_{ab}$HINBCS; see Section 1 B viii)and then diluted to 5ml with PBS. Two hundred microlitres of this preparation was added to an equal volume of a lymphocyte suspension (2×10^6 cells/ml) in an LP3 tube, incubated at 37°C for 10 minutes and then maintained at 4°C for 2 hours. Rosettes were enumerated as described previously (Sec. 11 D i).

Neuraminidase treated SRBC ($SRBC_n$)

Treatment of SRBC with neuraminidase enhances rosette stability and hence enumeration of T cells (Kaplan and Clarke, 1974). SRBC were washed 3 times in PBS (400g; 10 minutes). Neuraminidase (0.05 units) was added to 5ml of a 10% suspension of washed SRBC in PBS and incubated at 37°C for 30 minutes. The cells were subsequently washed 3 times with PBS and the packed cells diluted to give an 0.5% v/v suspension in PBS containing 10% v/v $SRBC_{ab}$HINBCS. The rosettes formed with $SRBC_n$ were incubated at 4°C for 1 hour.

Aminoethylisothiouronium bromide (AET) treated SRBC ($SRBC_a$)

A 4% w/v solution of AET was adjusted to pH 9.0 with 4M NaOH. SRBC were washed 3 times in PBS (400g; 10 minutes). The resulting packed SRBC were diluted in a ratio of 1:4 with the AET solution and thoroughly mixed. This suspension was incubated at 37°C for 15 minutes with mixing at 5 minute intervals. The cells were then washed 5 times with PBS. After the final wash, the packed cells were diluted to give an 0.5% v/v suspension in PBS containing 10% v/v $SRBC_{ab}$HINBCS. The rosettes formed with $SRBC_a$ were incubated at 4°C for 30 minutes.

iii)Mouse erythrocyte rosettes (MRBC)

MRBC were obtained from freshly drawn, heparinised blood from CFLP mice (Bath University; out-bred stock). Blood was washed 3 times with PBS and care was taken to remove the buffy coat layer. Rosettes were performed in exactly the same manner as those with SRBC (Sec. 1 D ii). Light microscopy photographs of Facb, SRBC and MRBC rosettes

are shown in Figure 2.3.

(E) Phenotypic characteristics identified by direct fluorescent techniques

i) Surface Ig detection

Mononuclear cells (MN) were incubated at 37°C for 30 minutes and subsequently washed 3 times with warm (37°C) PBS to remove passively adsorbed Ig from the cells. After the final wash, the lymphocytes were resuspended at a concentration of 2 x 10^6 cells/ml; 1ml aliquots being transferred to round bottomed tubes (LP4; Luckhams Ltd.) and the cells pelleted (400g; 10 minutes). The supernatant was carefully discarded and 1 drop of the relevant anti-human immunoglobulin-fluorescent conjugate was added. The cells were thoroughly resuspended in this antiserum and kept in ice for 30 minutes with occasional mixing. The cells were then washed 3 times with ice cold PBS. The cell pellet was resuspended in 1 drop of 5%PBS/95% glycerol and the preparation examined using a Leitz SM-LUX microscope fitted with a Leitz Ploemopak epifluorescence unit.

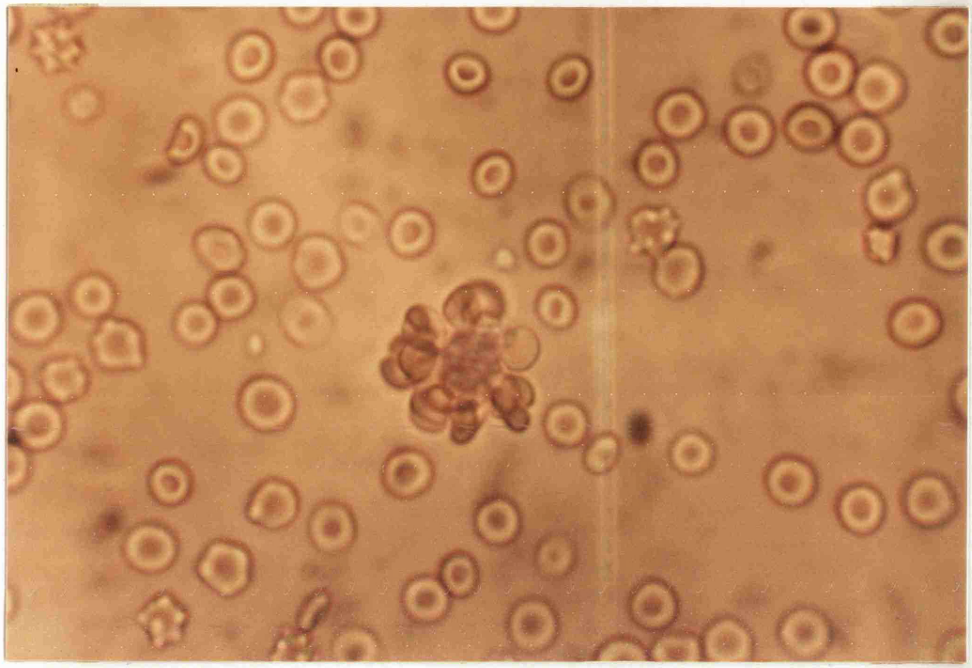
ii) HLA-DR and myeloid antigen detection

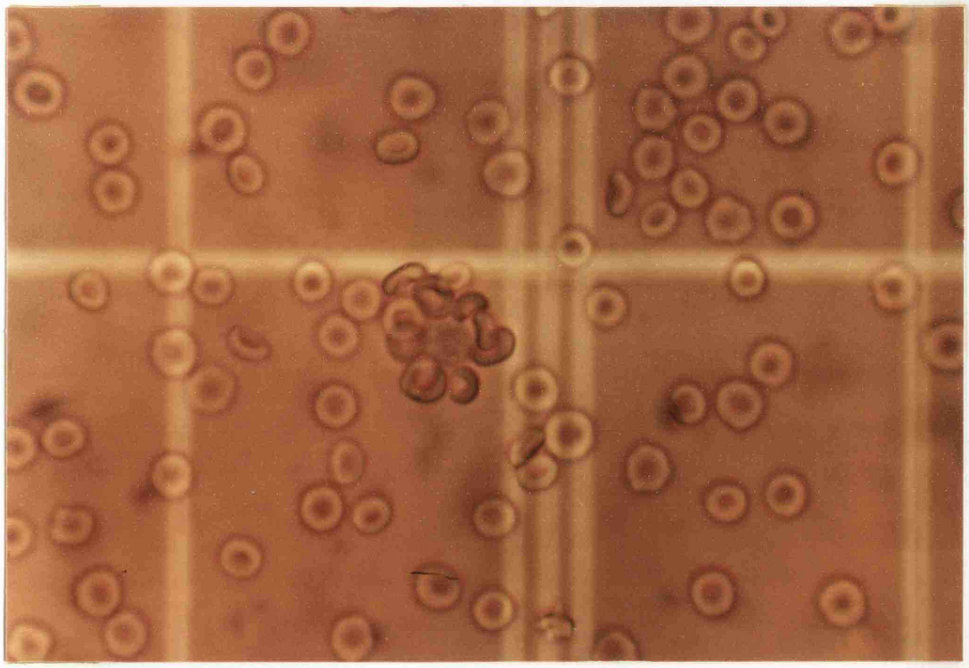
Anti-human HLA-DR and monocyte antigen monoclonal antibodies were diluted according to the manufacturers recommendations (Mouse monoclonal anti-human DR antigen - 1:10 000; Mouse monoclonal anti-human monocyte - 1:100; total IgG_1 concentration in both - 1.0mg/ml). One hundred microlitres of this dilution was added to a pellet of 10^6MN cells (isolated on Ficoll-Paque; Sec. 11 C ii), thoroughly mixed and left on ice for thirty minutes with occassional mixing. The cells were washed 3 times with ice cold PBS after incubation. One drop of rabbit anti-mouse IgG-FITC conjugate was added to the cell pellet, thoroughly mixed and left on ice for 30 minutes (with occassional mixing). After this time, the cells were washed 3 times with ice cold PBS (400g; 10 minutes). The cell pellet was subsequently resuspended in 1 drop of 5%PBS/95% glycerol and

Figure 2.3 Light microscopy photographs of various rosettes with human lymphocytes

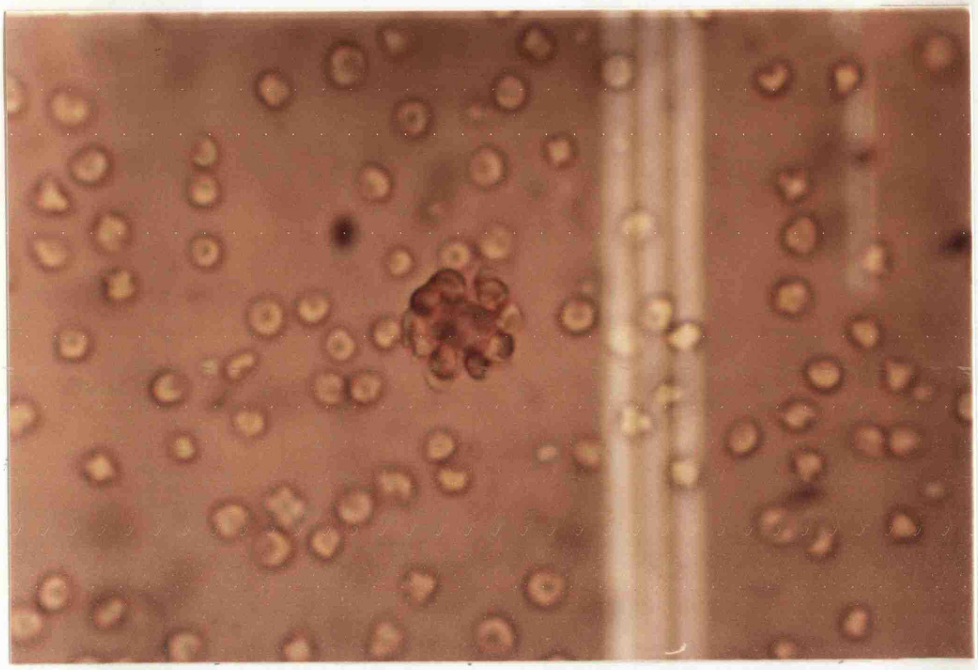
The photographs show rosettes formed with sheep, mouse and Facb coated calf RBC. The techniques used were those described in Chap. 11 Sec. 11 D i, ii and iii. The photographs were taken with a Canon 35mm SLR camera attached to a Zeiss SM-Lux microscope using direct illumination. Total magnification (on the microscope) x 400.

Human Lymphocytes Rosetted with Facb Coated CRBC

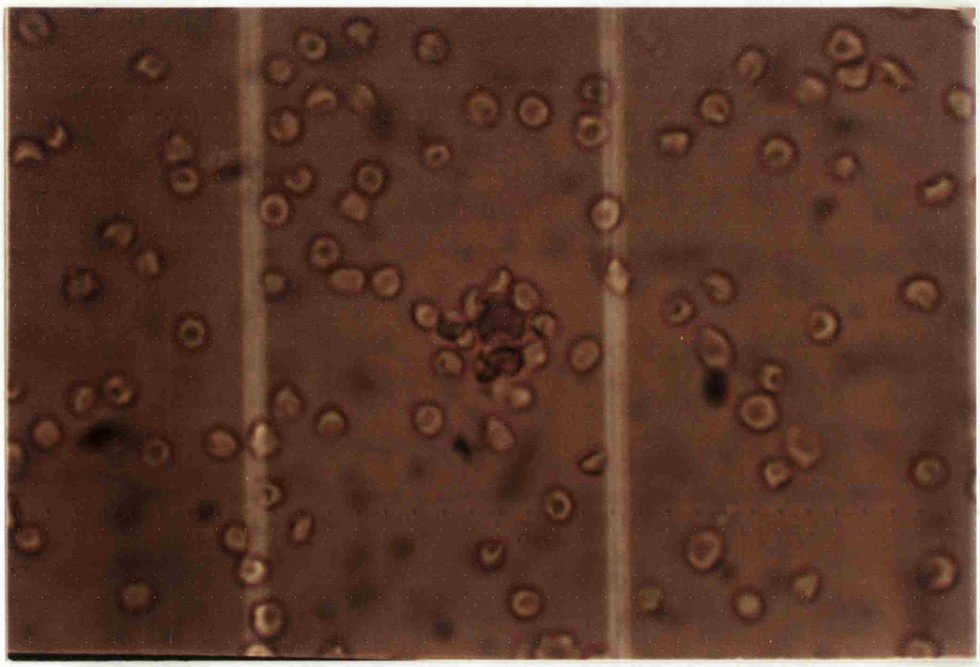




Human Lymphocytes Rosetted with SRBC



Human Lymphocytes Rosetted with MRBC



examined as described previously (Sec.11 E i).

(F) Mononuclear cell subpopulation separation techniques

i) T cells

Lymphocytes were rosetted with $SRBC_a$ as described previously (Sec. 11 D ii) except that because of the large volumes employed, rosetting was performed in sterile, 25ml conical bottomed bottles (Sterilin). After 1 hour, the cell pellets were gently resuspended with a pasteur pipette and 3ml of Ficoll-Paque was carefully layered beneath the cell suspension. The samples were then centrifuged for 30 minutes (400g; 20°C) and the T cell depleted interface cells were removed. These were washed 3 times with CMFSS (400g; 10 minutes) and assessed for T cell contamination by SRBC rosette formation as previously described (Sec. 11 D ii).

T cells could be recovered from the cell pellet after Ficoll-Paque separation by lysis of the SRBC with ammonium chloride solution. The cell pellet was thoroughly resuspended in 5ml ammonium chloride (Sec. 1 D). This was incubated for 10 minutes at 37°C and then washed 3 times with PBS (400g; 10 minutes).

ii) Facb R^+ cell depletion

Rosette depletion

Facb R^+ cells were depleted as described above (Sec 11 F i) using CRBC sensitised with the Facb fragment of rabbit anti-CRBC IgG.

Immobilised complexes

The technique used to deplete Facb R^+ cells by adherence to immobilised complexes was similar to that described by Horwitz et al., (1978). Two millilitres of a solution of bovine serum albumin (BSA;5mg/ml) in PBS was added to a plastic petri dish (5cm diameter; Sterilin) and

incubated at 37°C for 1 hour. The solution was discarded and the plates thoroughly washed with PBS. Two millilitres of an optimal dilution (in PBS) of anti-BSA IgG (predetermined in preliminary experiments) was added to the plate and incubated at room temperature for 1 hour. The plate was again thoroughly washed with PBS. Two millilitres of a lymphocyte suspension (4×10^6 cells/ml) were added to the plate and incubated at room temperature for 1 hour. Two hundred microlitres of this suspension was retained to determine the %Facb R^+ cells (Sec. 11 D i). After incubation, the plate was gently agitated and the non-adherent cells removed. Several volumes of PBS were used to gently wash the plate to remove any unbound cells. These were centrifuged (400g;10 minutes) and rosettes performed (Sec. 11 D i) to determine the degree of Facb R^+ cell depletion.

iii) Monocyte depletion

Carbonyl iron treatment

Carbonyl iron powder was added to freshly drawn heparinised blood at a concentration of 20mg/ml and mixed for 30 minutes at 37°C. The blood was then diluted 1:1 with CMFSS and separated on Ficoll-Paque as previously described (Sec. 11 C ii). The efficiency of monocyte depletion was investigated by performing differential stains on cytospin preparations of the lymphocyte preparations.

Plastic adherence

A mononuclear cell (MN) suspension prepared by Ficoll-Paque separation (Sec 11 C ii) was washed and resuspended in HBSS containing 10% v/v foetal calf serum (FCS). Two millilitres of this suspension (4×10^6 cells/ml) were incubated in plastic petri dishes (5cm diameter; Sterilin) for 30 minutes at 37°C. The plates were washed vigorously with several volumes of HBSS to obtain the non-adherent cells. These cells were then pelleted (400g;10 minutes) and resuspended at 4×10^6 cells/ml.

Monocyte contamination was assessed as described above and if necessary, the procedure was repeated to ensure complete removal of the monocytes.

Percoll gradient separation

A MN cell suspension was obtained from freshly drawn heparinised blood by separation over Ficoll-Paque (Sec. 11 C ii). The MN cells were layered over a cushion of 52% Percoll (density 1.062g/ml) and centrifuged at 400g for 20 minutes. The pelleted cells (lymphocytes) were washed 3 times in PBS (400g; 10 minutes) before being used further. The cells remaining above the Percoll layer were largely monocytes with a variable degree of lymphocyte contamination.

(G) Lymphocyte activation by mitogens

The in vitro activation of lymphocytes was studied using Concanavalin A (Con A), Phytohaemagglutinin (PHA) and Pokeweed mitogen (PWM). The freeze dried commercial preparations were all reconstituted with sterile PBS. Con A was reconstituted immediately before use and filter sterilised (0.22μm pore size; Millipore disposable filter unit); PHA (400μg/ml) and PWM were prepared, aliquotted and stored at -196^{o}C and at -20^{o}C respectively. Immediately before use these stock solutions were diluted in medium RPMI 1640.

Ficoll-Paque isolated MN cells were prepared under sterile conditions and suspended at a concentration of 5×10^5 cells/ml in RPMI 1640 culture medium containing 10% v/v FCS. Quadruplicate cultures were prepared in round-bottomed tissue culture microtitre plates (Nunc). Each well contained 200μl cell suspension and 10μl of either the mitogen solution or medium. In all experiments, a range of mitogen concentrations was used. The plates were incubated for 72 hours in a humidified atmosphere of 95% air/5% CO_2 at 37^{o}C. Four hours before harvesting, (Titertek Cell Harvester, Flow Laboratories Ltd.,) 0.5μCi of tritiated

thymidine (specific activity 5Ci/mmol) was added to each well. Radioactivity incorporated into trichloroacetic acid (TCA) precipitable material was measured in a Packard Tri-Carb liquid scintillation spectrometer using toluene/PPO/POPOP scintillation fluid.

(H) Measurement of Ig production

i) Human lymphocyte culture conditions

Two hundred microlitres of a sterile suspension of MN cells in RPMI 1640 (5×10^5 cells/ml) were added to each well of a flat-bottomed microtitre plate (Nunc). Quadruplicate cultures each contained 10 µl of either PWM solution or medium. A range of mitogen concentrations were used. The plates were incubated for 14 days at 37°C in a humidified atmosphere of 95% air/ 5% CO_2. At the end of the incubation period, the supernatants were harvested and stored at -90°C until assayed for Ig production by the enzyme-linked immunosorbent assay.

ii) Enzyme-linked immunosorbent assay (ELISA)

Two hundred microlitres of an optimal dilution of an anti-human polyvalent immunoglobulin was dispensed into each ELISA cuvette well (Gilford ELISA cuvette packs, Corning Ltd.). The cuvettes were incubated in a humid box for 1 hour at 37°C and subsequently overnight at 4°C. They were washed extensively with PBS/Tween, the final wash being aspirated with a mechanical pump. Two hundred microlitres of each doubling dilution of the control serum or test supernatants (in PBS/Tween) was added (in duplicate) to the cuvettes, which were subsequently incubated in a humid box at 37°C for 1 hour. After incubation, the washing procedure with PBS/Tween was repeated. Two hundred microlitres of an optimal dilution (in PBS/Tween) of the relevant alkaline phosphatase conjugate anti-human IgG or IgM (determined in preliminary experiments) was added to each cuvette and the plates incubated at 37°C for 1 hour in

a humid box. After extensive washing with PBS, 200µl of a solution of alkaline phosphatase substrate (p-nitrophenol phosphate) in ethanolamine buffer (1mg/ml), was added to each cuvette. The colour was allowed to develop at room temperature and when the positive control serum reached an absorbance value of 2.0 (at 405nm; Gilford Manual ELISA plate reader, Corning Ltd.,), the reaction was stopped by the addition of 200µl of 1.5M NaOH. The optical density of each cuvette was then determined at 405nm.

iii) Ig production by murine lymphocytes- the plaque forming cell (PFC) assay

A modification of the method described by Cunningham (1968) was used to determine the number of Ig secreting cells in a suspension of mouse spleen cells. A single cell suspension of murine splenic lymphocytes was prepared (Sec.11 C iii), filtered into LP2 tubes (Luckhams), diluted in HBSS and the cell concentration determined. The tubes were stored on ice until required. Indicator erythrocytes were washed 3 times in PBS, diluted to give a 40% v/v suspension and stored on ice. Glass microscope slides were thoroughly cleaned with alcohol and stuck together in pairs by 3 strips of double sided tape (Sellotape,England). Each pair of slides thus provided 2 chambers for duplicate samples. To each chamber was added 50µl 'incubation medium', This comprised 200µl cell suspension (added immediately before use), 200µl indicator cell absorbed guinea pig complement (1:10 dilution) and 100µl 40% v/v indicator cell suspension. The chamber was filled with 'chaser medium' composed of 1ml complement (1:10 dilution), 1ml HBSS and 0.5ml 40% v/v indicator cell suspension. The chambers were sealed with molten vaseline and allowed to settle on a leveling table. After incubation at 37°C for 90 minutes, the numbers of plaques in each chamber was determined using a Leitz laborlux 12 microscope fitted with an Hitachi CCTV camera attached to an Hitachi video monitor VM-129 (total magnification x 40).

Modifications

In some experiments it was necessary to determine the number of lipopolysaccharide (LPS) specific PFC. In order to do this, 2ml washed, packed sheep RBC were suspended in 4ml of a solution of LPS (1mg/ml) and incubated at 37°C for 1 hour. The cells were washed 3 times in PBS and diluted to give a 40% v/v suspension. These were then used as described above.

CHAPTER 111

CHARACTERIZATION OF THE FACB R^+ CELL

SECTION 1

(A) Introduction

The Facb R^+ cell has already been partially characterized (Hall, Winrow and Bacon, 1980; Winrow and Hall, 1980; Hall and Bacon, 1981; Winrow, 1982). It has been shown to be Sig^- (detected by direct fluorescence) E^-; C_3R^-; HP R^-; specific esterase negative. Thus, these cells appear to be 'null' cells when judged by the criteria discussed in Chap. 1 Sec. 1 C iii and may be similar to L cells described by Horwitz and Garrett (1977), Horwitz et al., (1978) and Niaudet et al., (1979). These workers reported that L cells have a deep nuclear cleft and express an antigen detected by the monoclonal antibody OKM1 and/or HLA-DR related antigens. The suggested that this may indicate that L cells are derived from the myeloid lineage stem cell. In order to investigate the similarity between Facb R^+ lymphocytes and L cells, various experiments were performed which examined the cellular morphology of Facb R^+ cells and their monocyte related characteristics.

(B) Electron microscopy of Facb R^+ cells

Facb R^+ cells were obtained by positive selection from freshly drawn heparinized blood. Cells from 6 patients with active RA and 6 healthy control subjects were obtained as described in Chap. 11 Sec. 11 F ii).

The cells were prepared for the electron microscope by Miss J. Price (Royal United Hospital, Bath). The specimens were embedded in 1% agar, stained with uranyl acetate and lead citrate and examined using a JEOL JEM-1008 transmission electron microscope. Some of the electron micrographs obtained are shown in Figures 3.1 and 3.2. Approximate cell dimensions were calculated from the electron micrographs at a total magnification of x 36000. Measurements in four different planes were recorded for each cell.

these are given in Table 3.1. The measurements show that both the cellular and nuclear diameters are significantly larger for Facb R^+ cells from patients with RA than for those from healthy control subjects ($p<0.001$). Also these rheumatoid cells were significantly smaller than monocytes from healthy controls ($p<0.001$). The ratio of cell:nucleus diameter was smaller in rheumatoid than control cells suggesting that the former have a larger volume of cytoplasm than the latter.

Structurally, the electron micrographs of Facb R^+ cells showed that both rheumatoid and control cells possessed deep nuclear clefts and a considerable degree of peripheral condensation of chromatin. The latter suggests that the cells are relatively mature. The cytoplasm of the control cells contained numerous isolated ribosomes and a few mitochondria (4-8). The rheumatoid cells also showed numerous ribosomes but in addition contained polysomes (Fig. 3.2 a;c) and rough endoplasmic reticulum (Fig 3.2 c). Greater numbers of mitochondria (9-20) were observed in these cells compared with control cells. The former also showed well developed Golgi complexes (Fig 3.2 a;b;c) and evidence of cytoplasmic fibrillar structures characteristic of myeloid cells (Fig 3.2 a;c).

(C) Steroid sensitivity of Facb R^+ cells

Monocytes have been shown to be highly sensitive to corticosteroids. Thus, hydrocortisone was used to examine the steroid sensitivity of Facb R^+ cells. A bolus dose of 100mg hydrocortisone sodium succinate (HCO) (Organon Laboratories) was given intravenously to a group of healthy control subjects and to a group of patients with classical, active, RA (selected by Dr.B. Cadge, RNHRD, Bath). A sample of venous blood was taken before administration of HCO and also at 2 and 4 hours thereafter. A total white cell count, a differential count, Facb, EA and E rosettes were performed on each sample (see Chap.11 Sec.11 C for methods). The results are given in Tables 3.2a,b and Figs. 3.3 and 3.4. Monocytes were significantly reduced in numbers in both rheumatoids and controls at 4 hours post HCO

Table 3.1 Cellular measurements from electron micrographs

	N	MEASUREMENTS µm		
		MEAN	RANGE	
CELL SIZE	24	5.1	4.5-5.7	C
NUCLEUS SIZE	32	3.8	3.2-4.5	C
NUCLEUS:CELL RATIO		0.75:1.0		C
CELL SIZE	36	8.2	7.1-9.3	RA
NUCLEUS SIZE	26	5.5	4.4-6.7	RA
NUCLEUS:CELL RATIO		0.67:1.0		RA
CELL SIZE	8	10.9	9.0-12.9	MONO
NUCLEUS SIZE	8	5.7	4.9-6.4	MONO
NUCLEUS:CELL RATIO		0.50:1.0		MONO

KEY

C -Control cell measurements

RA -Rheumatoid cell measurements

MONO -Monocyte measurements

Fig.3.1 Electron micrographs of Facb R^+ cells from control subjects

Facb R^+ cells were obtained by rosetting with Facb coated CRBC and centrifugation through Ficoll-Paque (Ch.3 Sec.A i). Micrographs of osmium tetroxide/uranyl acetate stained cells were obtained using a JEOL JEM-1008 transmission electron microscope.

KEY

E - CRBC

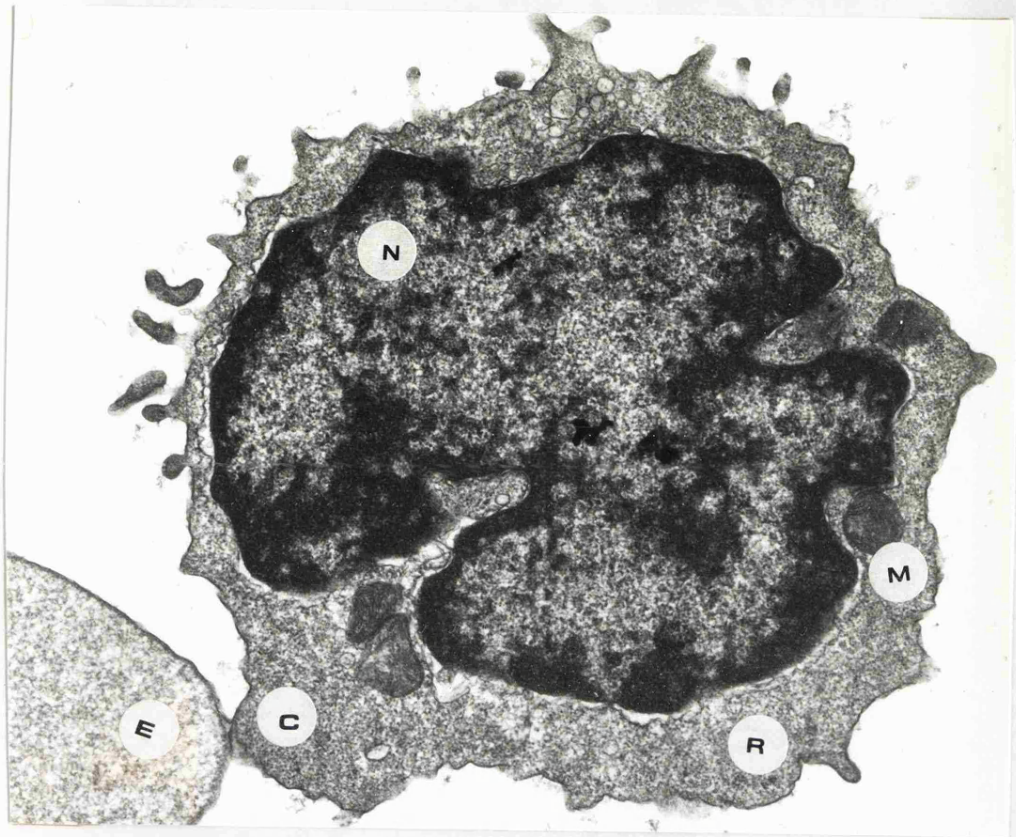
C - Cytoplasm

M - Mitochondrion

R - Ribosomes

N - Nucleus

NU - Nucleolus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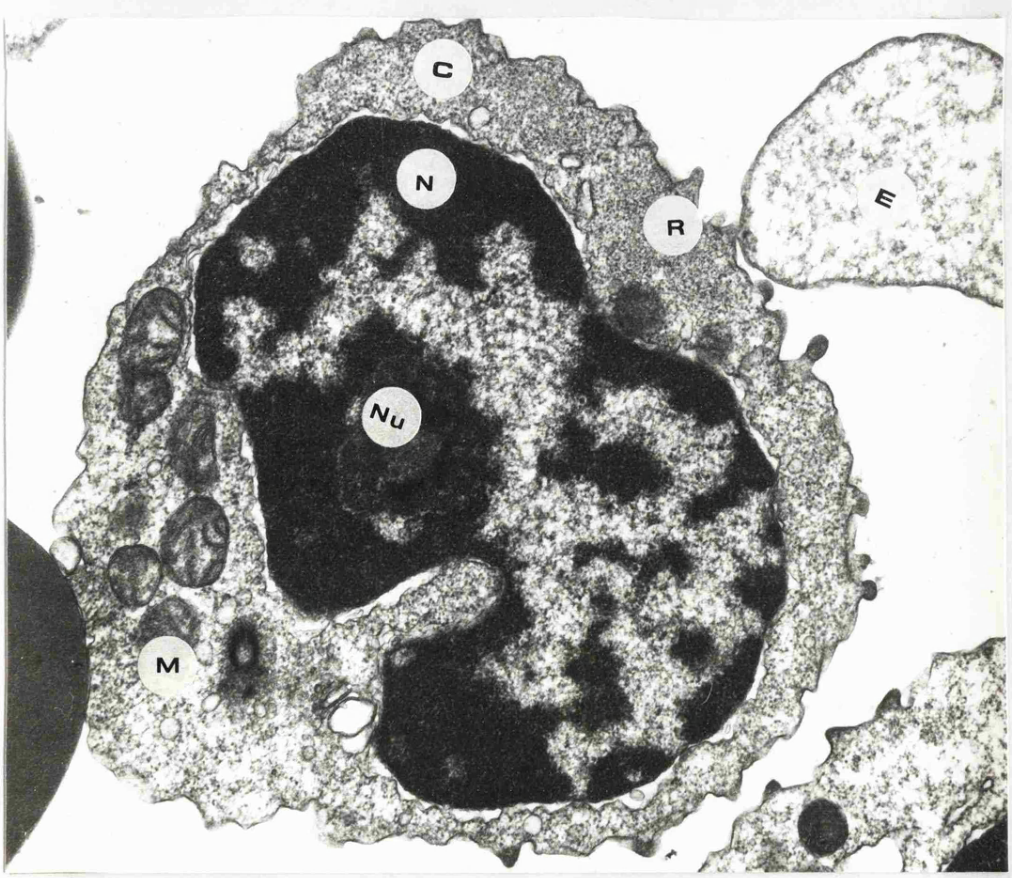
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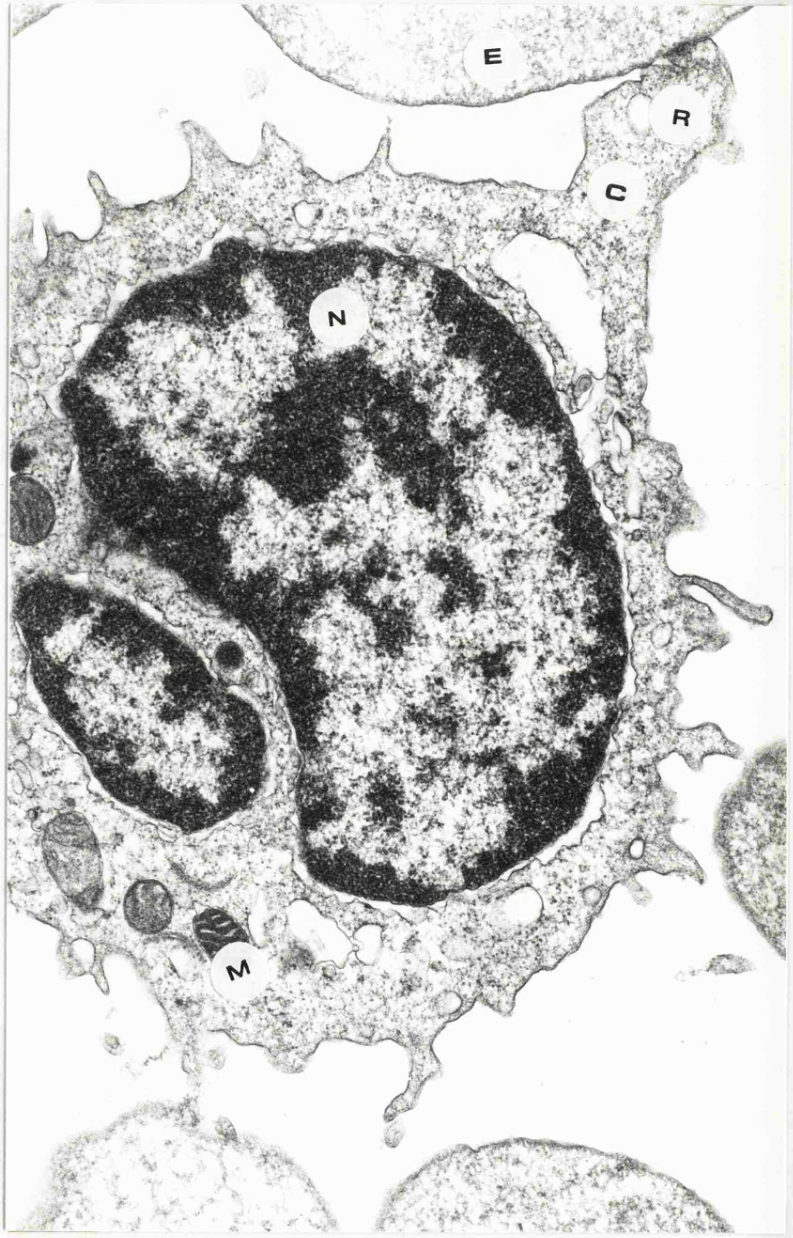
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Fig. 3.2 Electron micrographs of Facb R^+ cells from rheumatoid subjects

Facb R^+ cells were obtained by rosetting with Facb coated CRBC and centrifugation through Ficoll-Paque (Ch.3 Sec.A i). Micrographs of osmium tetroxide/uranyl acetate stained cells were obtained using a JEOL JEM-1008 transmission electron microscope.

KEY

E - CRBC

C - Cytoplasm

M - Mitochondrion

R - Ribosomes

P - Polysomes

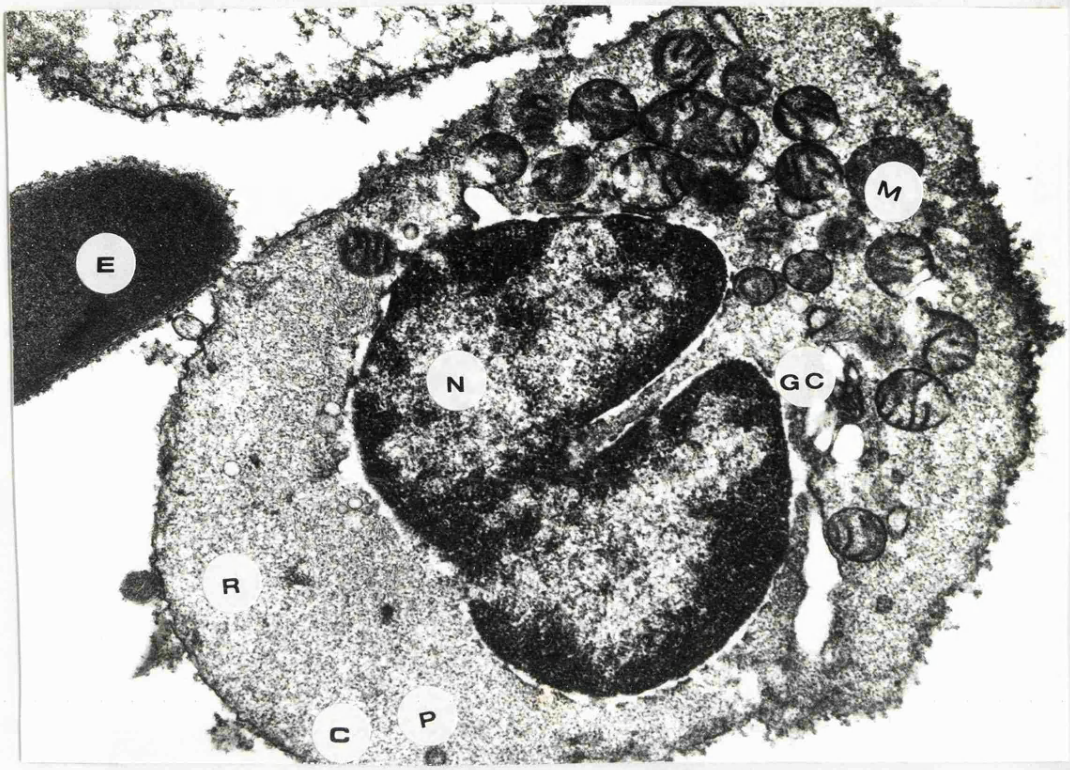
RER- Rough endoplasmic reticulum

GC - Golgi complex

CS - Centrosome

N - Nucleus

Nu - Nucleolus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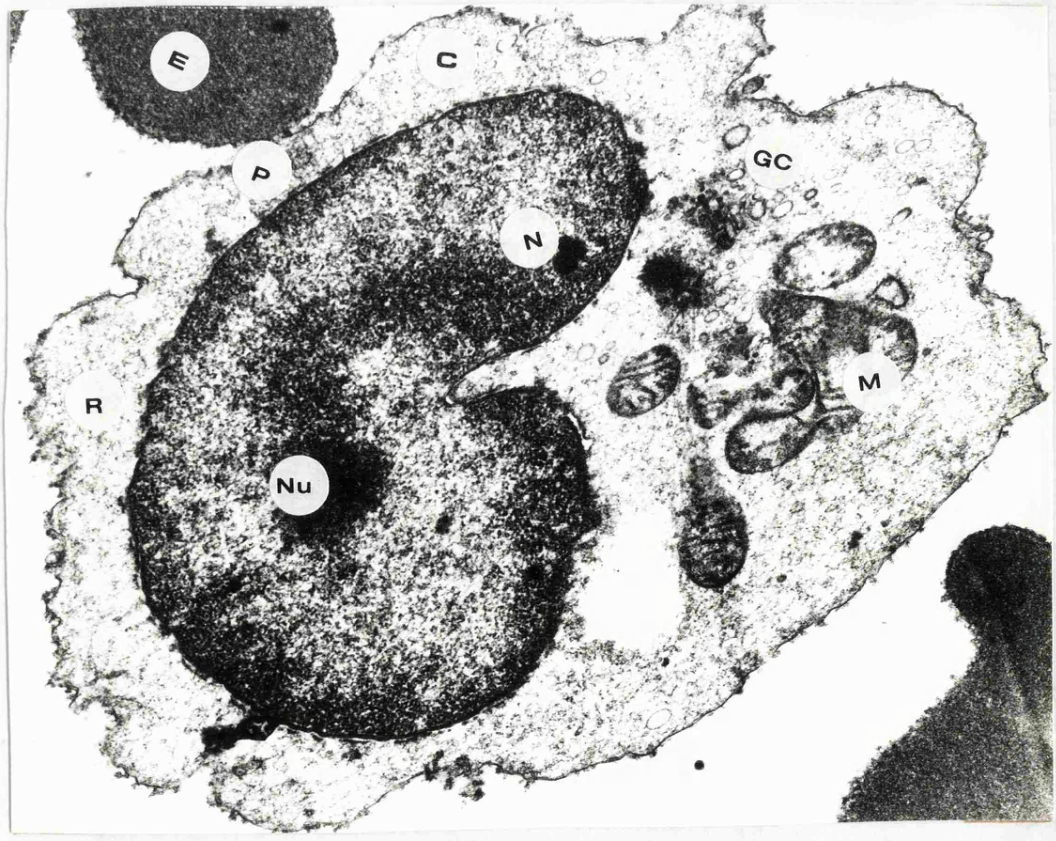
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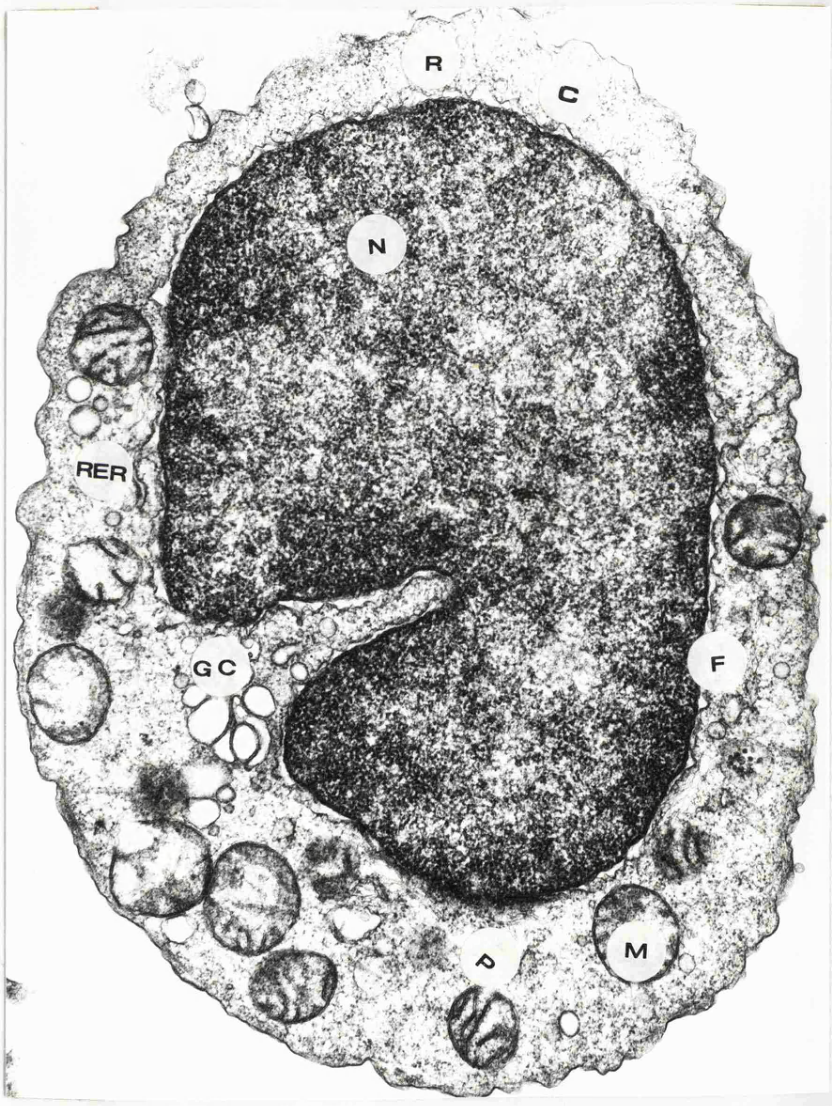
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administration, the effect being more pronounced in the latter ($p<0.001$) than the former ($p<0.01$). The number of circulating neutrophils was significantly increased at 4 hours in both patients with RA ($p<0.05$)and control subjects ($p<0.001$). A significant decrease in the number of circulating E R^+ cells (T cells) was also observed in both groups ($p<0.001$).HCO had no effect on the number of EA R^+ cells in the peripheral blood of either group. Similarly, the number of Facb R^+ cells in control blood was unaffected by the administration of HCO. However, RA subjects had significantly fewer circulating Facb R^+ cells 4 hours after HCO administration than at time 0. Thus, these results suggest that the Facb R^+ cell is steroid sensitive in patients with RA but not in control subjects.

(D) HLA-DR and monocyte antigens on Facb R^+ cells - an investigation with monoclonal antibodies

Lymphocytes obtained from freshly drawn heparinized blood (Chap.11 Sec. 11 C ii) were treated with anti-HLA-DR or anti-human monocyte antigen monoclonal antibodies as described in Chap.11 Sec 11 E ii. The results are shown in Table 3.3. The percentage of Facb R^+ cells in both rheumatoid and control subjects was significantly decreased by both the anti-HLA-DR (control:$p<0.005$;rheumatoid;$p<0.001$) and the anti-monocyte antigen monoclonal antibodies (control;$p<0.005$;rheumatoid:$p<0.001$). Although it was thought unlikely that this effect could be due to non-specific blocking of the Facb receptors (since both the antibodies were used at extremely low dilutions) an alternative method was used to investigate the presence of the antigens on Facb R^+ cells.

Lymphocytes were obtained from freshly drawn, heparinised blood (Chap.11 Sec. 11 C ii) and Facb rosettes were performed (Chap.11 Sec.11 D i). After incubation at 4^oC for 1 hour, the rosettes were gently resuspended, diluted in 3ml PBS/5%BSA (bovine serum albumin) and used to prepare slide mounted, dried specimens by cytocentrifugation (Shandon

Table 3.2a Effect of HCO on control leukocyte populations

	TIME (HOURS)		
MONOCYTES ($X10^{-4}$)	0	2	4
n	8	8	8
$\bar{x}\pm SD$	29.3± 12.8	8.1± 7.0	5.0± 4.4
p	-	<0.01	<0.001
NEUTROPHILS ($X10^{-5}$)			
n	8	8	8
$\bar{x}\pm SD$	23.8± 4.5	62.9±19.3	77.4±21.3
p	-	<0.001	<0.001
Facb R^+ ($X10^{-4}$)			
n	8	8	8
$\bar{x}\pm SD$	3.8± 2.3	4.3± 3.3	5.3± 3.4
p	-	NS	NS
EA R^+ ($X10^{-4}$)			
n	8	8	8
$\bar{x}\pm SD$	43.3±30.6	28.1±13.8	34.5±16.0
p	-	NS	NS
E R^+ ($X\ 10^{-5}$)			
n	8	8	8
$\bar{x}\pm SD$	12.9± 3.4	6.7± 2.6	4.8± 2.0
p	-	<0.05	<0.001

p determined by Student's t-test against T 0 hours.

Table 3.2b Effect of HCO on rheumatoid leukocyte populations

	TIME (HOURS)		
MONOCYTES ($X10^{-4}$)	0	2	4
n	17	11	16
$\bar{x}$±SD	36.2±24.0	19.4±14.3	11.2±12.5
p	-	<0.1	<0.01
NEUTROPHILS($X10^{-5}$)			
n	17	11	16
$\bar{x}$±SD	48.2±19.7	67.8±23.5	69.8±29.7
p	-	<0.05	<0.05
Facb R^+ ($X10^{-4}$)			
n	17	11	16
$\bar{x}$±SD	8.9± 5.7	5.7± 3.9	2.7± 3.3
p	-	NS	<0.001
EA R^+ ($X10^{-4}$)			
n	17	11	16
$\bar{x}$±SD	45.0 34.1	31.6±17.6	28.4±37.3
p	-	NS	NS
E R^+ ($X10^{-5}$)			
n	17	11	16
$\bar{x}$±SD	11.6± 5.7	7.3 ± 2.6	4.3 ± 3.2
p	-	NS	<0.001

p determined by Student's t-test against time 0 hours.

Fig. 3,3 Demonstration of the effect of hydrocortisone on lymphocyte subpopulations

A 100mg bolus dose of hydrocortisone sodium succinate (HCO) was given intra-venously (iv) to a group of healthy control subjects and to a group of patients with classical, active rheumatoid arthritis of not more than one year duration, All the patients were on non-steroidal drugs.

KEY

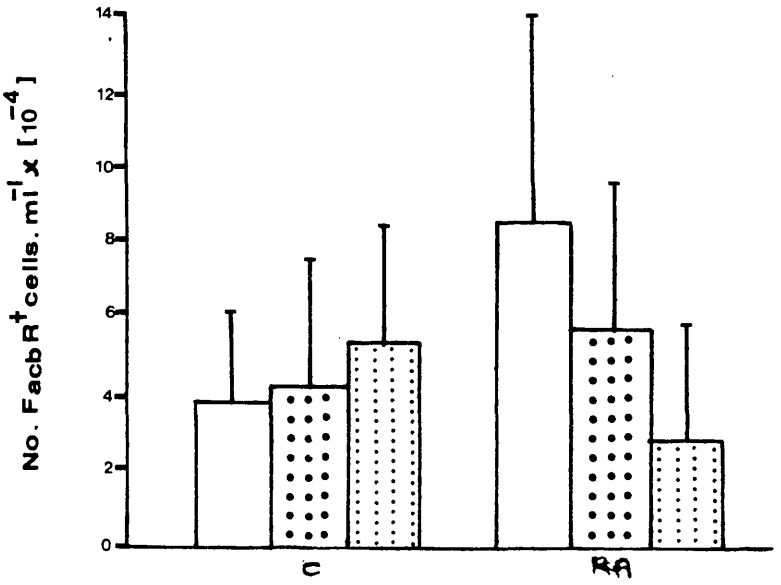
C - Control lymphocyte subpopulations

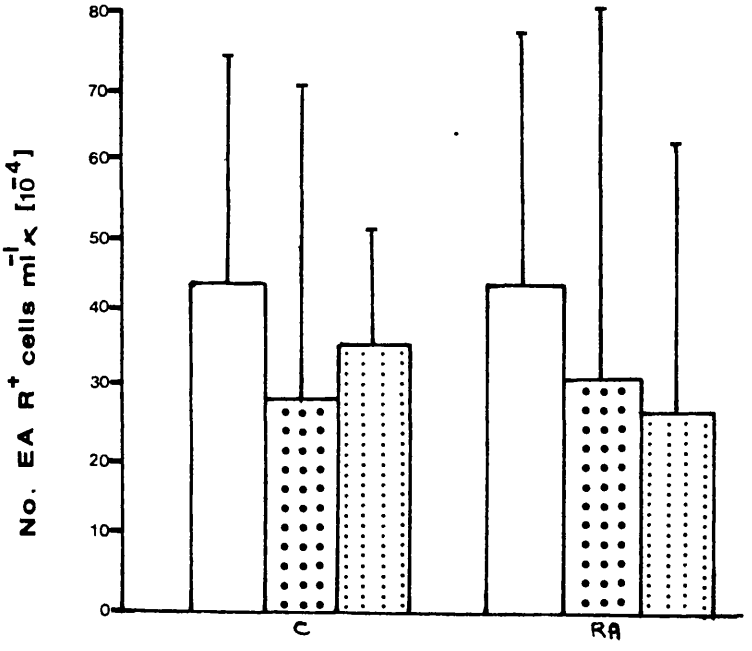
RA - Rheumatoid lymphocyte subpopulations

 - 0 Hours

 - 2 Hours

 - 4 Hours


No. FacbR+cells.ml-1 x [10-4]
14
12
10
8
6
4
2
0
C
RA


No. EA R+ cells ml-1 x [10-4]
80
70
60
50
40
30
20
10
0
C
RA

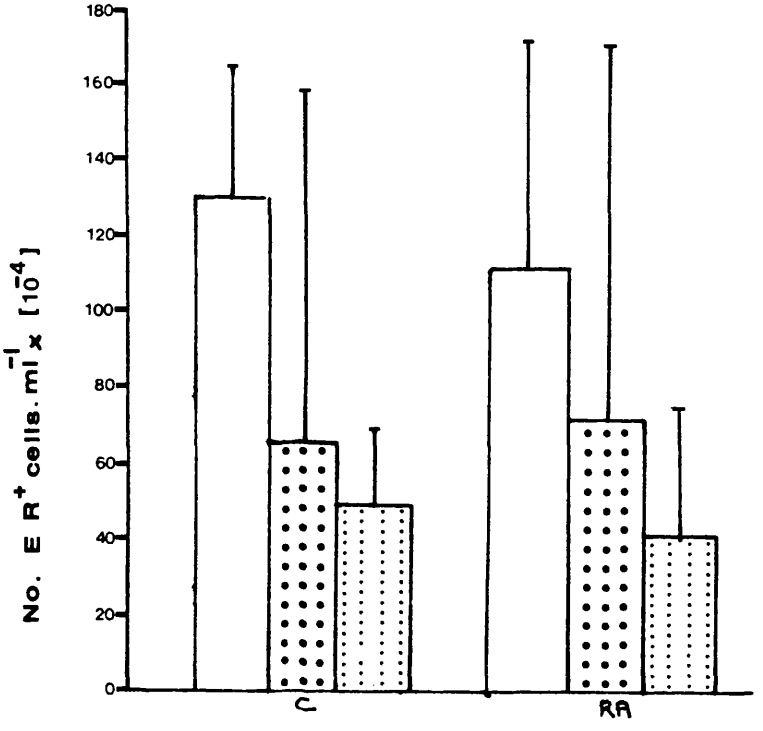

No. E R+ cells.ml-1 x [10-4]
180
160
140
120
100
80
60
40
20
0
C
RA

Fig. 3.4 Effect of hydrocortisone on other leukocyte populations

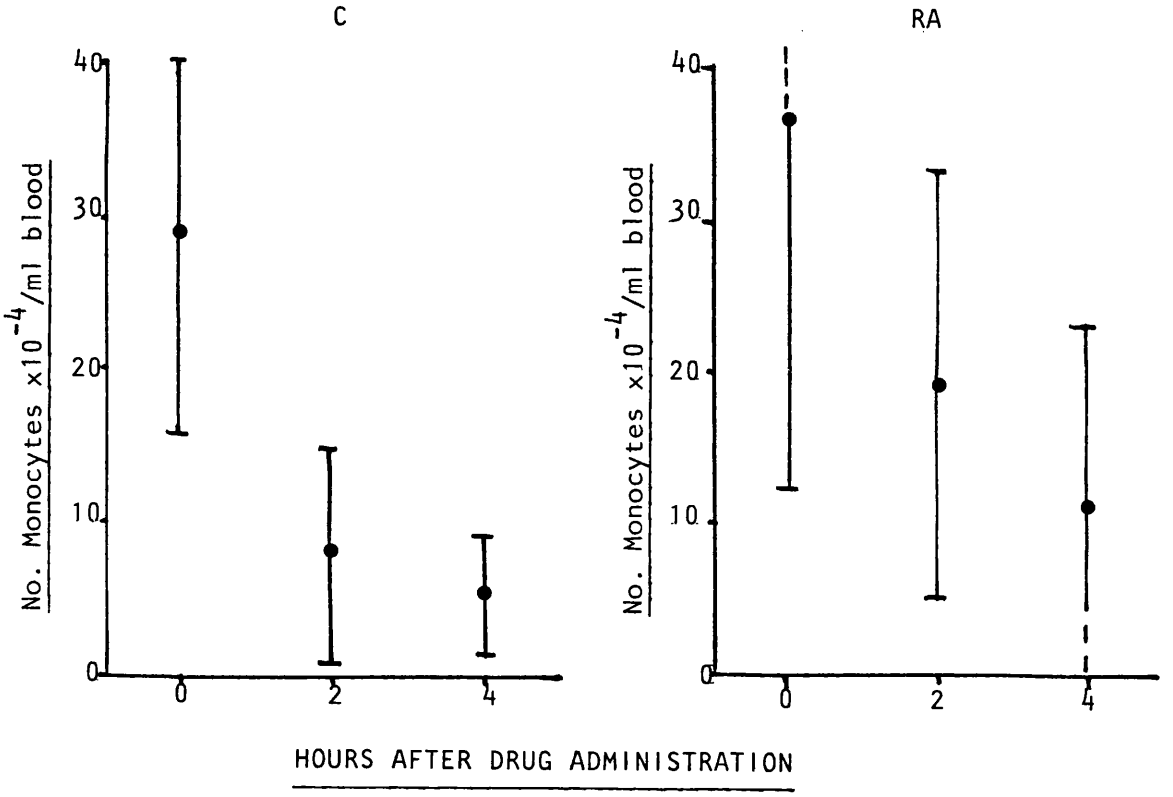
Each blood sample was subjected to a total white cell count (Ch.2 Sec.11 C iv) and a differential count.

KEY

C - Control cells

RA - Rheumatoid cells

The results are expressed as the mean +/- one standard deviation (sd).


C
RA
No. Monocytes x10^{-4}/ml blood
40
30
20
10
0
0
2
4
HOURS AFTER DRUG ADMINISTRATION

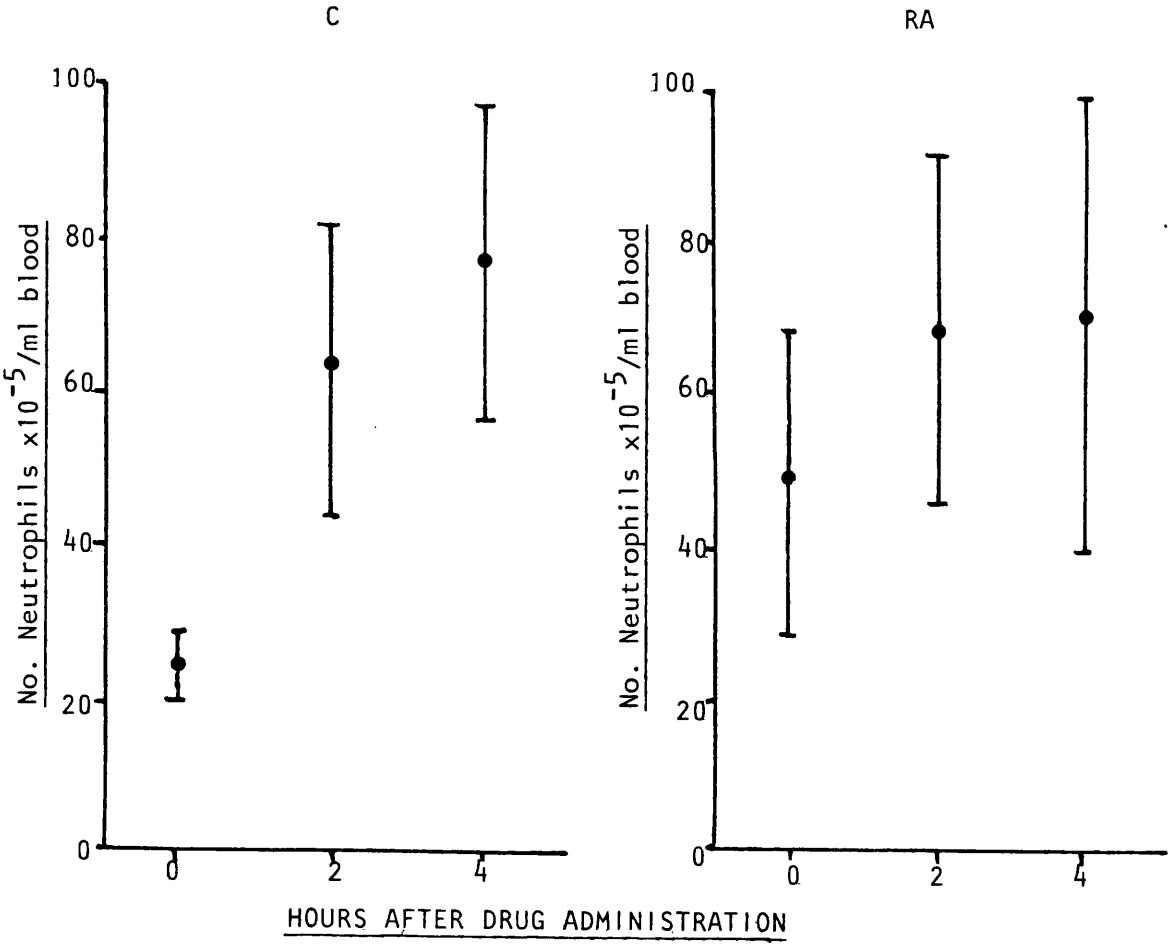

C
RA
No. Neutrophils x10^{-5}/ml blood
100
80
60
40
20
0
0
2
4
HOURS AFTER DRUG ADMINISTRATION

Table 3.3 a

Effect of monoclonal anti-DR or anti-monocyte on rheumatoid lymphocytes

SUBJECT	% Facb	% Facb post monoclonal + complement	
		Anti-DR	Anti-monocyte
KT	5	2	0
TC	6	1	1
MS	6	1	1
WS	8	2	2
NB	8	3	3
EW	5	1	1

Table 3.3 b

Effect of monoclonal anti-DR or anti-monocyte on control lymphocytes

SUBJECT	% Facb	% Facb post monoclonal + complement	
		Anti-DR	Anti-monocyte
LE	4	1	2
SL	2	1	1
MM	3	2	1
SF	2	0	1
DP	4	2	2
NF	2	1	1

Table 3.4 Effect of monoclonal anti-DR or anti-monocyte antigen on cytospin preparations of rheumatoid and control Facb rosettes

SUBJECT	Ratio: Fluorescent Facb rosettes / Non-fluorescent rosettes	
	Anti-DR	Anti-monocyte
KT (RA)	3/3	4/4
TC (RA)	2/2	4/4
MS (RA)	4/4	3/3
WS (RA)	4/4	5/5
NB (RA)	6/6	4/4
EW (RA)	5/5	3/3
LE (C)	2/2	1/1
SL (C)	1/1	2/2
MM (C)	1/1	2/2
SF (C)	1/1	1/1
DP (C)	2/2	3/3
NF (C)	1/1	2/2

Elliot; 400g for 10 minutes). The specimens were fixed in alcohol and flooded with a drop of the recommended dilution of one of the monoclonal antibodies. After incubation at room temperature for 30 minutes, the specimens were thoroughly washed in PBS and were covered with a drop of rabbit anti-mouse IgG-FITC (fluorescein isothiocyanate) conjugate. This was left for 30 minutes at room temperature. The specimens were again washed on PBS and then left to dry. A drop of 95% glycerol/5% PBS was placed over each specimen and they were examined as described previously (Chap.11 Sec. 11 E i). The results are shown in Table 3.4. All the rosettes counted bore both HLA-DR and monocyte markers.

(E) Investigation of the density of Facb R^+ cells using Percoll

In functional studies, Percoll (density 1.062g/ml) was used to separate monocytic cells from mononuclear cell preparations. In one experiment it was noted that the %Facb R^+ cells in the lymphocyte preparation was severely depleted after removal of monocytes. It was decided to investigate this further. Mononuclear cells (MN) were obtained from freshly drawn venous blood by centrifugation through Ficoll (see Chap. 11 Sec 11 C ii). Facb rosttes were performed (Chap.11 Sec. 11 D i) and the remaining MN cell preparation was separated over 52% v/v Percoll (density 1.062g/ml). The two populations obtained (sample 2 : density $<$1.062g/ml; sample 3 : density $>$ 1.062g/ml) were washed and Facb rosettes were performed on both.

The results are shown in Table 3.5. Those subjects who had levels of Facb R^+ cells in the initial MN cell preparation above the normal range (0-4%; see Chap. vi Sec 1 A) showed a significant decrease ($p < 0.01$) in the total number of Facb R^+ cells in sample 3 compared with the initial MN cell preparation (sample 1). The number of these cells in sample 2 was not significantly different to that in sample 1. Thus, in this group, 64% of the total number of Facb R^+ cells

Table 3.5 Investigation of the density of Facb R^+ cells using Percoll

Initial number of Facb R^+ cells within normal range				Initial number of Facb R^+ cells above normal range			
	Sample				Sample		
Pa	1	2	3	Pa	1	2	3
B	0.21	0.10	0.15	A	1.70	1.12	0.51
D	1.04	0.32	0.54	C	1.24	0.68	0.35
E	0.73	0.22	0.32	F	0.97	0.48	0.18
G	0.27	0.14	0.19	I	1.70	1.27	0.10
H	0.95	0.21	0.41				
J	1.48	0.44	1.02				
n	6	6	6		4	4	4
$\bar{x}$	0.78	0.24 *	0.44		1.40	0.89	0.29 **
sd	0.48	0.12	0.32		0.36	0.37	0.18

Statistical analysis using Student's t-test

Significance was determined for each sample in relation to sample 1.

* $p < 0.05$

** $p < 0.01$

The results are express as total numbers of Facb R^+ cells X 10^{-4}.

KEY.

Pa Patient

Sample 1 - Total number Facb R^+ cells $\times 10^{-4}$ recovered from Ficoll.

2 - Total number Facb R^+ cells $\times 10^{-4}$: density < 1.062 g/ml.

3 - Total number Facb R^+ cells $\times 10^{-4}$: density > 1.062 g/ml.

applied to the Percoll separated out with the monocytes (ie. those cells with a density $< 1.062 g/ml$). In contrast, those subjects who had levels of Facb R^+ cells in sample 1 within the normal range showed a significant decrease in the total number of Facb R^+ cells ($p < 0.05$) in sample 2 compared with sample 1. The number of these cells in sample 3 was not significantly different to that in sample 1. Thus, in this group, 72% of the total number of Facb R^+ cells applied to the Percoll separated out with the lymphocytes, (ie. those cells with a density $> 1.062 g/ml$). Therefore, these results indicate that in patients with raised percentages of Facb R^+ cells, the latter are less dense than in normal, healthy controls or in patients with %Facb R^+ cells within the normal range.

SECTION 11

(A) Introduction

Studies of the functions mediated via the Facb receptor (or the cell on which it is expressed) were difficult to achieve owing to the problems encountered trying to produce an Facb R^+ cell-free lymphocyte suspension. Depletion of Facb R^+ cells by rosetting (Chap. 11 Sec. 11 F i) was inefficient owing to the fragility of the rosettes and removal of these cells by immobilized complexes (Chap.11 Sec, 11 F ii) resulted in a critical reduction of monocytes. Thus, an alternative method of Facb R^+ cell depletion was sought. Winrow (1982) has shown that the receptor which is capable of binding Facb exhibits different enzyme sensitivities to those receptors detected by whole IgG. These results indicated that the Facb receptor is biochemically different from other Fc receptors and thus it is feasible that it may also be antigenically distinct. Thus, an attempt was made to produce an antiserum specific for the Facb receptor. In order to do this it was necessary to produce a relatively pure preparation of the Facb receptor. This was initially attempted by subjecting lymphocytes to 'temperature shock' as described by Sarmay et al., (1978;1979).

(B) Fc R shedding induced by 'temperature shock'

MN cells were obtained as described previously (Chap. 11 Sec. 11 C ii) and Facb and EA rosettes were performed (Chap. 11 Sec. 11 D i). The cells were maintained at 0^oC for 1 hour and then transferred to an incubator at 37^oC for various lengths of time. After incubation, they were washed 3 times with PBS at 37^oC and Facb and EA rosettes performed. It was found that Fc receptors measured by EA rosettes decreased to a minimum value after 45 minutes incubation at 37^oC (see Fig 3.5). This decrease was significant in both rheumatoid ($p<0.005$) and control subjects

Figure 3.5. Effect of temperature shock on the %Facb and %EA rosettes

Lymphocytes were incubated at 0°C for 1 hour and then quickly transferred to 37°C where they were kept for 45 minutes. The cells were then washed and Facb and EA rosettes were performed as described previously (Chap. 11 Sec. 11 D ii). Facb and EA rosettes were also performed on duplicate samples which were not subjected to the 37°C incubation period.

Results

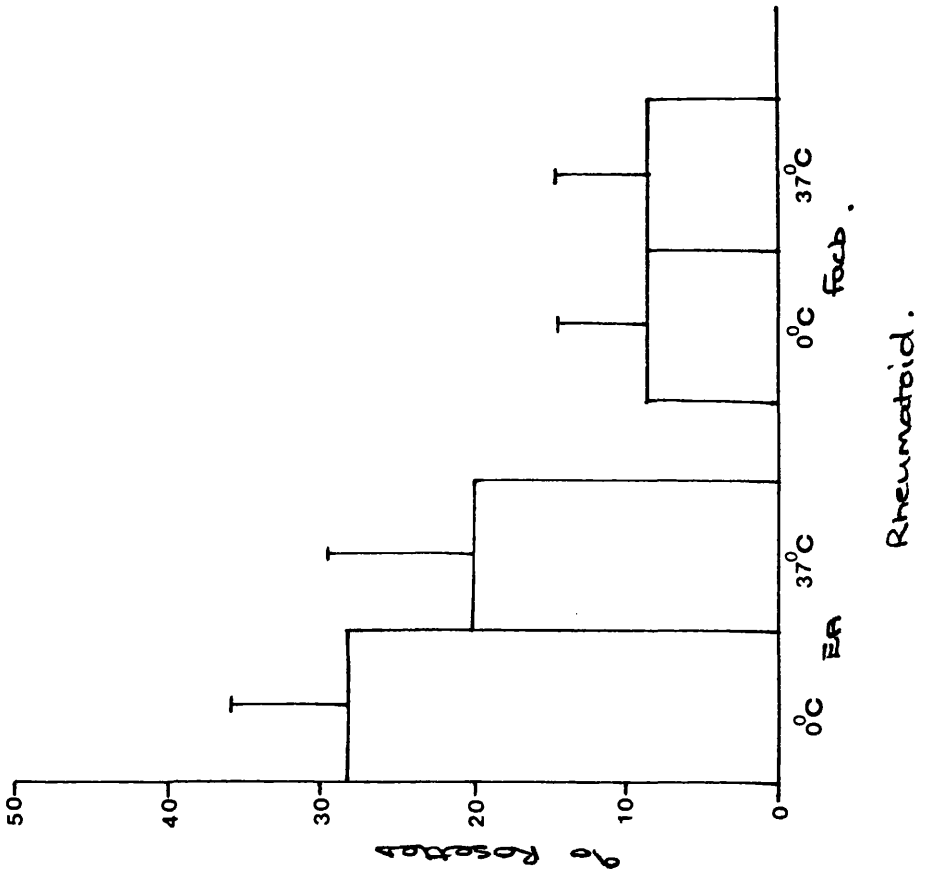
	Control				Rheumatoid			
	EA		Facb		EA		Facb	
	0°C	37°C	0°C	37°C	0°C	37°C	0°C	37°C
n	8	8	8	8	23	23	24	24
$\bar{x}$	23.6*	14.4 *	5.9**	5.3**	28.3+	20.2+	8.5 **	8.5**
sd	6.7	7.6	2.6	1.8	7.8	9.4	6.2	6.3

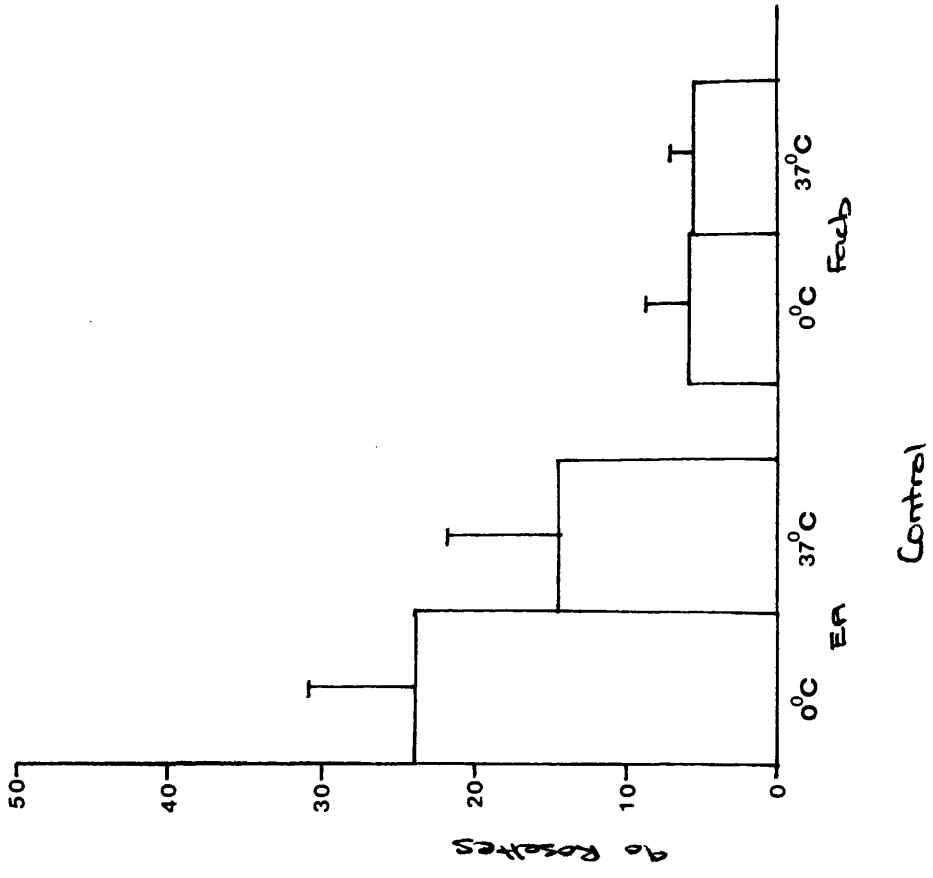
Significant results determined by Student's t-test

*- $p < 0.025$

**- p not significant

+- $p < 0.005$


% rosettes
50
40
30
20
10
0
0°C
37°C
EA
0°C
37°C
Fcab
Rheumatoid.


% rosettes
50
40
30
20
10
0
0°C
37°C
EA
0°C
37°C
Fcab
Control

($p < 0.025$). However, the Fc receptors which bound Facb could not be induced to shed. These results gave a further indication that the Facb receptor is different to the majority of Fc receptors. However, since neither the Facb receptors were shed, nor could the remaining Fc receptors be removed from the other lymphocytes, it was not possible to use such a preparation to produce an antiserum. Thus, an alternative source of Facb receptor was sought.

(C) Investigation of an antiserum to the Fc receptor on a K562 cell line

Winrow (1982) reported that K562 cells did not bear a receptor which bound Facb. However, this author found that 52% of the cells from a second K562 cell line formed Facb rosettes (see Table 3.6). An antiserum to this line was prepared in rabbits by Mr K Narendran (Bath University Biochemistry Group) by repeated i.m injections of K562 FCR (obtained by non-ionic detergent solubilization of K562 cells) bound to Sepharose coupled rabbit IgG.

Facb and EA rosettes were performed (Chap. 11 Sec. 11 D i) on several lymphocyte preparations (obtained by Ficoll-Paque separation of heparinized blood; Chap.11 Sec. 11 C ii). Different volumes of the $F(ab')_2$ fragment of the anti-K562 FcR IgG were added to 2×10^6 lymphocytes from each subject. The cells were incubated at room temperature for 30 minutes, washed 3 times in PBS and Facb and EA rosettes were performed.

The results show that the anti-K562 Fc R antiserum significantly inhibited Facb rosettes in both rheumatoids ($p < 0.01$) and controls ($p < 0.05$). It did not affect the percentage of EA rosettes detected (Table 3.7). In each group, at the highest concentration of antiserum tested, the decrease in the percentage of Facb rosettes (4.8 in rheumatoids; 2.3 in controls) is comparable to the decrease in %EA rosettes (5.2 in rheumatoids; 1.8 in controls). Thus, these results suggest that the rabbit anti-K562 FcR antiserum specifically inhibited Facb rosettes.

Table 3.6 The %Facb R^+ cells in two separate K562 cell lines

One cell line was grown at the Royal National Hospital for Rheumatic Diseases (Hosp.) and the other was donated from the Biochemistry Department of Bath University (Univ.).

	K562 Hosp		K562 Univ	
	Facb	EA	Facb	EA
	57	57	4	56
	57	65	6	64
	54	77	8	53
	36	39	2	54
	55	58	10	68
n	5	5	5	5
$\bar{x}$	51.8	59.2	6.0	59.0*
sd	8.9	13.8	3.2	6.6

Statistical analysis using Student's t-test

* $p<0.001$ EA v Facb K562 Univ.

Table 3.7 Effect of rabbit $F(ab')_2$ anti-K562 FcR on Facb and EA rosettes

	Volume antiserum (μl)						
	0	10	20	25	50	100	200
Facb							
Rheumatoid							
n	9	4	-	4	9	-	-
$\bar{x}$	6.6	2.8	-	2.0	1.8*	-	-
sd	3.5	1.0	-	1.4	1.7	-	-
Control							
n	5	-	3	-	5	3	3
$\bar{x}$	3.4	-	2.3	-	1.2	1.7	0.7**
sd	0.9	-	1.2	-	1.1	0.6	1.2
EA							
Rheumatoid							
n	9	4	-	-	9	-	-
$\bar{x}$	22.0	15.0	-	-	16.8	-	-
sd	8.8	3.9	-	-	6.7	-	-
Control							
n	5	-	3	-	5	3	3
$\bar{x}$	11.8	-	11.3	-	11.4	10.7	10.0
sd	2.9	-	3.8	-	3.2	4.0	4.6

Statistical analysis using Student's t-test

The significance of the results is determined with reference to the initial rosette values where no antiserum was present.

* $p < 0.01$

** $p < 0.05$

CHAPTER IV

Facb R^+ cells in mice - a functional study

(A) Introduction

Animals have been used to examine various immunological phenomena for a long time. Whilst no-one would suggest that observations made in animal models give all the answers about the analogous human situation, such observations are extremely useful in indicating the direction in which human studies should progress.

The presence of null cells in mice had been reported by several workers (Warr et al., 1978; Schrader and Nossal, 1980). Since mice are relatively easy to manipulate, it was decided to look for the presence of Facb R^+ cells in mice.

(B) Facb R^+ cells in mice

Single cell suspensions of mouse splenic lymphocytes were obtained from male BALB/c (Olac,Bicester) and CFLP (Bath University out-bred stock) mice (Chap. 11 Sec. 11 C iii) and Facb rosettes were performed (Chap.11 Sec. 11 D i). Facb R^+ cells were found in both BALB/c and CFLP mice (Table 4.1).

The presence of Facb R^+ cells in mice meant that studies could be undertaken to determine a possible function for these cells. Winrow (1982) reported the findings from a preliminary experiment in which human control subjects were given a skin test (Tine test for tuberculosis associated antigens). Those individuals who showed a positive skin test response also showed an increase in %Facb R^+ cells in the peripheral blood. It was decided to investigate this response more fully in mice.

(C) Effect of a delayed-type hypersensitivity (DTH) response on the %Facb R^+ cells in mouse spleen

Table 4.1 Titration of Facb with BALB/c and CFLP lymphocytes

	Concentration of Facb			
	0	1/50	1/100	1/150
BALB/c	% Rosette positive cells			
n	5	5	5	5
$\bar{x}$	0	2.6	2.4	0.6
s±d	0	1.1	1.1	0.5
CFLP	% Rosette positive cells			
n	10	10	10	10
$\bar{x}$	0	1.3	1,1	0.3
s±d	0	0.5	0.6	0.5

i)DTH response to oxazolone (4-ethoxymethylene-2-phenyl-oxazol-5-one)

A solution was prepared of oxazolone in 50% acetone/50% ethanol (30mg/ml). The hair was removed from the abdominal wall of CFLP mice and 0.1ml of the oxazolone solution was spread over the nude area. After 5 days, 250 μg oxazolone in 50% acetone/50% olive oil was painted on the right (test) ear of each mouse. The left (control) ear was painted with solvent only. Groups of animals were sacrificed on days 0,1,2,3 and 6 after challenge, their ear thickness measured and the percentage Facb R^+ splenic lymphocytes determined (Chap. 11 Sec. 11 C iii; 11 D i).

The mean thickness of the test ear was significantly increased 24 hours after challenge ($P<0.005$) and had returned to a normal value by day 6 (Figure 4.2). No significant change was observed in the %Facb R^+ cells.

ii)DTH response to SRBC

CFLP mice were injected intra-peritoneally (ip) with 10^6 SRBC in 0.2ml PBS and were challenged 5 days later with 10^6 SRBC (in 0.1ml PBS) in the test foot pad and with 0.1ml PBS in the control foot pad. Animals were sacrificed on days 0,1,3,4,5 and 7 after challenge, their foot pad thickness measured and the %Facb $R^{\pm}$ splenic lymphocytes determined (Chap. 11 Secs. 11 C iii; 11 D i)

The mean thickness of the test foot pads was significantly increased 24 hours after challenge ($P<0.005$) and had returned to normal by day 7. No significant change in %Facb R^+ cells was observed (Figure 4.2). These results and those in Section 1 C i showed that it was not possible to elicit a change in the %Facb R^+ splenic lymphocytes in mice by a simple DTH response. Recently, it has been shown that the response in humans to the purified protein derivative of tuberculin (PPD) involves both a humoral and a cell-mediated response (Neveu, Buscot and Soulillou, 1980; Ringden et al., 1979). For this reason, primary and secondary humoral responses were induced in mice to investigate the effect this might have on the %Facb R^+ splenic lymphocytes.

Figure 4.1 Effect of a DTH reaction to oxazolone on the %Facb R^+ cells in mouse spleen

Male CFLP mice were sensitised epicutaneously to oxazolone and challenged 5 days later on the right ear. The left ear was treated with solvent only as a control. Mice were sacrificed by cervical dislocation, their spleens removed, (For %Facb R^+ cell determination) and their ear thickness measured (by micrometer gauge)

Results

The results are expressed as the mean +/- one standard deviation.

Day	n	%Facb	Control ear mm	Test ear mm	*p
0	7	2.4±1.1	0.26±0.05	0.26±0.05	NS
1	7	1.6±0.5	0.26±0.05	0.70±0.10	<0.005
2	7	2.7±1.0	0.26±0.08	0.56±0.05	<0.005
3	7	2.4±0.8	0.26±0.05	0.31±0.04	NS
6	7	2.3±0.7	0.25±0.05	0.25±0.05	NS

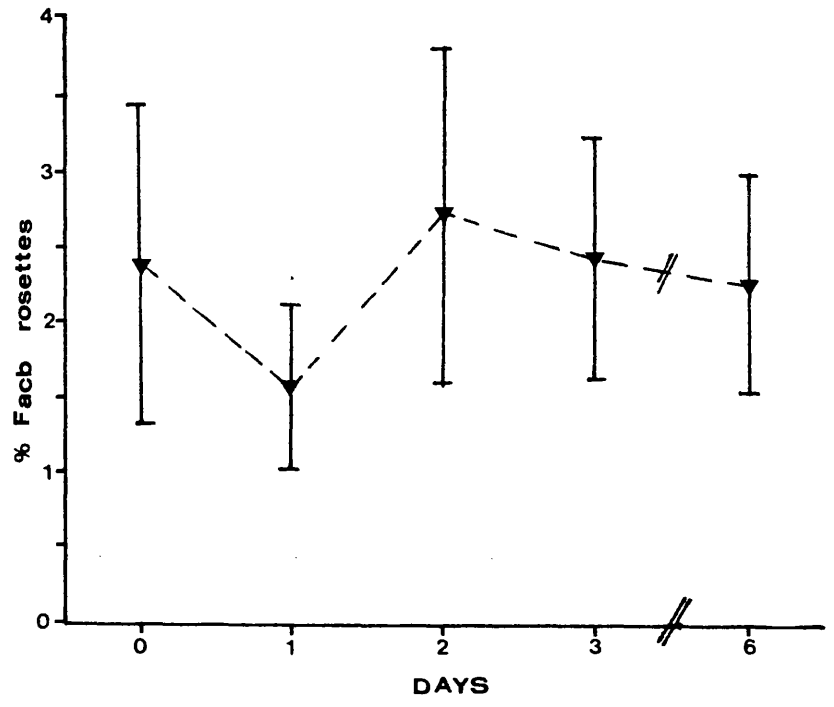
* Determined by Student's t- test

KEY

▼ %Facb R^+ cells

△ Test ear thickness (mm)

● Control ear thickness (mm)


% Facb rosettes
0
1
2
3
4
0
1
2
3
6
DAYS

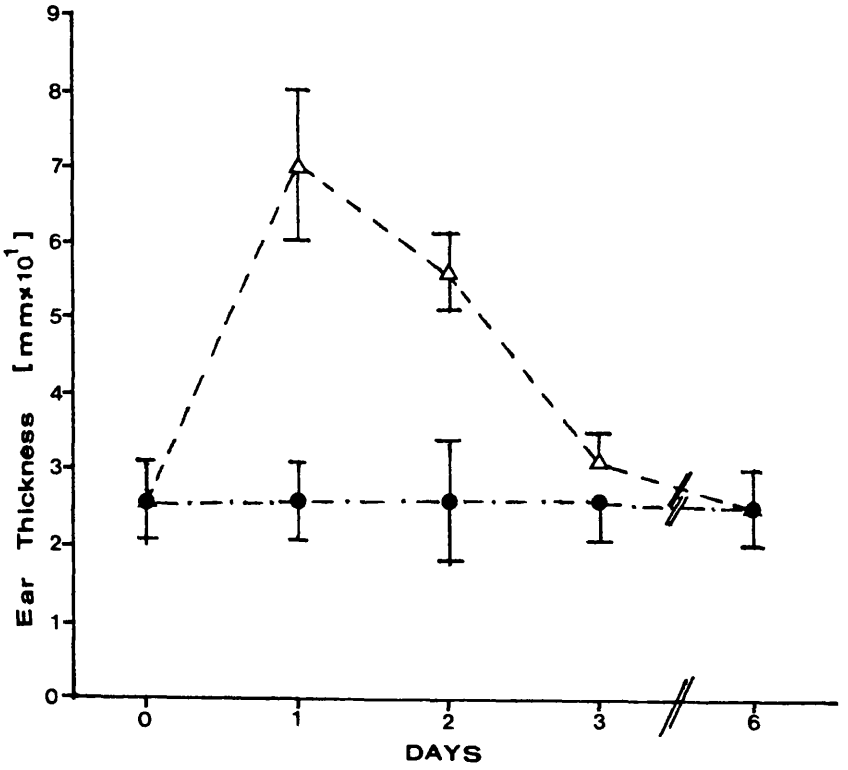

Ear Thickness [mm x 10⁻¹]
0
1
2
3
4
5
6
7
8
9
0
1
2
3
6
DAYS

Figure 4.2 Effect of a DTH reaction to SRBC on %Facb R^+ cells in mouse spleen.

CFLP mice were injected ip with 10^6 SRBC and challenged in the foot pad 5 days later with either 10^6 SRBC (test foot pad) or PBS (control foot pad). Animals were sacrificed by cervical dislocation, their spleens removed (for %Facb R^+ cell determination) and their foot pad thickness measured (by micrometer gauge).

Results

All results are expressed as the mean +/- one standard deviation.

Day	n	%Facb	Control ear	Test ear	*p
0	6	3.2±1.7	2.8±0.2	2.8±0.2	NS
1	6	-	2.7±0.4	5.5±0.5	< 0.005
3	6	2.5±1.4	2.8±0.6	3.3±0.4	NS
4	6	3.3±1.0	-	-	
5	6	3.0±1.1	3.0±0.5	2.9±0.5	NS
7	6	2.7±1.0	2.6±0.6	2.6±0.3	NS

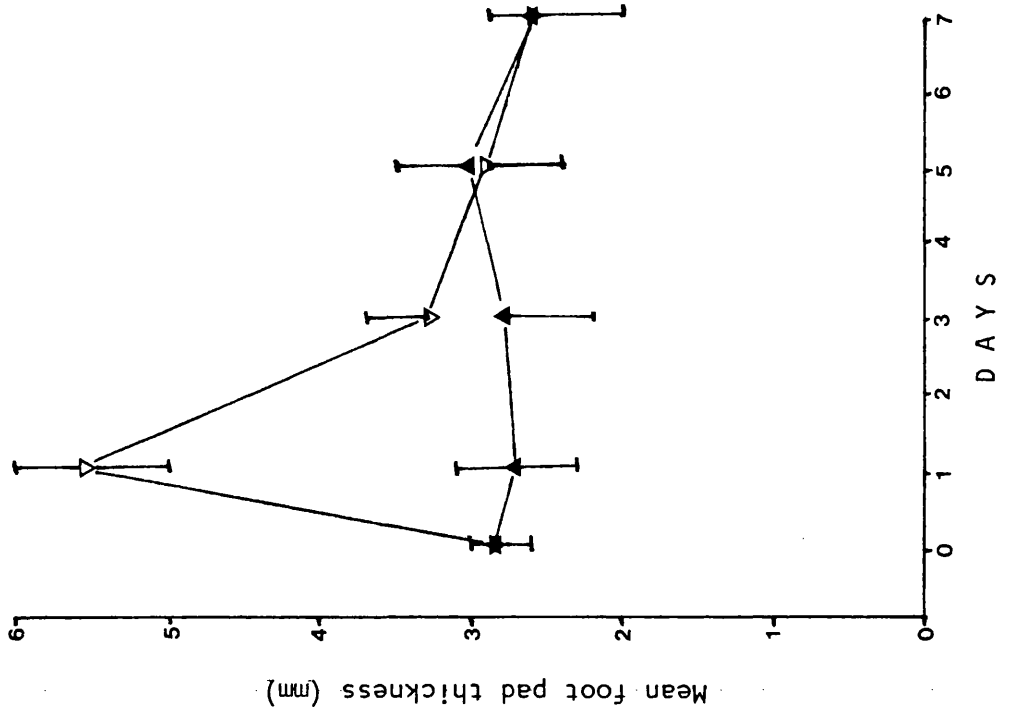
* Determined by Student's t-test.

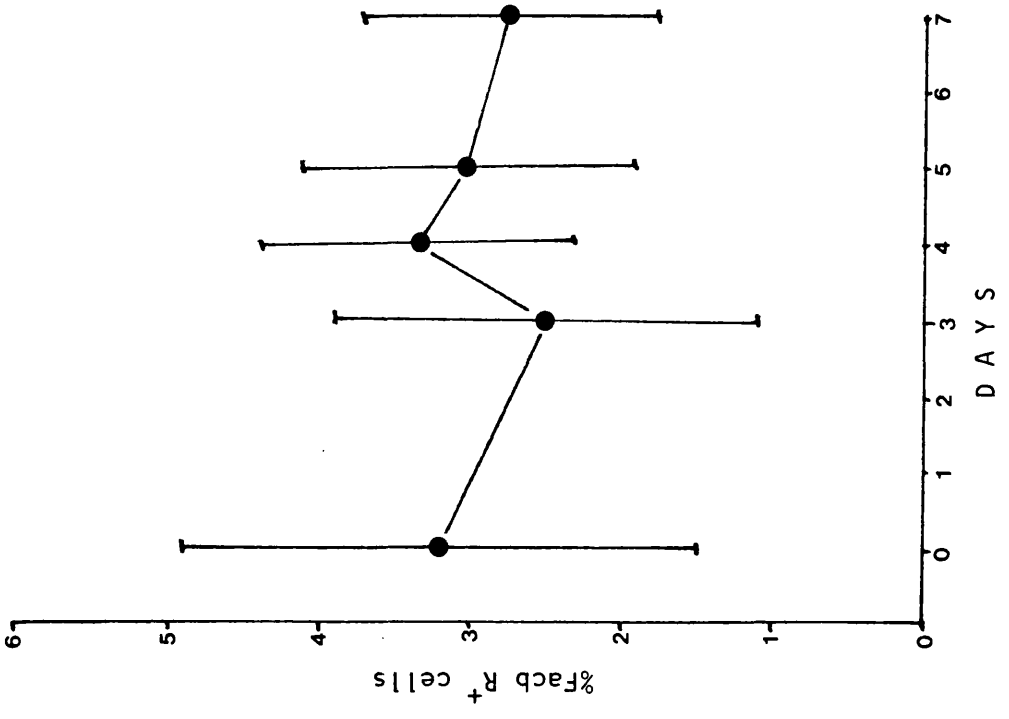
KEY

● %Facb R^+ cells

▲ Control foot pad thickness (mm)

▽ Test foot pad thickness (mm)


Mean foot pad thickness (mm)
0
1
2
3
4
5
6
7
DAYS


%Facb R+ cells
0
1
2
3
4
5
6
7
DAYS

(D) The effect of primary and secondary immunization on the %Facb R^+ splenic lymphocytes.

i) SRBC as immunogen

The immunization schedule and experimental protocol are described in the legend to Figure 4.3. Both CFLP and BALB/c mice were used. Briefly, mice were given either a primary of secondary immunization with SRBC and were sacrificed at regular intervals after the final injection. The %Facb R^+ splenic lymphocytes and the numbers of direct and indirect PFC were determined (Chapter 11 Sections 11 D i: 11 H iii).

Neither CFLP nor BALB/c mice showed any change in the %Facb R^+ splenic lymphocytes after primary immunization with SRBC (Figures 4.4a and 4.5a). The number of SRBC-specific direct PFC in BALB/c spleens showed a significant rise by day 1 after immunization which had not peaked by day 4 (Figure 4.4b). These results indicated that a primary immune response to SRBC had been elicited. Three days after secondary immunization, both CFLP and BALB/c mice showed a significant increase ($P<0.05$ and $p<0.001$ respectively) in %Facb R^+ cells (Figures 4.4a and 4.5b). The numbers of SRBC-specific direct and indirect PFC in BALB/c spleens showed significant increased 2 days after challenge; the direct PFC reaching a peak at day 3 (Figure 4.4b) whilst the number of indirect PFC was still increasing on day 5 (Figure 4.4c). These results suggested that a secondary immune response had occurred. Thus, the %Facb R^+ splenic lymphocytes in mice increased 3 days after a secondary challenge with SRBC. However, this alteration may have simply been non-specific owing to the recent priming of the mice (Shortman, Howard and Baker, 1978; Shortman, Howard, Teale and Baker, 1979). In order to determine whether or not this was true, mice were challenged with either a cross-reacting or non cross-reacting antigen after first being primed with SRBC.

Figure 4.3 Schematic representation of primary and secondary immunization in mice

Male CFLP and BALB/c mice were injected intraperitoneally (ip) with either 0.2ml of PBS or 0.2ml of an SRBC suspension in PBS containing 5×10^9 SRBC/ml. Fourteen days later all mice were given 10^9 SRBC in 0.2ml PBS (ip). Mice were sacrificed at various intervals after the second injection by cervical dislocation. Their spleens were removed and single cell suspensions obtained (Chap.11 Sec. 11 C iii) from which the %Facb R^+ splenic lymphocytes was determined (Chap.11 Sec. 11 D i). The numbers of direct and indirect plaque forming cells (PFC) in the BALB/c splenocyte suspensions were determined (Chap.11 Sec. 11 H iii).

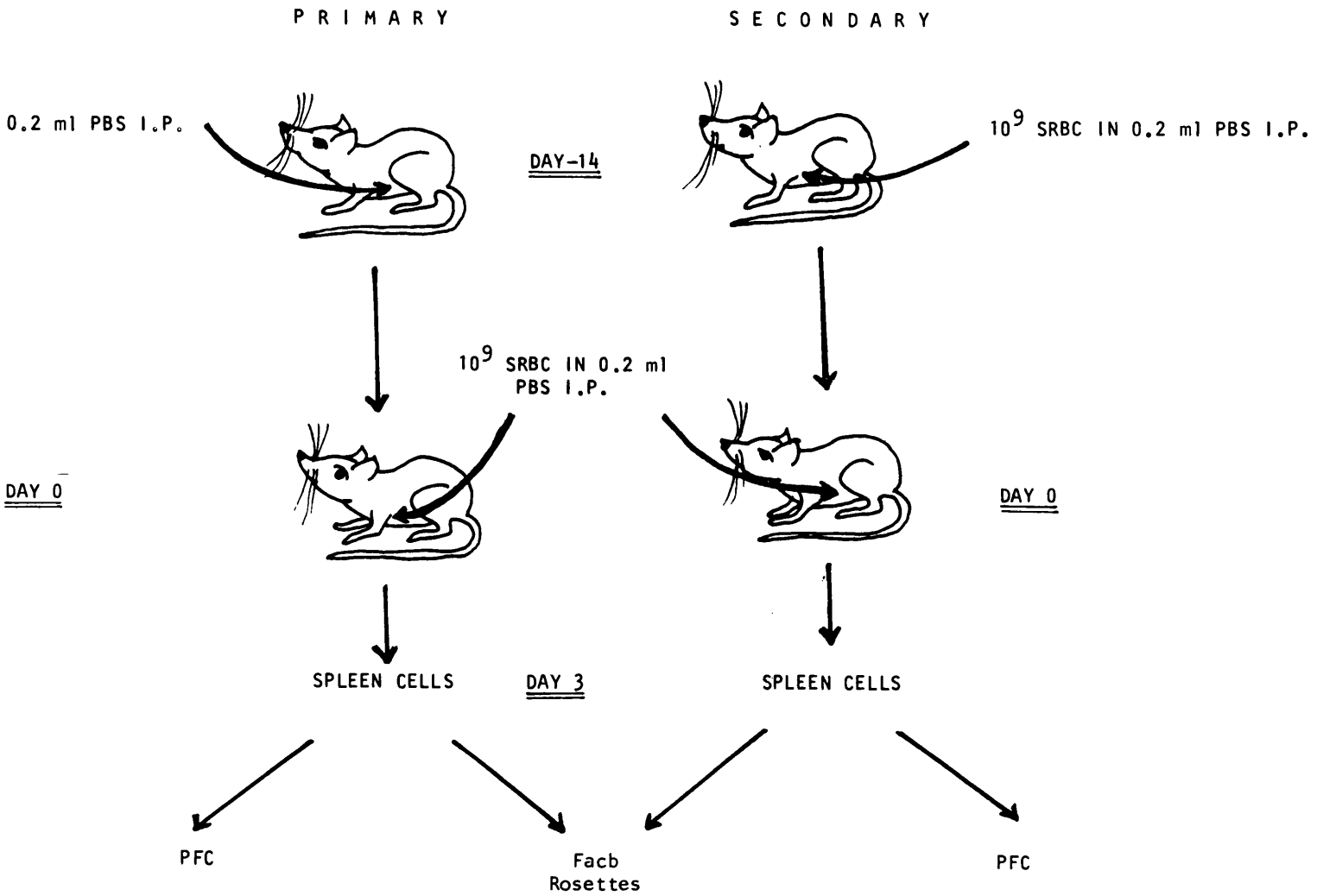

PRIMARY
SECONDARY
0.2 ml PBS I.P.
DAY-14
10^9 SRBC IN 0.2 ml PBS I.P.
10^9 SRBC IN 0.2 ml PBS I.P.
DAY 0
DAY 0
SPLEEN CELLS
DAY 3
SPLEEN CELLS
PFC
Facb Rosettes
PFC

Figure 4.4 Effect of primary and secondary immunization on the %Facb R^+ splenic lymphocytes in BALB/c mice.

Male BALB/c mice were immunized with SRBC as descrided in the legend to Figure 4.3. After sacrifice, the %Facb R^+ cells and the number of direct and indirect PFC in each splenocyte suspension were determined (Chap. 11 Secs. 11 D i; 11 H iii).

Results

All results are expressed as the mean +/- 1 standard deviation.

Primary immunization

Day	n	%Facb	Direct PFC/10^6 spleen cells	Indirect PFC/10^6 spleen cells
0	10	1.8±0.9	360 ± 118	-
1	10	1.7±0.7	1012 ± 598 *	-
3	10	1.6±0.5	3550±1298 *	-
4	10	1.4±1.0	8265±2622 *	-

Secondary immunization

0	10	2.2±0.6	184 ± 70	160 ± 91
2	10	2.1±0.9	201 ± 62	120 ± 71
3	10	6.1±1.1 *	2957±1027 *	2481 ± 803 *
4	10	4.0±0.9 *	2868±1141 *	4437±1634 *
5	10	2.5±1.0	2271±1056 *	4466±1813 *

Statistical analysis using Student's t-test

* $p<0.001$ when compared with day 0 values.

KEY

● Primary immunization

▲ Secondary immunization

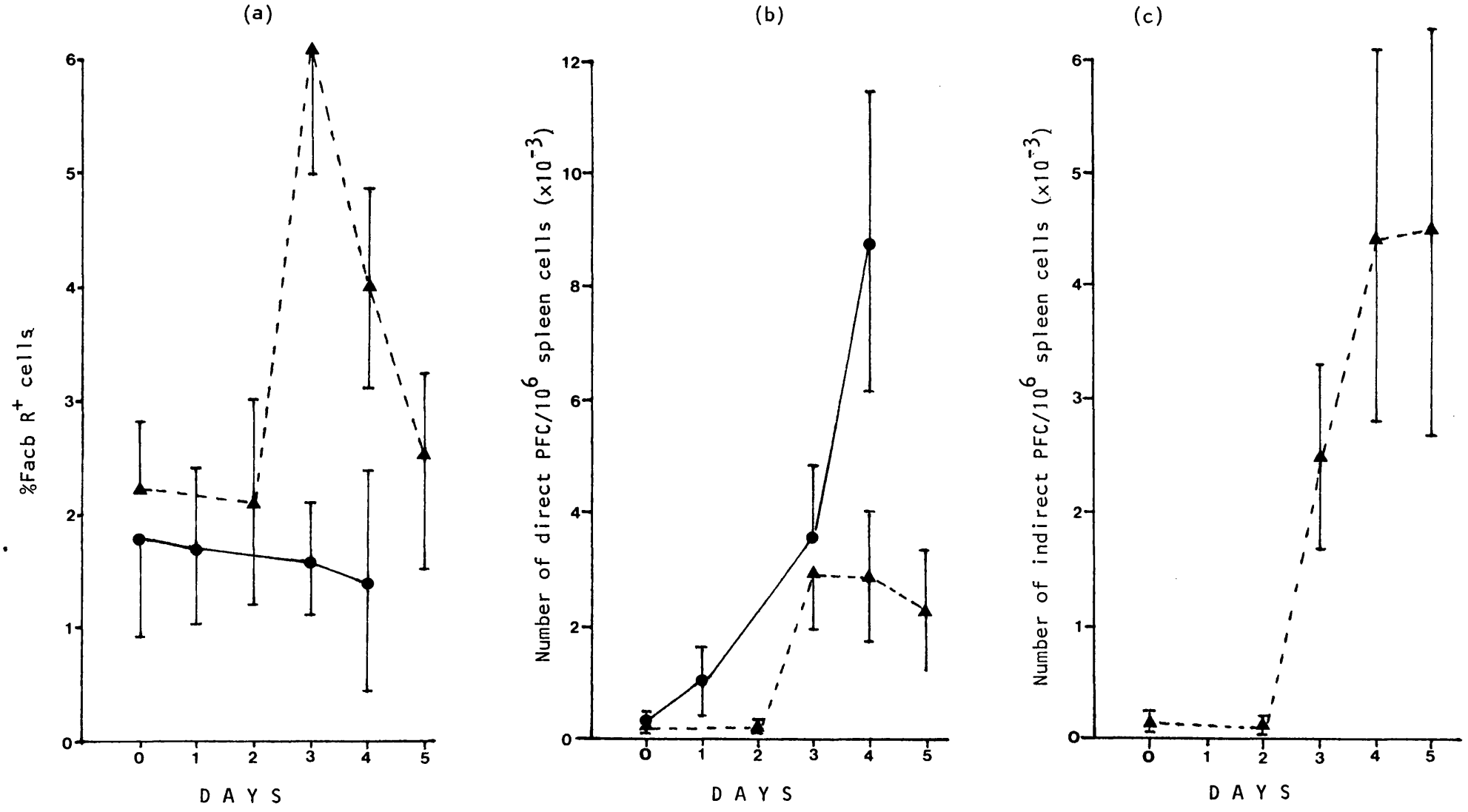

(a)
%Facb R+ cells
0
1
2
3
4
5
6
DAYS
(b)
Number of direct PFC/10^6 spleen cells (x10^-3)
0
2
4
6
8
10
12
DAYS
(c)
Number of indirect PFC/10^6 spleen cells (x10^-3)
DAYS

Figure 4.4. Effect of primary and secondary immunization on the %Facb R^+ cells in the spleens of CFLP mice

Male CFLP mice were immunized with SRBC as described in the legend to Figure 4.3. After sacrifice the %Facb R^+ cells was determined (Chap.11 Sec. 11 D ii)

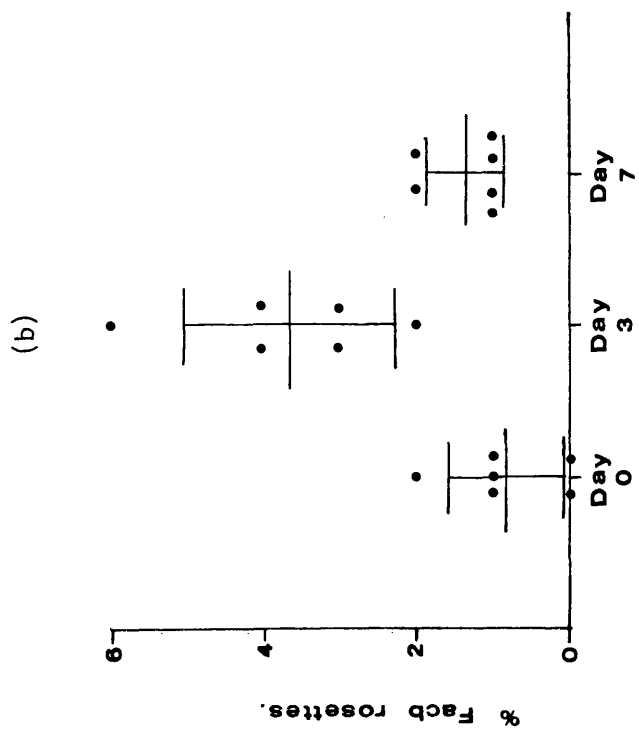
Results

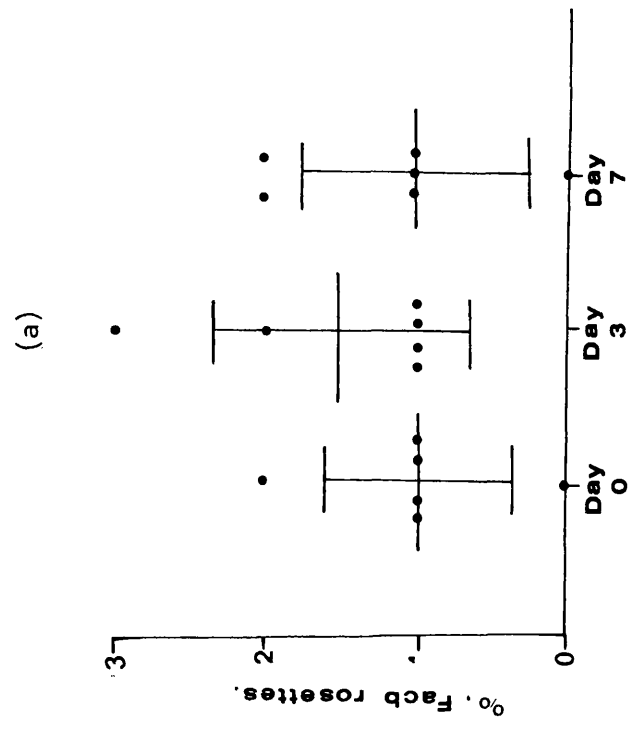
All the results are expressed as the mean +/- 1 standard deviation.

Day	n	%Facb R^+ cells	
		Primary response	Secondary response
0	6	1.0±0.6	0.8±0.8
3	6	1.5±0.8	3.7±1.4 *
7	6	1.0±0.8	1.3±0.5

Statistical analysis using Student's t-test

* $p<0.05$ when compared with day 0 values.


(b)
% Facb rosettes.
0
2
4
6
Day 0
Day 3
Day 7


(a)
% Facb rosettes.
0
1
2
3
Day 0
Day 3
Day 7

ii) The effect of the identity of the challenging antigen on the %Facb R^+ splenic lymphocytes in mice

Male BALB/c mice were given ip injections of 10^9 SRBC in 0.2ml PBS. Fourteen days later groups of animals were challenged with either 10^9 SRBC, 10^9 goat RBC (GRBC; cross-reacting) or 10^9 chicken RBC (ChRBC; non cross-reacting). Animals were sacrificed on days 0 and 3 after challenge. A single cell suspension was prepared from the spleen of each animal (Chap. 11 Sec. 11 C iii) and the %Facb R^+ cells was determined (Chap. 11 Sec. 11 D i). The numbers of direct and indirect PFC were enumerated (Chap. 11 Sec. 11 H iii) using as target cells the same type of RBC used for the secondary challenge.

After 3 days, those animals which received a challenge dose of SRBC or the cross-reacting antigen GRBC, showed a significant increase in the %Facb R^+ cells and in the number of direct and indirect PFC (Figure 4.6; $p < 0.005$). At this time, no significant change was observed in the %Facb R^+ cells or in the numbers of direct and indirect PFC as a consequence of boosting the mice with ChRBC. Thus, those mice boosted with either SRBC or GRBC mounted a secondary response to these antigens and showed a significant increase in the %Facb R^+ cells. In contrast, those challenged with ChRBC only mounted a primary response and showed no change in the %Facb R^+ cells. These results indicate that the %Facb R^+ splenic lymphocytes increased as a result of secondary immunization and not merely because of a non-specific effect due to recent priming. The observed increase in %Facb R^+ cells may have been related to the induction of IgG synthesis or to the development of antigen specific memory cells. In order to determine which of these two alternatives was correct, lipopolysaccharide (LPS) from Escherichia coli was used as an immunogen. LPS is a polyclonal B cell activator (PBA) which stimulates B cells to produce both IgM and IgG (Anderson et al., 1972).

Figure 4.6 Effect of the identity of the challenging antigen on %Facb R^+ splenic lymphocytes in BALB/c mice

Mice were immunized with 10^9 SRBC and challenged with either SRBC, GRBC (cross-reacting) or ChRBC (non cross-reacting), 14 days later. The %Facb R^+ splenic lymphocytes and the numbers of direct and indirect PFC were determined 0 and 3 days after challenge (Chap.11 D i; 11 H iii).

Results

All results are expressed as the mean +/- 1 standard deviation.

Challenge antigen	n	%Facb		Number of PFC/10^6 spleen cells			
				Direct		Indirect	
		Day 0	Day 3	Day 0	Day 3	Day 0	Day 3
Sheep	10	1.8±0.9	4.1±1.2*	360±118	2455 ± 506*	137±160	4728±2162*
Goat	10	1.8±0.9	3.7±1.3*	360±118	3859±2612*	137±160	4515± 679*
Chicken	10	1.8±0.9	1.1±0.6+	360±118	686± 623+	137±160	148± 137+

Statistical analysis using Student's t-test

* $p < 0.005$

} —— when compared with day 0 values.

+ p not significant

KEY

■ Goat

▲ Sheep

● Chicken

} —— challenging antigen.

%Facb R+ cells
Number of direct PFC/10^6 spleen cells (x10^-3)
Number of indirect PFC/10^6 spleen cells (x10^-3)
1
2
3
4
5
6
0
3
DAYS

iii) LPS as antigen

Male BALB/c mice were immunized with 50μg LPS in 0.2ml PBS using the schedule described in Sec.1 D i (Figure 4.3). The numbers of direct and indirect anti-LPS PFC were enumerated using LPS-coupled SRBC (Chap.11 Sec. 11 H iii), the %Facb R^+ cells was determined as described previously (Chap.11 Sec. 11 D i).

The %Facb R^+ cells showed a slight increase on day 4 after a single injection of LPS and also after a second injection (Figure 4.7; $p<0.05$). This increase was significantly lower than that observed after secondary immunization with SRBC ($p<0.001$). Direct and indirect PFC were enhanced after both the first and the second injections of LPS. However, the peak indirect PFC response after the second injection was significantly lower ($p<0.05$) than that after a single injection.Thus, the %Facb R^+ cells increased after a single dose of LPS and therefore did not require the development of memory cells. Also, the reduced peak PFC responce observed after a second injection of LPS suggests that this antigen either leaves the cells refractory to a second challenge or that it does not efficiently elicit the development of memory cells. This would also suggest that the observed increase in %Facb R^+ cells is not dependent on an Ag specific response, but merely on the production of IgG. Thus, experiments were performed in order to determine whether or not Facb R^+ cells play a role in IgG production.

(E) The effect of Facb R^+ cell depletion on the secondary immune response of adoptively transferred splenic lymphocytes

The experimental protocol for the adoptive transfer is shown in Figure 4.8. Briefly, BALB/c mice were immunized with 10^9 SRBC ip. Fourteen days later mice were challenged with 10^9 SRBC ip and then sacrificed 3 days later, single cell suspensions being produced from their spleens (Chap.11 Sec. 11 C iii). 5×10^6 Facb R^+ cell depleted or 5×10^6 sham depleted lymphocytes were injected ip with 10^9 SRBC in naive recipients.

Figure 4.7 Effect of LPS on the %Facb R^+ splenic lymphocytes in BALB/c mice.

BALB/c mice were injected ip with either 0.2ml PBS or 50 g LPS in 0.2ml PBS. Fourteen days later all mice were given 50μg LPS in 0.2ml PBS ip. Groups of mice were sacrificed by cervical dislocation on days 0,2,3 and 4 after the second injection. The spleens were removed and the single cell suspensions obtained (Chap,11 Sec. 11 C iii) were used to determine the %Facb R^+ cells and the numbers of direct and indirect PFC (Chap.11 Sec. 11 D i; 11 H iii).

Results

All results are expressed as the mean +/- 1 standard deviation.

Primary injection

Day	n	%Facb	Number of PFC/10^6 spleen cells	
			Direct	Indirect
0	8	2.0±0.9	2113± 342	869 ± 573
2	10	2.1±0.3	3049± 979	1081±1015
3	10	2.6±1.2	4501±1618	4754±2968
4	10	3.2±1.2*	3207± 2493	2431±1331

Secondary injection

0	8	2.0±0.9	1086± 383	260± 243
2	10	2.2± 0.4	3332±1244	1211± 888
3	10	3.1±2.0	4731±2202	1972±1859
4	10	3.3±1.1*	2520±1214	1466±1210

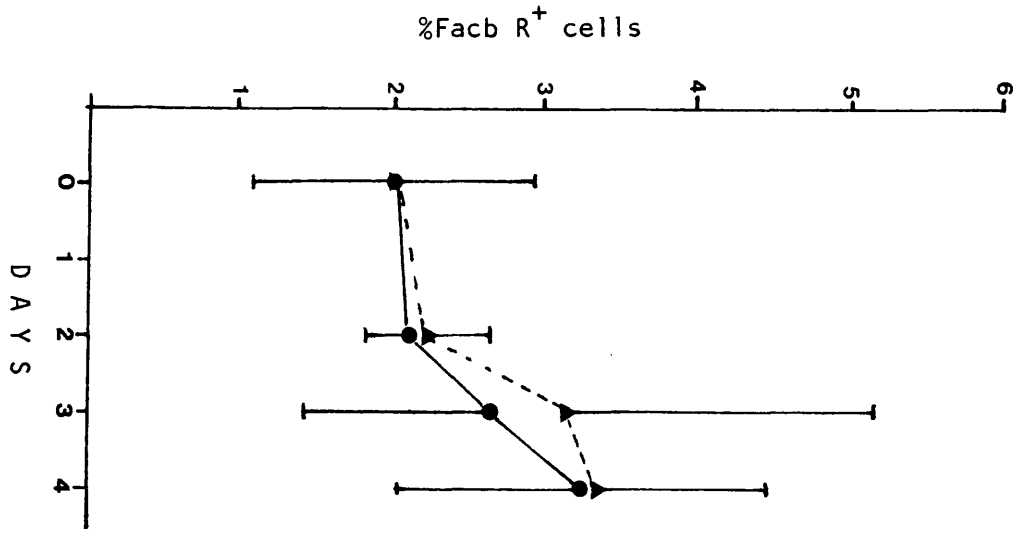
Statistical analysis using Student's t-test

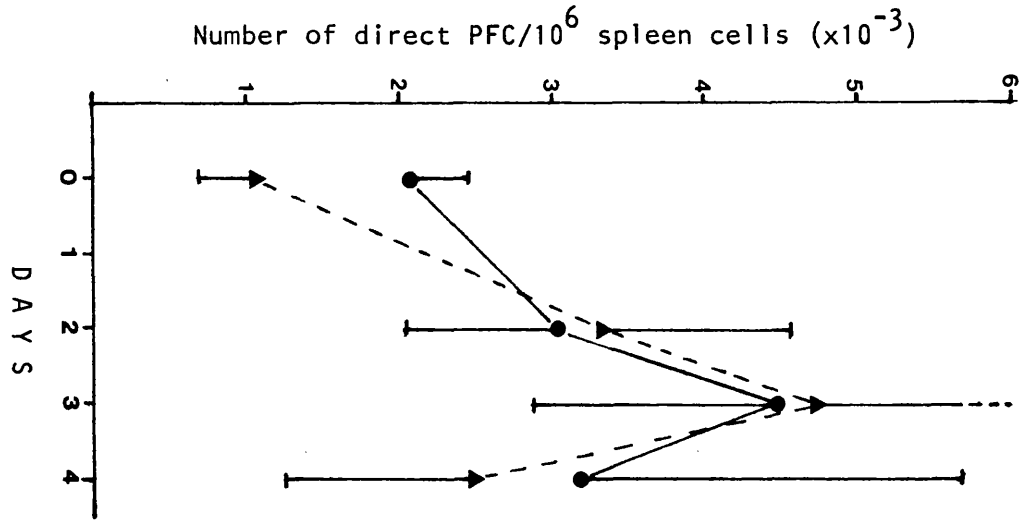
* $p<0.05$ compared with the day 0 value.

KEY

● Single injection of LPS

▲ Two injections of LPS


%Facb R+ cells
1
2
3
4
5
6
DAYS
0
1
2
3
4


Number of direct PFC/10^6 spleen cells (x10^-3)
1
2
3
4
5
6
DAYS
0
1
2
3
4

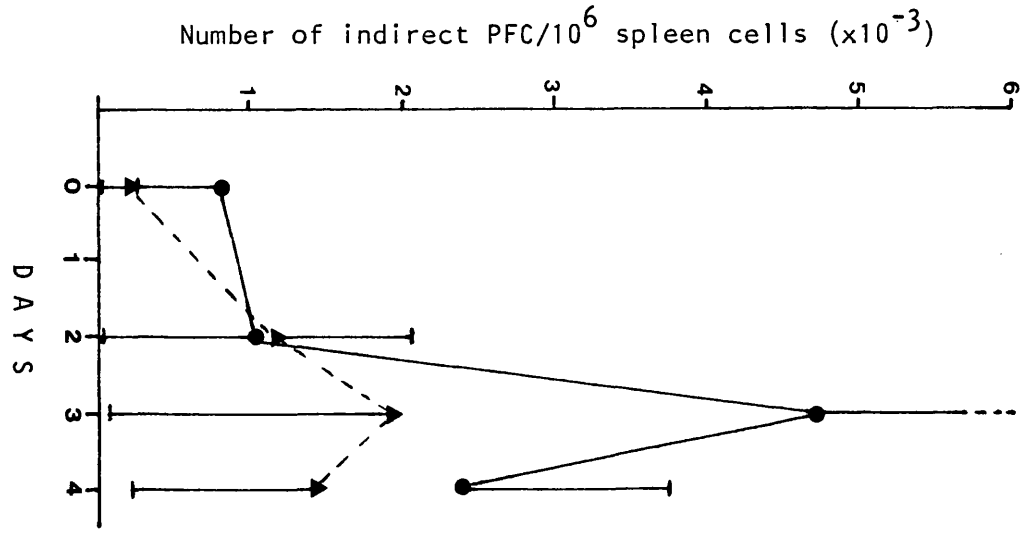

Number of indirect PFC/10^6 spleen cells (x10^-3)
1
2
3
4
5
6
DAYS
0
1
2
3
4

Figure 4.8 Experimental protocol for the adoptive transfer of splenic lymphocytes

Male BALB/c mice were injected ip with 10^9 SRBC in 0.2ml PBS. Fourteen days later they were challenged with 10^9 SRBC in 0.2ml PBS ip. After 3 days, the mice were sacrificed by cervical dislocation, their spleens removed and single cell suspensions of splenic lymphocytes were prepared (Chap.11 Sec. 11 C iii). Each suspension was divided into two aliquots one being depleted of Facb R^+ cells by separation of rosettes over Ficoll-Paque (Chap.11 Sec. 11 D i; 11 F ii) and the other being 'sham depleted' by rosetting with non-sensitized CRBC. The cells at the buffer-Ficoll interface were harvested, washed 3 times in PBS (10 minutes; 400g) and then injected ip into naive recipients (5×10^6 cells in 0.2ml PBS) with 10^9 SRBC. Eight days after transfer, the recipients were sacrificed by cervical dislocation and the numbers of direct and indirect PFC in single cell suspensions of splenic lymphocytes were determined (Chap.11 Sec. 11 H ii).

ADOPTIVE TRANSFER

Day -14

10^9 SRBC I.P. IN 0.2 ml PBS

Day 0

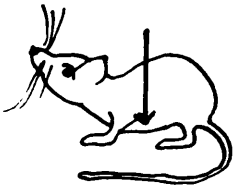
10^9SRBC I.P. IN 0.2 ml PBS

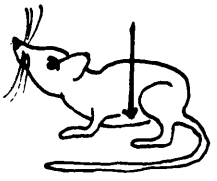
Day 3

SPLEEN CELLS

5×10^6 SHAM DEPLETED LYMPHOCYTES + 10^9 SRBC, I.P.

5×10^6 FacbR DEPLETED LYMPHOCYTES + 10^9 SRBC, I.P.





Day 8

SPLEEN CELLS

SPLEEN CELLS

PFC

Facb Rosettes

PFC

Facb Rosettes

Control primary and secondary responses were also performed. 5×10^6 lymphocytes from naive mice or from day 3 secondarily challenged mice were transferred into naive recipients with 10^9 SRBC. Eight days later all the mice were sacrificed and the numbers of direct and indirect PFC determined (Chap.11 Sec. 11 H ii).The results are shown in Fig. 4.9.

Those mice injected with naive cells (Group A) or with cells which had been primed 3 days before transfer (Group B) showed normal primary and secondary responses (respectively) to SRBC. Although those mice which received sham depleted lymphocytes (Group C) produced lower levels of direct and indirect PFC than those of Group B, this was not satistically significant. Those mice which received Facb R^+ cell depleted lymphocytes (Group D) produced similar levels of direct and indirect PFC to Group B mice, but a significantly higher number of indirect PFC ($p<0.05$) compared to Group C mice. If Group C is considered to be the true control for Group D (since the cells underwent similar experimental procedures before adoptive transfer), the results appear to suggest that Facb R^+ cells limit the production of IgG. Since it has been shown that suppression of a humoral response may be mediated by Fc R present on the surface of lymphocytes (see Chap.1 Sec 11 D iii), experiments were designed to investigate the role of the Facb R in such phenomena.

(F) The effect of antigen specific Facb on the secondary immune response

Groups of BALB/c mice were immunized ip with 10^9 CRBC in 0.2ml PBS. Fourteen days later the mice were given ip injections of 10^9 CRBC in 0.2ml PBS either alone or with 4µg rabbit Facb anti-CRBC or 2µg rabbit IgG anti-CRBC. The mice were sacrificed before, and 3 days after challenge by cervical dislocation and single cell suspensions were prepared from their spleens (Chap.11 Sec. 11 C iii). The %Facb R^+ cells (Chap.11 Sec. 11 D i) and the numbers of direct and indirect PFC (Chap. 11 Sec. 11 H ii) were determined.

Figure 4.9 The effect of Facb R^+ cell depletion on the secondary immune response of adoptively transferred splenic lymphocytes

Naive mice were immunized with 10^9 SRBC and either naive cells, cells from mice challenged 3 days previously, Facb R^+ cell depleted lymphocytes or sham depleted lymphocytes.

Results

All results are expressed as the mean +/- 1 standard deviation.

Donor	Group	n	Number of PFC/10^6 spleen cells	
			Direct	Indirect
Naive mice	A	5	3029±1221	489± 739
D3 primed	B	4	1391± 561	2293±1670
Sham depleted D3 primed	C	5	969± 207	1172± 347
Facb depleted D3 primed	D	5	1224± 482	2514±1286

Statistical analysis using Student's t-test

Direct PFC: $p_{AvB}<0.05$

Indirect PFC: $p_{AvB}<0.05$; $p_{DvC}<0.05$

Key

 Indirect PFC

 Direct PFC

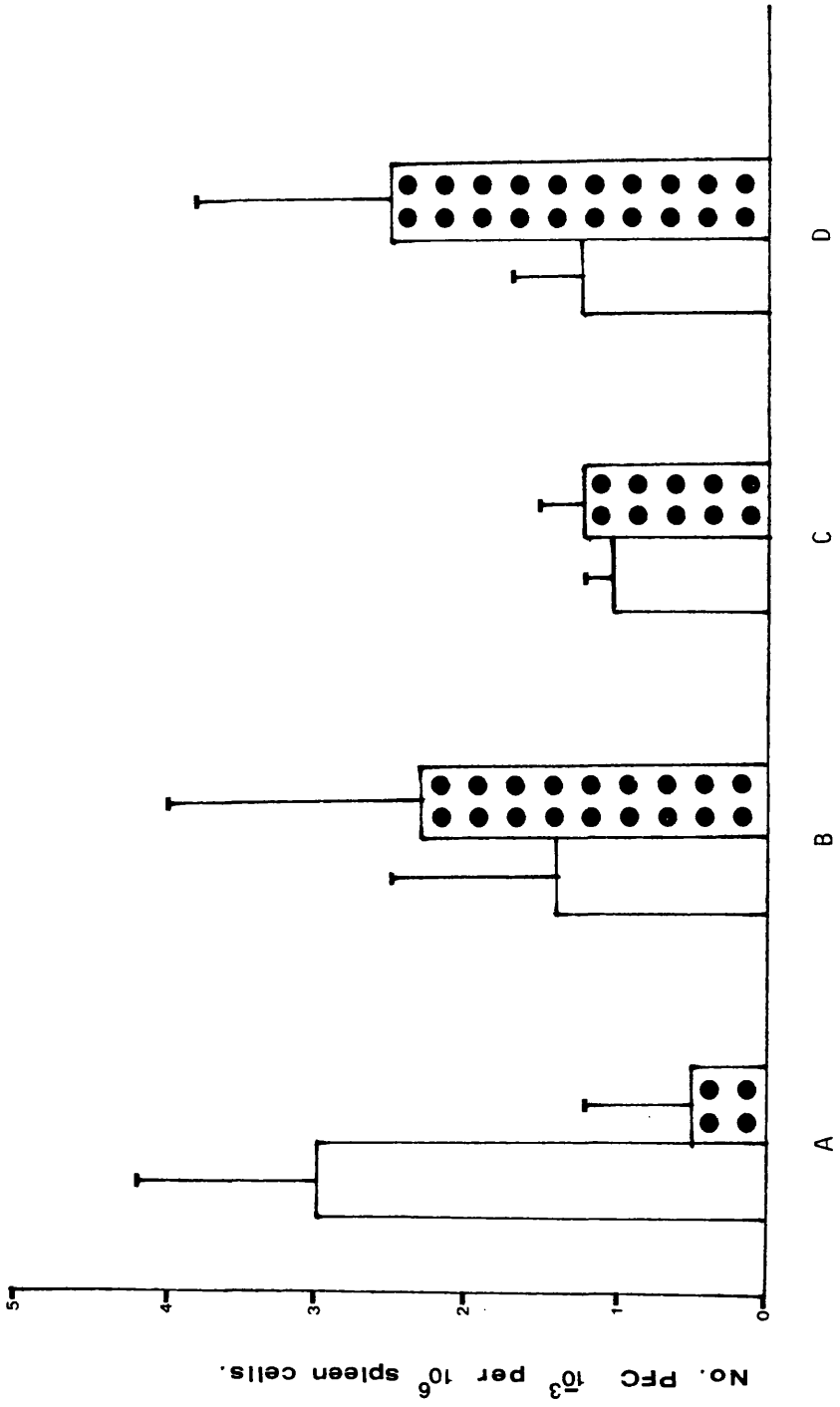

No. PFC 10^{-3} per 10^6 spleen cells.
0
1
2
3
4
5
A
B
C
D

Figure 4.10 The effect of antigen specific IgG and Facb on the secondary immune response of BALB/c spleen cells

BALB/c mice immunized with CRBC were challenged with CRBC alone or in conjunction with CRBC specific Facb or IgG. The %Facb R^+ splenic lymphocytes and the numbers of direct and indirect PFC were determined before and 3 days after challenge.

Results

All results are expressed as the mean +/- one standard deviation.

Group	n	%Facb		Number of PFC/10^6 spleen cells			
				Indirect		Direct	
		Day 0	Day 3	Day 0	Day 3	Day 0	Day 3
A CRBC	10	1.4±0.4	3.1±1.1	147±75	2806±660	23±35	3531±1478
B Facb	10	1.4±0.4	4.2±1.2	147±75	463±356	23±35	29± 61
C IgG	10	1.4±0.4	4.0±1.8	147±75	665±561	23±35	450± 836

Statistical analysis using Student's t-test

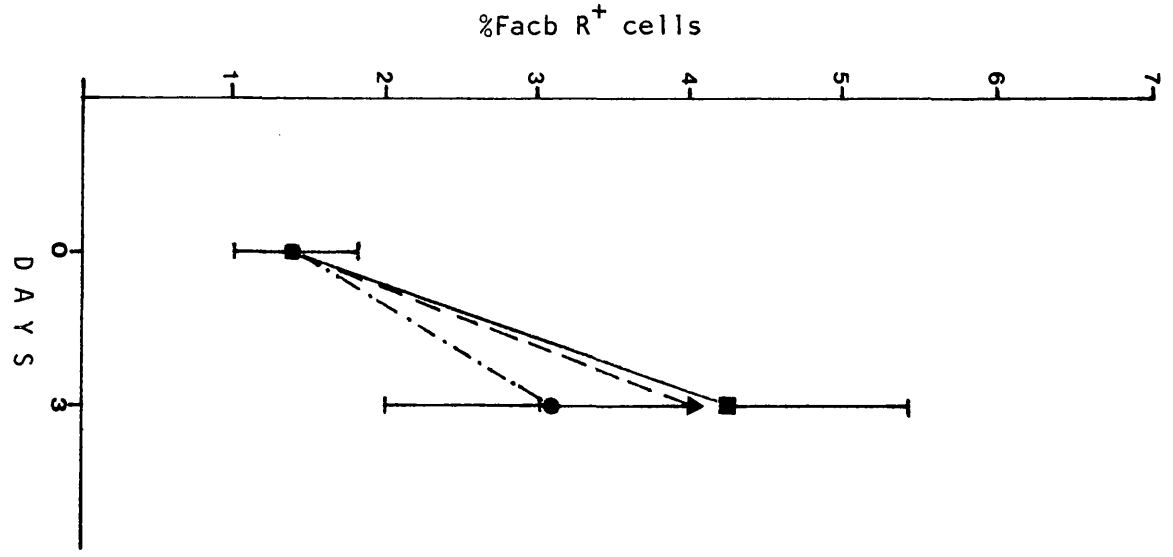
		%Facb	Indirect	Direct
P_{d0vd3}	A	< 0.005	< 0.005	< 0.005
	B	< 0.005	< 0.05	NS
	C	< 0.005	< 0.05	NS
Day 3				
p AvB		NS	< 0.005	< 0.005
p BvC		NS	NS	NS
p AvC		NS	< 0.005	< 0.005

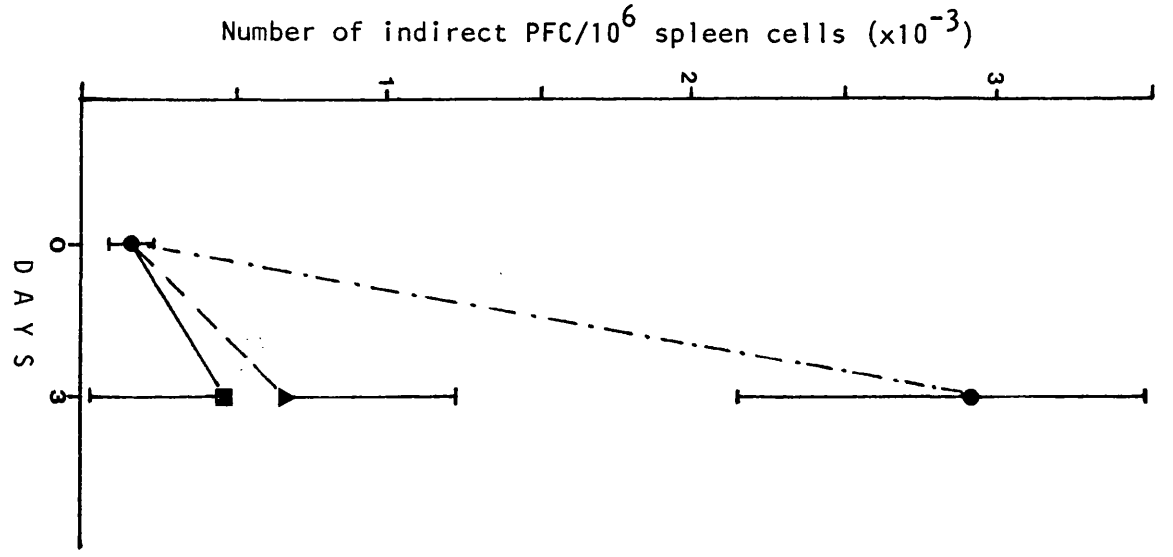
KEY

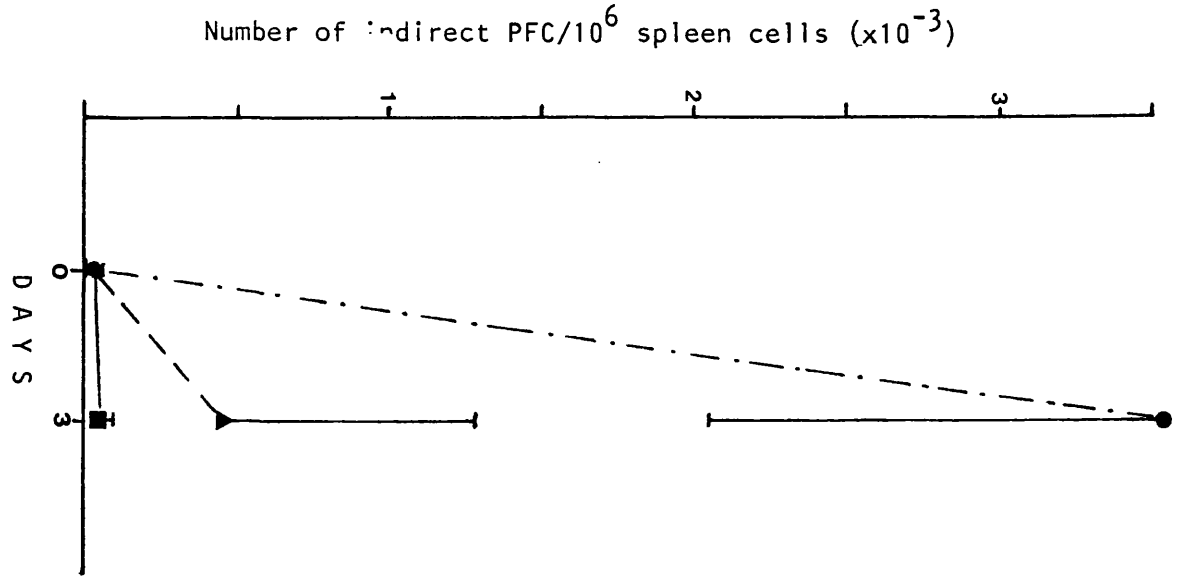
Challenge injection: ● CRBC

■ CRBC + Facb

▲ CRBC + IgG


%Facb R+ cells
1
2
3
4
5
6
7
DAYS
0
3


Number of indirect PFC/10^6 spleen cells (x10^-3)
1
2
3
DAYS
0
3


Number of indirect PFC/10^6 spleen cells (x10^-3)
1
2
3
DAYS
0
3

The %Facb R^+ cells was significantly increased on day 3 compared with day 0 in all groups (Figure 4.10). The day 3 %Facb R^+ cells in those mice injected with Ag+Facb (Group B) was slightly higher than that in those mice injected with Ag alone (Group A). All the groups showed a significant increase in the number of direct PFC on day 3 when compared with day 0 values, only Group A mice produced significantly higher numbers of indirect PFC (Figure 4.10). When compared to Group A, the day 3 PFC levels in Groups B and C (those mice injected with Ag+IgG) were significantly reduced. Thus, these results indicate that inoculation of Ag specific IgG or Facb concomitantly with the Ag significantly reduces the number of IgG and IgM secreting cells. This suppression could be due to Ag masking by the specific IgG and Facb or to an alteration in the kinetics of the response. The former is unlikely since only very low levels of these proteins were inoculated. Also, a significant increase in %Facb R^+ cells was observed on day 3 after challenge suggesting that a secondary response had occurred. However, a second experiment was performed (using CFLP mice) in which Group C mice were inoculated with 8µg $F(ab')_2$ anti-CRBC instead of IgG. The Facb and $F(ab')_2$ were prepared from the same sample of rabbit IgG anti-CRBC and the protein content of the Facb preparation was twice that of the $F(ab')_2$ solution.

Mice were sacrificed 8 days after challenge and the numbers of direct and indirect PFC per 10^6 spleen cells were determined (Chap.11 Sec. 11 H ii). The results showed that the presence of Ag specific $F(ab')_2$ (Group C) with CRBC in the second injection had no effect on the numbers of direct and indirect PFC elicited by Ag alone (Group A). In contrast, the presence of Facb considerably reduced both these parameters (direct PFC:$p<0.02$; indirect PFC:$p<0.02$; Figure 4.11). These results were obtained 8 days after challenge. Since the number of indirect PFC reached a peak by day 4 (Sec. 1 D i) in mice challenged with SRBC, it is unlikely that the suppression observed in this experi-

Figure 4.11 The effect of antigen specific Facb or $F(ab')_2$ on the secondary immune response of CFLP spleen cells

CRBC primed CFLP mice were challenged with CRBC either alone of in conjunction with antigen specific Facb of $F(ab')_2$. The numbers of splenic anti-CRBC indirect and direct PFC were determined 8 days after challenge.

Results

All results were expressed as the mean +/- one standard deviation.

Group	n	Number of PFC/10^6 spleen cells	
		Indirect	Direct
A	5	2322± 881	1641± 883
B	5	847± 359	779± 117
C	5	1784± 505	1553± 602

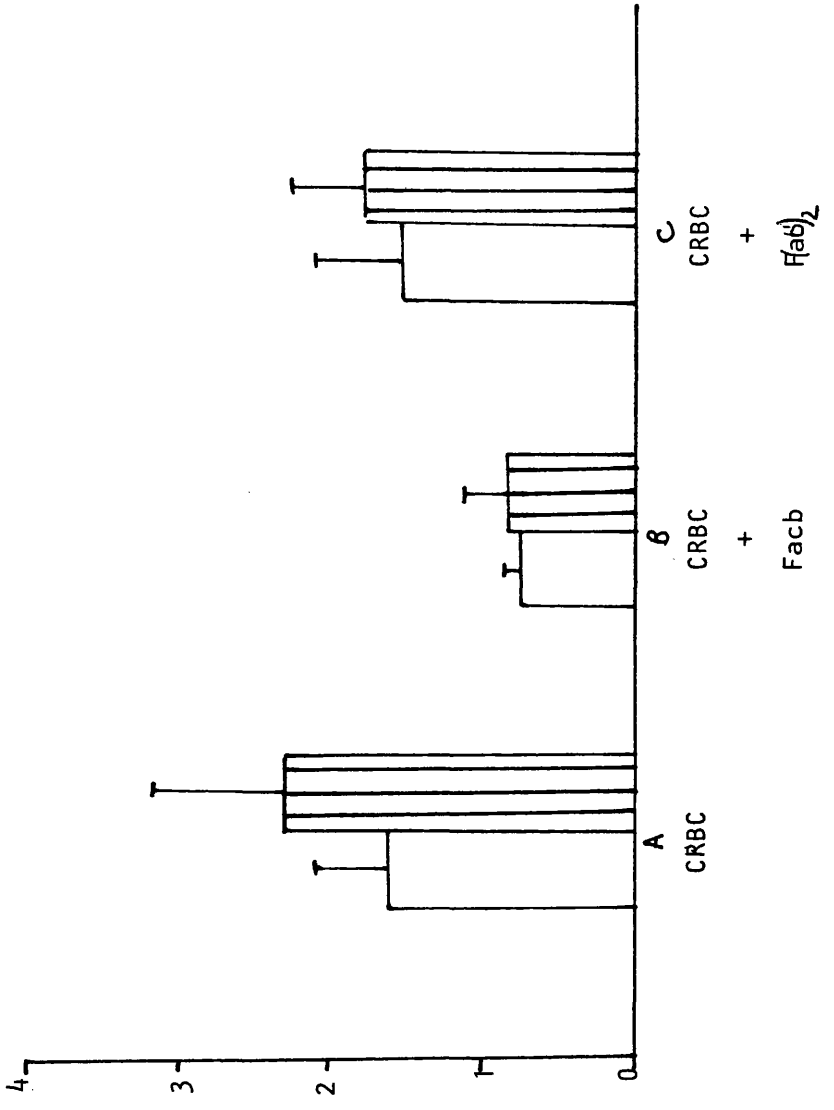
Statistical analysis using Student's t-test

	Direct	Indirect
p (AvB)	NS	< 0.02
p (BvC)	< 0.05	< 0,02
p (AvC)	NS	NS

KEY

Direct

Indirect


4
3
2
1
0
A
CRBC
B
CRBC
+
Facb
C
CRBC
+
F(ab')2

ment was due to alteration in the kinetics of the secondary response. Also, since no suppression was observed when $F(ab')_2$ was added at the time of challenge, antigenic masking was probably not occuring.

CHAPTER V

FUNCTIONAL STUDIES OF THE FACB R^+ CELL IN HUMANS

SECTION 1

Response of Facb R^+ cells to antigenic stimulation

(A) Introduction

Winrow (1982) reported the findings of preliminary experiments using the Tine test which suggested that an increase in the %Facb R^+ peripheral blood lymphocytes could be induced by antigenic challenge in immune individuals. Experiments were performed to substantiate this finding.

(B) The effect of a Tine test on the %Facb R^+ peripheral blood lymphocytes

A group of healthy controls were given a Tine test (a skin test for Mycobacterium tuberculosis related antigens using tuberculin). Samples of venous blood were taken before the skin test and on 1,2,3,4 and 7 days thereafter. A total white cell count and a differential count (Chap. 11 Sec. 11 C iv) were performed on each sample and the %Facb R^+ cells determined (Chap. 11 Sec. 11 D i). The response to the skin test was recorded on day 3 and the subjects were grouped as either positive or negative. Only 3 of the 11 controls showed no response to the skin test. The eight individuals who had a positive skin response, showed a significant increase in the mean number of Facb R^+ peripheral blood lymphocytes by day 2 after challenge ($p < 0.05$) which peaked on day 3 ($p < 0.02$). The mean number of Facb R^+ cells had returned to control levels by day 7. Thus, these results appear to confirm the preliminary observations of Winrow (1982). However, the group of skin test negative individuals had a higher initial mean number of Facb R^+ cells compared with the skin test positive group (although this may have been due to

Figure 5.1 The effect of a skin test for Mycobacterium tuberculosum associated antigens on the number of Facb R^+ peripheral blood lymphocytes.

Healthy controls were given a Tine test. The number of Facb R^+ peripheral blood lymphocytes was determined before the skin test and on days 1,2,3,4 and 7 thereafter.

Results

All results are expressed as the mean +/- standard deviation.

Day	n	Number of Facb R^+ cells ($x10^{-4}$)/ml blood
Skin test negative		
0	3	9.4 ± 1.7
1	3	3.8 ± 0.6
2	3	6.7 ± 6.7
3	3	10.4 ± 2.5
4	3	11.2 ± 6.4
7	3	7.6 ± 3.1
Skin test positive		
0	8	4.0 ± 2.2
1	6	6.0 ± 2.9
2	6	12.2 ± 8.1*
3	8	20.2 ± 10.4**
4	5	11.5 ± 10.4
7	7	6.0 ± 3.8

Statistical analysis using Student's t-test

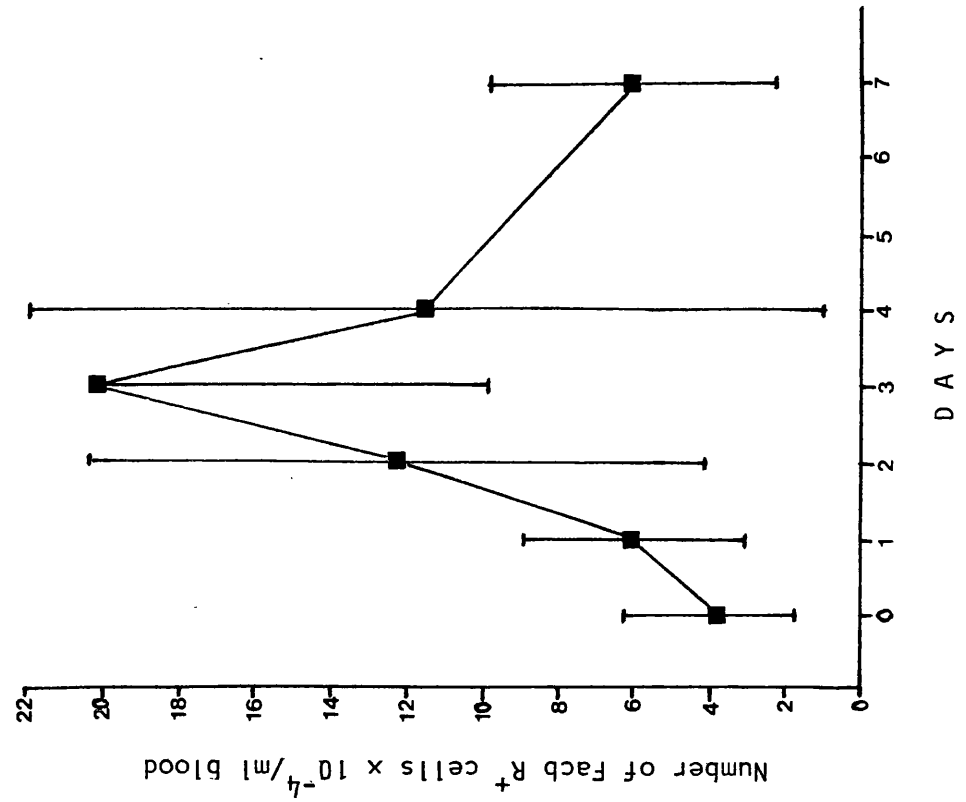
* $p<0.05$

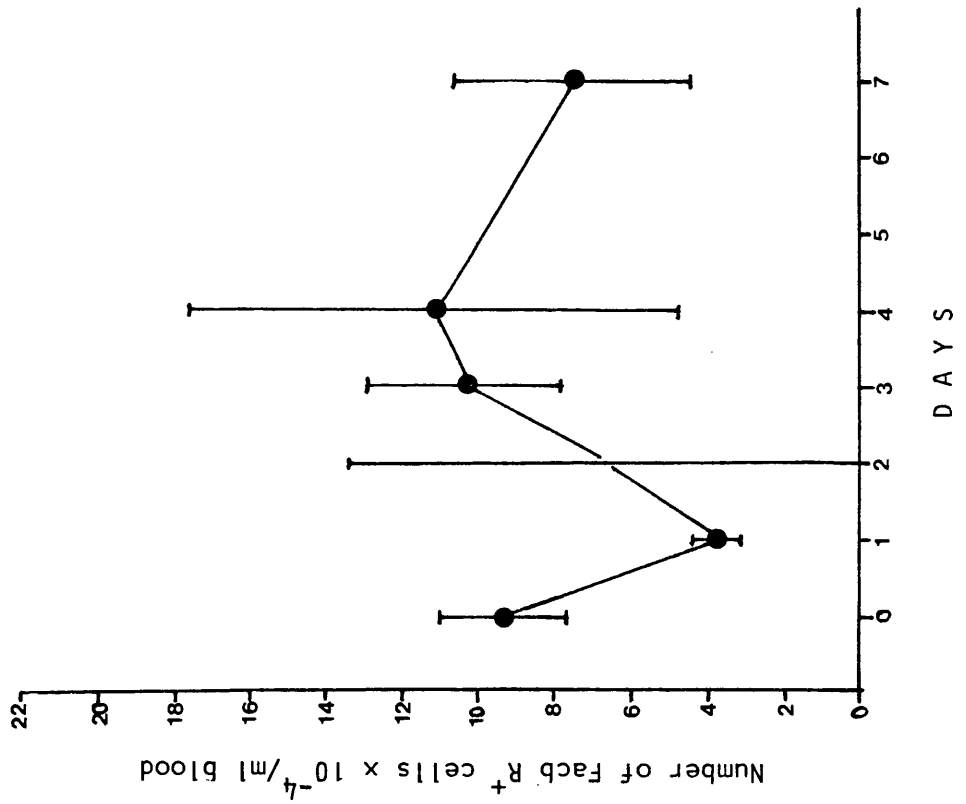
** $p<0.002$

KEY

● Skin test negative

■ Skin test positive


Number of Facb R+ cells x 10-4/ml blood
0
2
4
6
8
10
12
14
16
18
20
22
0
1
2
3
4
5
6
7
DAYS


Number of Facb R+ cells x 10-4/ml blood
0
2
4
6
8
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12
14
16
18
20
22
0
1
2
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4
5
6
7
DAYS

the small sample size of the skin test negative group) which might explain the apparent lack of increase of Facb R^+ cells in these individuals.

In order to examine the response of these cells in individuals already undergoing an inflammatory response, the experiment was repeated using a group of patients with active RA. Only 2 of the 9 patients tested showed positive skin responses to the Tine test. The results show that a significant decrease in the mean value of the number of Facb R^+ cells in non-immune individuals occurred 3 days after the skin test ($p < 0,05$; Table 5.1). Since only 2 individuals showed a positive response it was not possible to analyse the results statistically. However, the mean day 0 and day 3 values were very similar. All the patients studied were on non-steroidal anti-inflammatory drugs at the time of skin testing and were on bed rest during the 4 days of the trial. The significant decrease in the number of Facb R^+ cells seen in the non-responder group was probably due to the effects of chemotherapy and bed rest rather than to the skin test. If this supposition is true, the lack of decrease in those 2 patients who had positive skin responses was probably due to antigenic challenge in immune individuals. Both of these patients (and one individual in the non-responder group) could recall having been immunized against tuberculosis at least 20 years previously. Clearly, more work is required to confirm these results but the preliminary investigation suggests that Facb R^+ cells in patients with RA respond to antigenic challenge in a similar way to those in control subjects.

Since it was necessary to perform *in vitro* functional studies the phenomenon of antigen induced Facb R^+ cell proliferation observed *in vivo* was examined *in vitro* using the purified protein derivative of tuberculin (PPD) and cells from those control subjects who had shown a positive skin test response. Lymphocyte suspensions in RPMI 1640

Table 5.1 The effect of a skin test for Mycobacterium tuberculosum associated antigens on the number of Facb R^+ peripheral blood lymphocytes in patients with RA

Skin test response	n	Number of Facb R^+ cells ($x10^{-4}$)/ml blood (Mean +/- S.D.)	
		Day 0	Day 3
Positive	2	21.4±14.9	22.3±6.2
Negative	7	18.6± 8.8	8.9±6.5*

Statistical analysis using Student's t-test

* $p<0.05$

were prepared as described previously (Chap. 11 Sec. 11 C ii) . One x 10^6 cells in 1 ml of medium were cultured in each well of a flat-bottomed tissue culture plate (24 wells / plate; Nunc) at 37°C in a humidified atmosphere of 95% air / 5% CO_2. To each well was added either various concentrations of PPD in 10µl of medium or 10µl of medium alone. The plates were cultured for 0,2,3 or 4 days, the cells washed and the %Facb R^+ cells determined (Chap. 11 Sec. 11 D i). Preliminary experiments showed that 50 µg/ml PPD produced an optimal increase in the %Facb R^+ cells and this concentration was used in all subsequent experiments.

Those cells cultured alone showed a decrease in the %Facb R^+ cells which was statistically most significant on day 4 ($p < 0.05$; Figure 5.2). In contrast, the PPD stimulated cultures showed a significant increase in the %Facb R^+ cells on day 3 ($p < 0.005$). Thus, an increase in the %Facb R^+ cells from healthy individuals previously immunized in vivo can be induced by incubation with PPD <u>in vitro</u>. Since these cells appear to respond <u>in vitro</u> to an antigenic stimulus in a similar way to that observed <u>in vivo</u>, it is feasible that they may be stimulated (either by mitogens or by antigens) <u>in vitro</u> to perform those functions which they normally perform <u>in vivo</u>.

Figure 5.2 The effect of in vitro culture with PPD on the %Facb R^+ cells

One x 10^6 lymphocytes were cultured in vitro alone or with 50µg/ml PPD. The %Facb R^+ cells were estimated before culture and on 2,3 and 4 days thereafter. All results are corrected for viability and are expressed as the mean +/- standard deviation.

Results

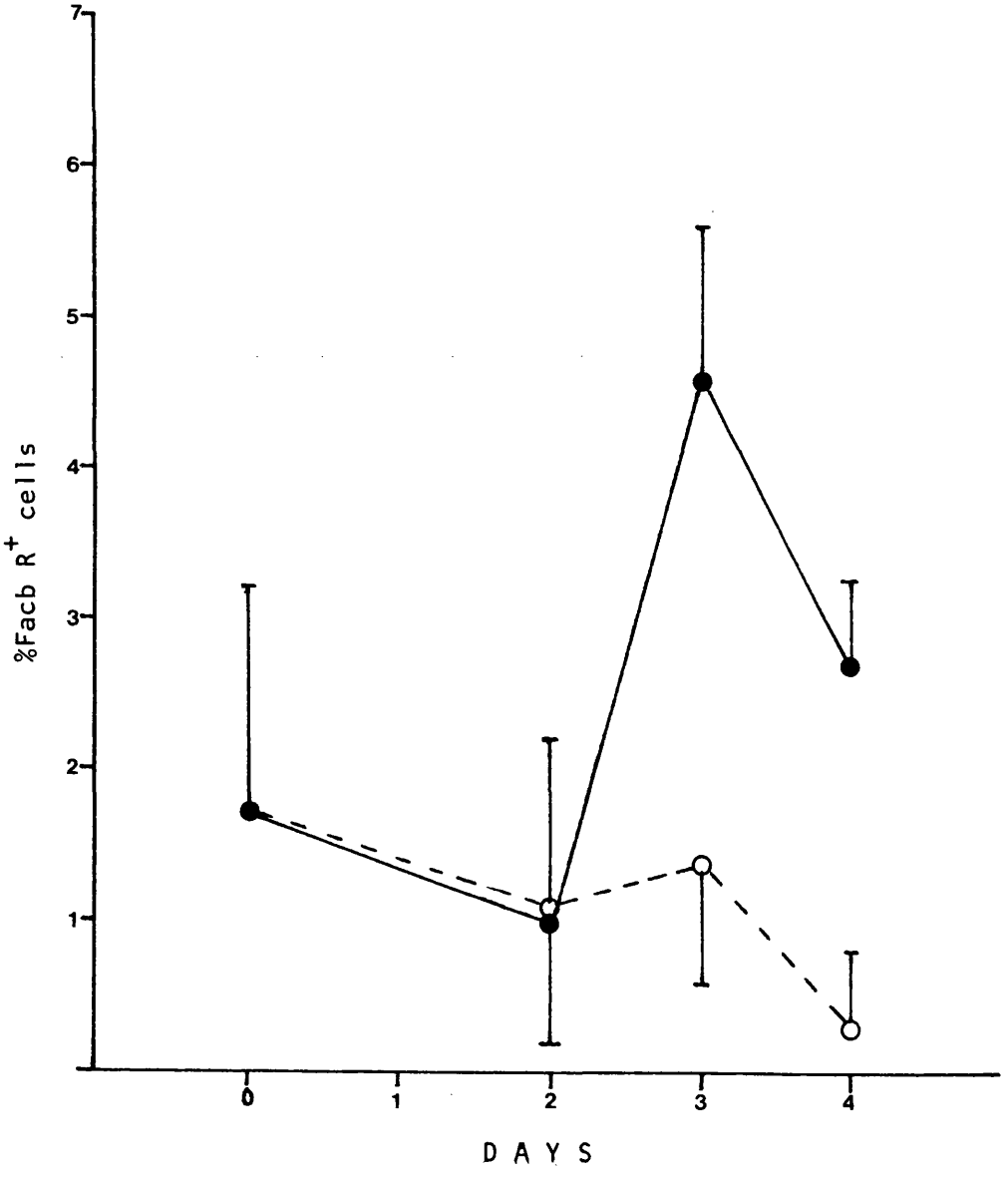
Day	n	%Facb R^+ cells	
		Control No PPD	Test 50µg PPD/ml
0	7	1.7±1.5	1.7±1.5
2	7	1.1±1.1	1.0±0.8
3	7	1.4±0.8	4.6±1.0*
4	7	0.3±0.5	2.7±0.8*

Statistical analysis using Student's t-test

* $p<0.005$

○ Control

● Test


7
6
5
4
3
2
1
%Facb R+ cells
0
1
2
3
4
DAYS

SECTION 11

The in vitro effect of mitogens on Facb R^+ cells

(A) Introduction

Mitogens are commonly used to investigate T and B cell activity. Many workers have shown previously that null cells do not proliferate in response to mitogen stimulation although they do enhance blastogenesis in other subpopulations in the cultures (Horwitz and Garrett, 1977; Despont, Steimer and Banderet, 1981; Lobo, 1981; Caraux et al., 1982). They have also been shown to enhance Ig synthesis in a PWM stimulated system (Lobo,1981). Thus, the effect of mitogens on Facb R^+ cells was investigated.

(B) The effect of mitogens on Facb R^+ cells

i) The in vitro effect of pokeweed mitogen and T cell growth factor on the %Facb R^+ cells

Lymphocyte suspensions were obtained from healthy volunteers and from patients with active RA as described previously (Chap.11 Sec.11 C ii). To each well of a 24 well flat-bottomed tissue culture plate (Nunc) containing 1 x 10^6 lymphocytes in 1ml RPMI 1640 was added either 100 μl medium, 100μl PWM (final concentration in the well; 1/40) or 100μl T cell growth factor (TCGF; a soluble product from Concanavalin A stimulated lymphocytes which is capable of supporting the long term growth of T cells in vitro; reviewed by Watson and Mochizuki, 1980). The plates were incubated at 37°C in a humidified atmosphere of 95% air/ 5% CO_2. After 3 days, the total number of viable Facb R^+ cells in each well was calculated (Chap. 11 Sec.11 D ii).

The results show that neither PWM nor TCGF caused proliferation of Facb R^+ cells (Table 5.2).

Table 5.2 The effect of PWM and TCGF on Facb R^+ cells

	Number of viable Facb R^+ cells ($x10^{-4}$)		
	C	PWM	TCGF
Control	1.72	2.69	1.02
	0.84	1.35	0.00
	0.00	1.13	0.00
	1.68	1.16	0.99
	0.95	0.00	1.34
n	5	5	5
$\bar{x}$	1.04	1.27	0.67
sd	0.71	0.96	0.63
Rheumatoid	2.70	1.70	-
	0.70	0.70	-
	0.95	0.00	-
	0.00	1.24	-
	0.00	0.00	-
n	5	5	
$\bar{x}$	0.87	0.73	
sd	1.11	0.75	

ii)The effect of Facb R^+ cell depletion on mitogen induced blastogenesis

Although Facb R^+ cells were not stimulated by mitogens it was possible that they might enhance mitogen induced blastogenesis in other cells. Peripheral blood lymphocytes were obtained from healthy volunteers and from patients with active RA as described previously (Chap.11 Sec. 11 C ii). Cultures containing various concentrations of Concanavalin A (Con A), Phytohaemagglutinin (PHA), or Pokeweed mitogen (PWM) were prepared (Chap.11 Sec 11 G) and incubated at 37^0C in a humidified atmosphere of 95% air/5% CO_2. In some cultures, lymphocyte suspensions were separated into 2 aliquots one of which was depleted of Facb R^+ cells (Chap.11 Sec. 11 F ii) whilst the other was subjected to a sham depletion by rosetting with unsensitized CRBC. Mitogen induced proliferation was measured by tritiated thymidine incorporation as described previously (Chap. 11 Sec. 11 G).

The results show that mitogen stimulated tritiated thymidine incorporation was unaffected by Facb R^+ cell depletion in both rheumatoid and control subjects (Tables 5.3 and 5.4). Thus, Facb R^+ cells do not enhance mitogen induced blastogenesis in other lymphocyte subpopulations.

(C) The effect of Facb and $F(ab')_2$ on in vitro Ig synthesis

Mononuclear cells from healthy controls and patients with active RA were obtained by centrifugation through Ficoll-Paque (Chap.11 Sec. 11 C ii). Cultures were prepared as described previously (Chap. 11 Sec. 11 H i). Each well was supplemented with either 10μl Facb (final concentration 25μg/ml), 10μl $F(ab')_2$ (final concentration 25μg/ml) or 10μl medium RPMI 1640. The Facb and $F(ab')_2$ were produced from the same preparation of rabbit IgG anti-CRBC and the concentration of Facb used was the same as that used to form Facb rosettes with 1×10^5 lymphocytes. The cells were incubated for 14 days as described previously (Chap.11 Sec. 11 H i) and the supernatants were assayed for IgG production by an enzyme linked

Table 5.3 The effect of Facb R^+ cell depletion on the mitogen responses of control lymphocytes

Mitogen		n	*^{3}H-thymidine incorporation (cpm; mean sd)	
			+ Facb R^+ cells	- Facb R^+ cells
Con A	100	8	937±1060	642± 392
	50	8	2521±1981	2265±1682
	10	8	2249±1966	1121± 645
PHA	5	8	3768±1918	3442±1901
	1	8	4953±2508	4129±1324
	0.2	8	4313±2684	3196±1267
PWM	1/20	4	1348± 349	1602± 658
	1/40	8	1049± 252	1475± 451
	1/200	6	1504± 876	780± 600

* ^{3}H-thymidine incorporation for each culture was determined from the equation:

Counts per minute (cpm) mitogen stimulated cells - cpm cells alone.

Statistical analysis using Student's t-test

All the results were analysed using Student's t-test. There was no statistically significant difference between those cultures lacking Facb R^+ cells and those containing them.

Table 5.4 The effect of Facb R^+cell depletion on the mitogen responses of rheumatoid lymphocytes

Mitogen		n	^{*3}H-thymidine incorporation (cpm; mean sd)	
			+ Facb R^+ cells	- Facb R^+ cells
Con A	100	6	2966±3406	3451±3221
	50	6	3915±3324	4159±2360
	10	6	3207±2564	3084±3800
PHA	5	6	2451±1358	3054±3136
	1	6	3673±2318	4453±3787
	0.2	6	2488±1110	2371±1466
PWM	1/20	6	2415±1862	2291±1385
	1/40	6	2936±3371	2196±1621
	1/400	6	4142±5168	2880±3269
	1/2000	6	2645±3572	807±1687

^{*3}H-tritiated thymidine incorporation for each culture was determined from the equation:

Counts per minute (cpm) mitogen stimulated cells - cpm cells alone.

Statistical analysis using Student's t-test

No statistical difference was observed between those cultures containing Facb R^+ cells and those without these cells.

immunosorbent assay (ELISA; Chap. 11 Sec. 11 H ii).

The results show that the presence of Facb in control cultures significantly decreased the amount of PWM induced IgG ($p<0.05$), whilst $F(ab')_2$ had no effect (Table 5.5). In contrast, there was no statistical difference between the levels of IgG produced in any of the cultures of rheumatoid cells. However, the amount of IgG produced in those cultures containing Facb was much less than those containing PWM alone. This difference did not reach significance owing to the large standard deviations observed with the rheumatoid cells. Also, the amount of IgG produced by rheumatoid cells stimulated with PWM alone was lower than that produced by control cells (although not significantly lower). It is feasible that if Facb R^+ cells are capable of suppressing IgG production (as suggested by the results with control cells), these cells in rheumatoids may have been activated in vivo (possibly by immune complexes) and hence were relatively refractive to further stimulation by Facb in vitro. Clearly, many of these problems would be resolved by repeating these experiments on a larger number of patients and controls.

Table 5.5 The effect of Facb and $F(ab')_2$ on the pokeweed mitogen induced synthesis of IgG

	Amount of immunoglobulin G ($ng/10^6$ cells)		
	PWM	PWM + Facb	PWM + $F(ab')_2$
Control			
n	5	5	5
$\bar{x}$	938	129*	1673
sd	663	161	1481
Rheumatoid			
n	5	5	5
$\bar{x}$	522	92	222
sd	712	119	217

Statistical analysis using Student's t-test

* $p<0.05$ (PWM+Facb compared with PWM alone)

The results are expressed as 'delta' values i.e.

immunoglobulin in test culture - Ig in control.

CHAPTER VI

CLINICAL STUDIES OF FACB R^+ CELLS IN RHEUMATOID ARTHRITIS

SECTION 1

Facb R^+ cells in early rheumatoid disease

(A) Introduction

Winrow (1982) has previously shown that in a cross-sectional study of rheumatoid patients there was no correlation berween the %Facb R^+ cells in the peripheral blood and disease activity. This study did not investigate this relationship in different disease categories, nor did it take into account the inherent variation of %Facb R^+ cells between individuals. Thus, a longitudinal study was undertaken to investigate the variation in %Facb R^+ cells with the development of rheumatoid disease.

(B) Early rheumatoid arthritis study

i) Investigation of control levels of Facb R^+ cells

A group of 17 healthy control subjects were bled at regular intervals over a period of one year. On each occasion, the %Facb R^+ peripheral blood lymphocytes was determined (Chap. 11 Secs. 11 C ii; 11 D ii). The mean %Facb R^+ cells in control peripheral blood was determined from these results and was found to be 2.4% with a standard deviation of 1.5% (Figure 6.1). Twelve of these subjects were used to investigate the variation of %Facb R^+ cells with time in controls. All twelve were bled on the same day and subsequently after 4, 21 and 22 weeks. However, if any of them had any form of infection (either on the day of bleeding or within a week before or after bleeding) their results were not included. It was found that there was no significant variation in the percentage of control Facb R^+ peripheral blood lymphocytes over the period studied (Figure 6.1).

Figure 6.1 Investigation of control levels of Facb rosette forming cells

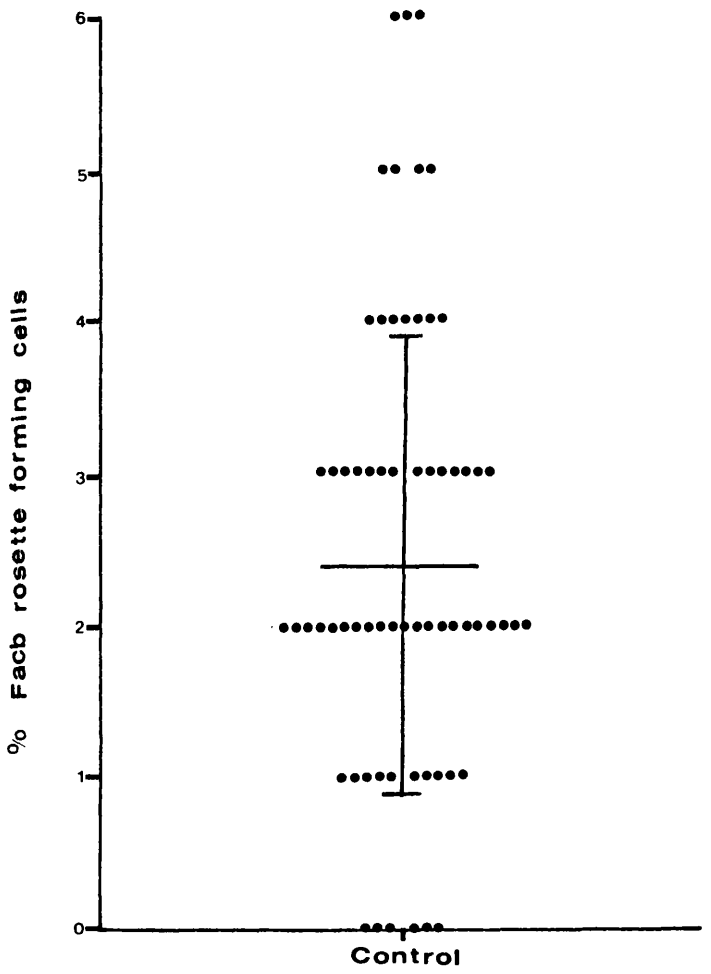
A group of 17 control subjects were studied over a period of 1 year. The %Facb R^+ cells for each control were used to determine the control mean and standard deviation. Also serial observations were made on a group of individuals showing the variation over a period of 1 week, 1 month and 5 months in the control mean %Facb R^+ peripheral blood lymphocytes.

Results

	Total %Facb R^+	Variation in %Facb R^+			
		WEEK			
		0	4	21	22
n	63	12	8	10	11
$\bar{x}$	2.4	2.7	2.1	2.5	2.7
sd	1.5	2.0	1.2	1.4	1.7

Statistical analysis using the analysis of variance (ANOVAR) test

The 'F' value calculated from these results was not significant at the 5% level.


% Facb rosette forming cells
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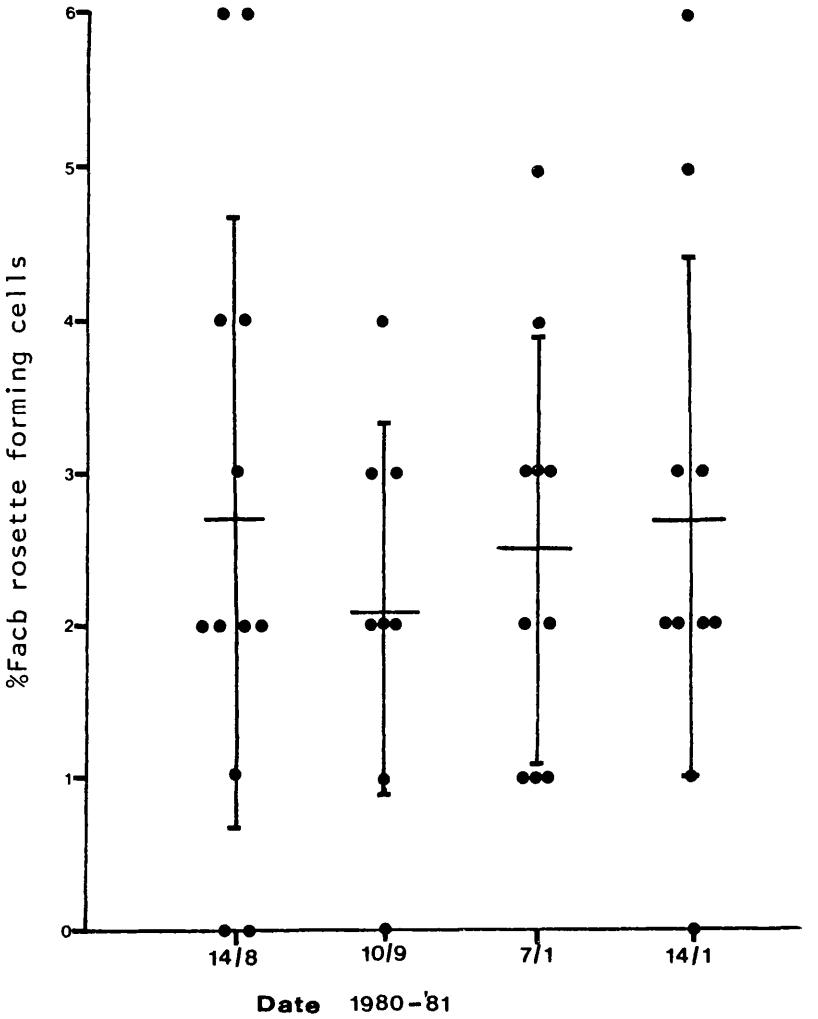
Control


%Facb rosette forming cells
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2
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14/8
10/9
7/1
14/1
Date 1980-'81

ii) A study of Facb R^+ cells in early rheumatoid disease

Selection of patients

Letters were sent to local General Practitioners announcing the formation of a clinic to investigate the development of rheumatoid disease. They were requested to refer to this clinic any patients presenting with non-specific joint pains of not more than 3 months duration. The patients were assessed on their first visit and were given subsequent appointments of either 1 week, 1 month or 3 months depending on the severity of their symptoms. All appointments were given with the proviso that should the patient feel worse at any time, they could telephone for an earlier appointment. Thus, between regular visits it can be assumed that the clinical state of each patient either remained static or improved.

Clinical assessment

This was performed by Drs. D. Blake and R. Waterworth (Royal National Hospital for Rheumatic Diseases, Bath). Assessment depended on the patients own report of their clinical state and on clinical evidence of disease activity (such as the number of joints showing evidence of synovitis). The patients were categorized as either sero-positive, sero-negative or palindromic RA, transient polysynovitis, Reiter's syndrome, polymyalgia rheumatica or osteoarthrosis.

The highest percentage Facb rosettes observed in this study was within 6 times the upper limit of control levels (mean + 2 standard deviations). Thus, in order to compare the disease activity of patients within any diesase category with the serial results of %Facb rosettes, the clinical assessment for each patient was expressed on a scale from 0-5 (ie from no disease activity to extremely active erosive disease).

One hundred patients were initially included in this study, 38 of whom either failed to attend for subsequent appointments or were referred elsewhere since their symptoms were not related to rheumatoid disease.

The correlations between %Facb R^+ cells and disease activity; plasma viscosity and disease activity and %Facb R^+ cells and serum IgG were investigated in each disease category.

Results

The results show that the %Facb R^+ cells correlated significantly with clinical assessment of disease activity (CA) in all disease categories except palindromic RA (PRA) and osteoarthrosis (OA), (Figures 6.2, 6.4, 6.5;6.6 and Table 6.1). Plasma viscosity (commonly used as a laboratory parameter for disease activity in RA) correlated significantly with CA only in the sero-positive RA (RA^+) group (Table 6.1, Figure 6.3). This correlation was not as significant ($p<0.05$) as the correlation of %Facb rosettes with this parameter ($p<0.001$).

The lack of correlation between %Facb R^+ cells and CA in the OA group confirms the observations of Hall, Winrow and Bacon (1980) which showed that these cells were not raised in patients with this disease. The lack of correlation in patients with PRA probably results from the nature of the disease itself in that patients who had high levels of Facb R^+ cells but were assessed to have very little disease activity, often had exacerbations of their disease just prior to, or immediately after their hospital appointment (Table 6.2). This suggests that a study of the %Facb R^+ cells in these patients may help to indicate when an exacerbation of the disease was going to occur. This obviously requires further investigation.

The observations (reported in Chapters 4 and 5 of this thesis) that Facb R^+ cells were involved in suppression of IgG synthesis provoked the investigation of the relationship berween %Facb R^+ cells and serum IgG in rheumatoid patients. However, no correlation was found between these two parameters in this study (Table 6.1).

Thus, these results indicate that levels of Facb R^+ cells above the normal range are an indication of active rheumatoid disease. However,

Table 6.1 Correlation between laboratory parameters and clinical assessment of disease activity in early RA

Disease Category	Correlation coefficients		
	FACBvCA	VISCvCA	FACBvIgG
RA^+	0.68*	0.29****	-0.18
RA^-	0.70*	0.29	0.40
TPS	0.58***	0.10	-0.19
Reiters	0.74**	0.22	0.36
PMR	0.72*	0.18	0.29
PRA	-0.02	-0.18	-0.13
OA	0.37	0.03	-0.41

Statistical analysis

The significance of the correlation coefficients (r) was determined using the equation

$$t = \frac{r}{\sqrt{1 - r^2}} \sqrt{n - 2}$$

where n is the number of observations used to calculate r and t is the Student statistic (with n - 2 degrees of freedom).

* $p < 0.001$

** $p < 0.01$

*** $p < 0.02$

**** $p < 0.05$

KEY

$RA^{+/-}$	Sero- postive/negative RA
TPS	Transient polysynovitis
Reiters	Reiter's syndrome
PMR	Polymyalgia rheumatica
PRA	Palindromic RA
OA	Osteoarthrosis
VISC	Plasma viscosity
CA	Clinical assessment

Figure 6.2 Correlation between clinical assessment and %Facb R^+ cells in patients with sero-positive RA

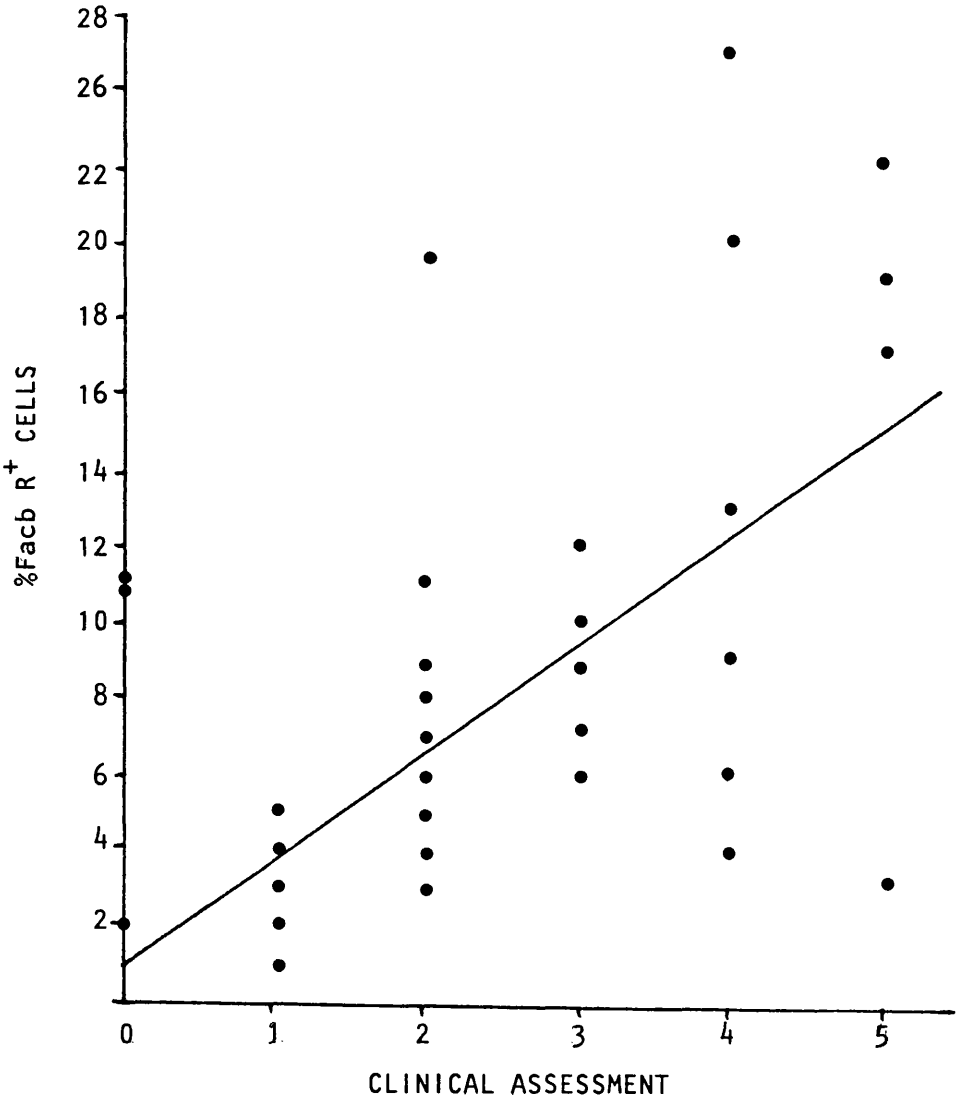
The figure shows a scatter diagram of %Facb R^+ cells against clinical assessment.

Results

Linear regression analysis showed that the data fit the straight line:

$$y = 1.1 + 2.9x$$

with a correlation coefficient of 0,68


%Facb R+ CELLS
28
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2
3
4
5
CLINICAL ASSESSMENT

Figure 6.3 Correlation between clinical assessment and plasma viscosity in patients with sero-positive RA

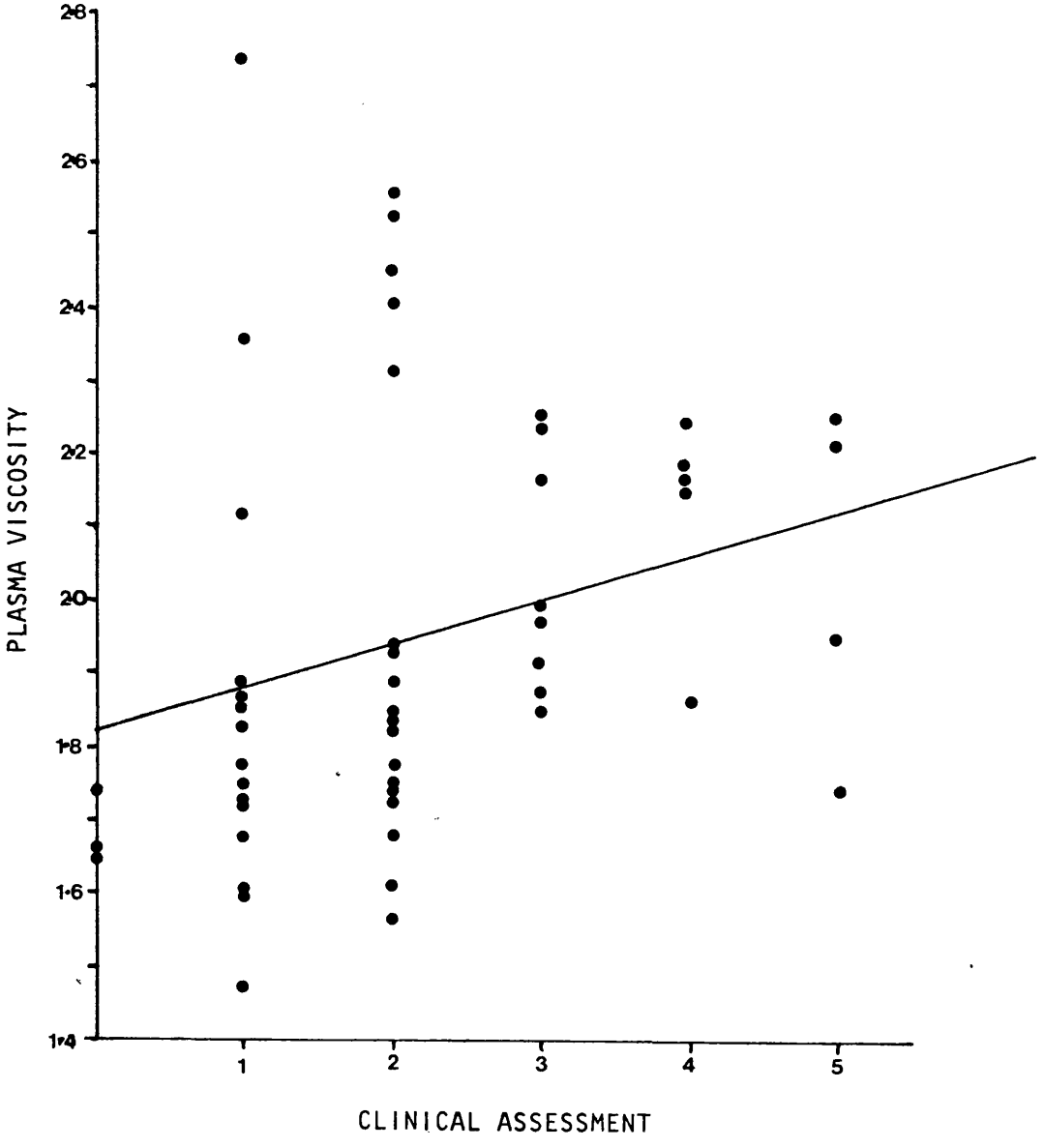
The figure shows a scatter diagram of plasma viscosity against clinical assessment.

Results

Linear regression analysis showed that the data fit the straight line:

$$y = 1.8 + 0.1x$$

with a correlation coefficient of 0.29


PLASMA VISCOSITY
2·8
2·6
2·4
2·2
2·0
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1·4
1
2
3
4
5
CLINICAL ASSESSMENT

Figure 6.4 Correlation between clinical assessment and %Facb R^+ cells in patients with sero-negative RA

The figure shows a scatter diagram of %Facb R^+ cells against clinical assessment.

Results

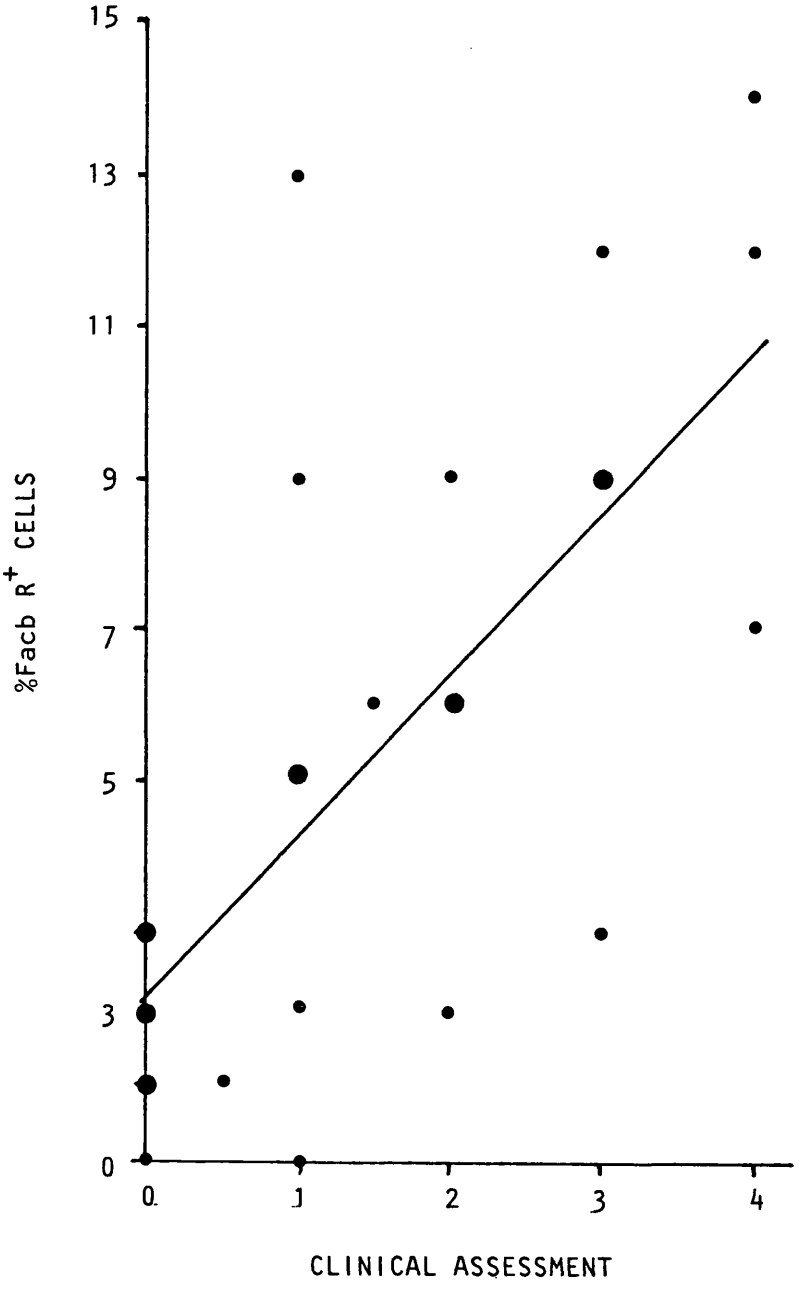
Linear regression analysis showed that the data fit the straight line:

$$y = 2.2 + 2.2x$$

with a correlation coefficient of 0.70.

KEY

- A single result
- More than one result at that value


15
13
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%Facb R+ CELLS
0
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CLINICAL ASSESSMENT

Figure 6.5 Correlation between clinical assessment and %Facb R^+ cells in patients with transient polysynovitis and Reiter's syndrome

The figures show scatter diagrams of %Facb R^+ cells against clinical assessment.

Results

Linear regression analysis showed that the data for patients with transient polysynovitis fit the straight line:

$$y = 1.8 + 2.1x$$

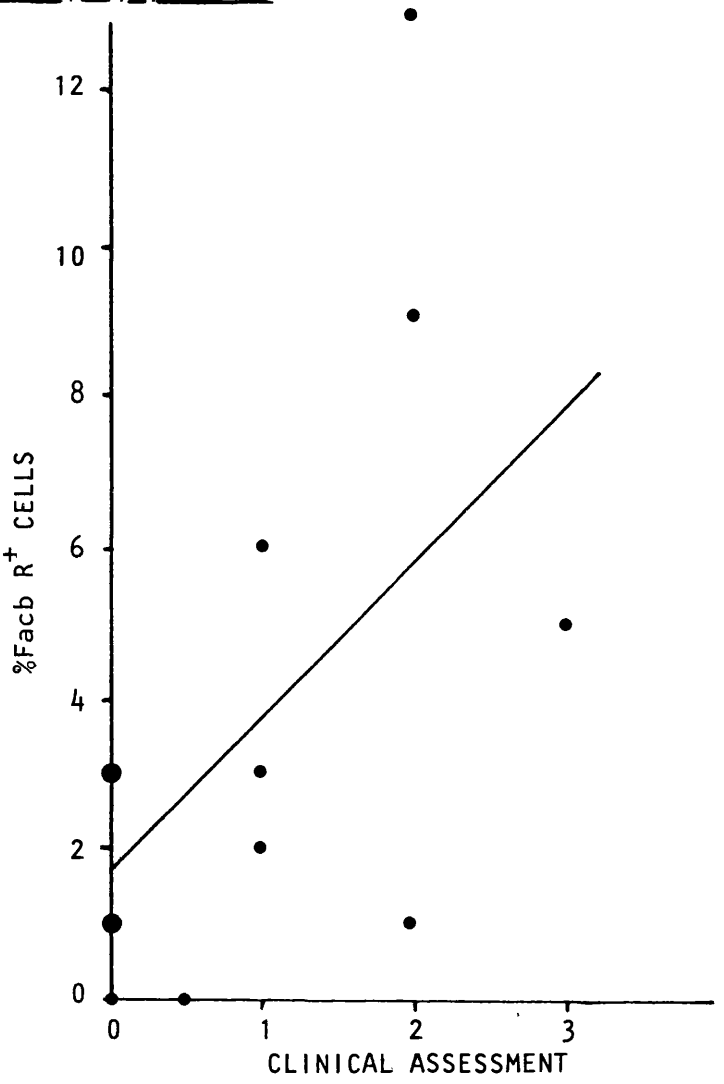
with a correlation coefficient of 0.58. The data for those patients with Reiter's sundrome fit the straight line: $y = 1.4 + 2.6x$

with a correlation coefficient of 0.74.

KEY

- A single result
- More than one result at that value.

Transient polysynovitis



Reiter's syndrome

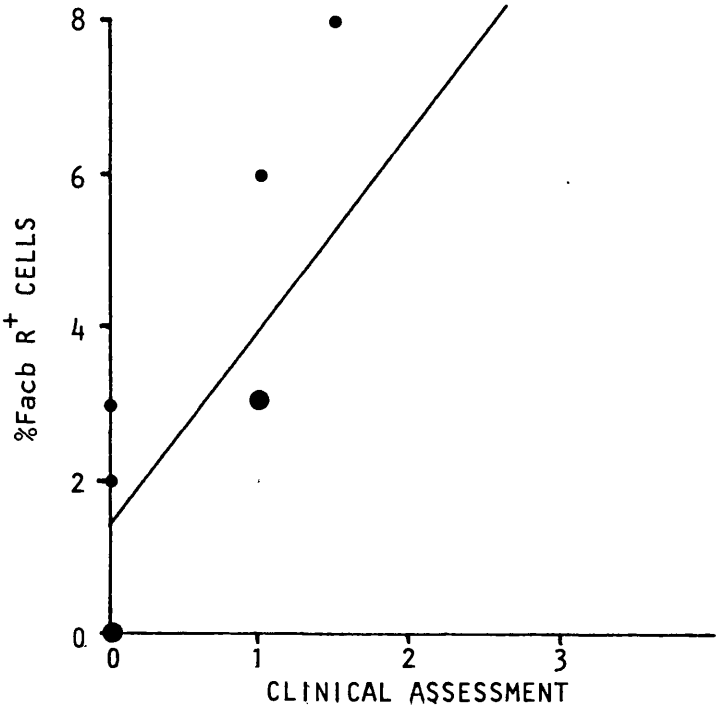


Figure 6.6 Correlation between clinical assessment and %Facb R^+ cells in patients with polymyalgia rheumatica

The figure shows a scatter diagram of %Facb R^+ cells against clinical assessment.

Results

Linear regression analysis showed that the data for patients with polymyalgia rheumatica fit the straight line:

$$y = 2.3 + 2.7x$$

with a correlation coefficient of 0.72.

KEY

- A single result
- More than one result at that value

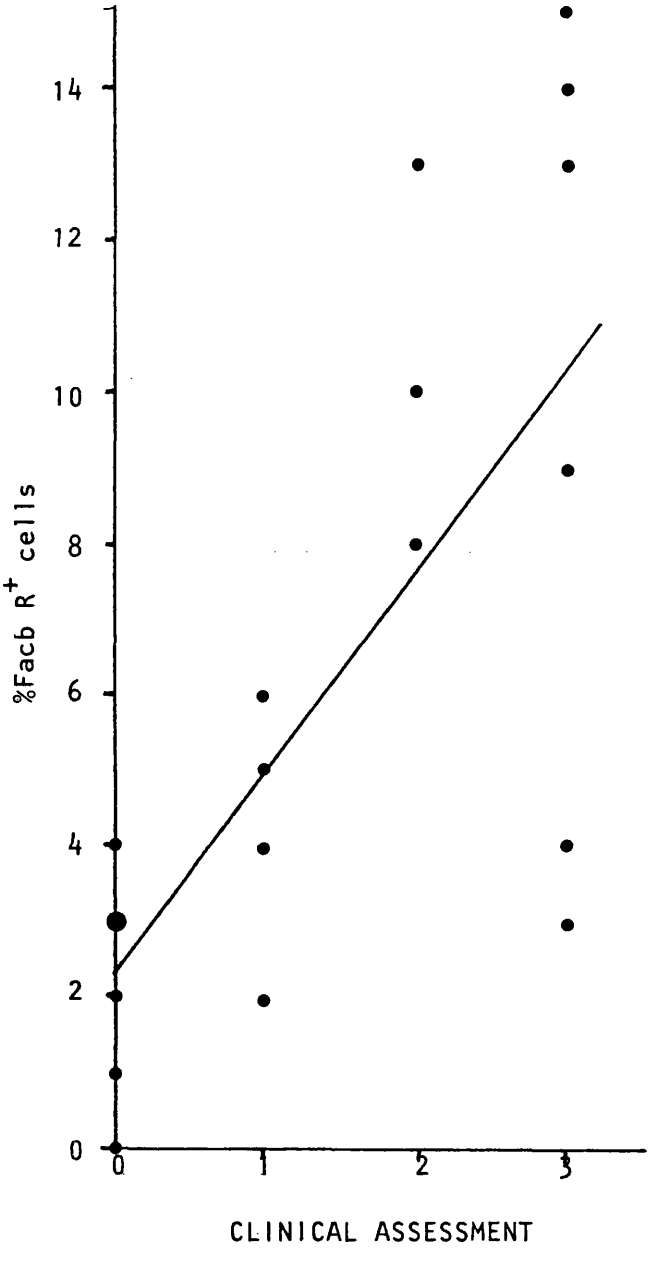

14
12
10
8
6
4
2
0
%Facb R+ cells
0
1
2
3
CLINICAL ASSESSMENT

Table 6.2 The relationship between %Facb R^+ cells and disease activity in palindromic RA

Patient	Date	%Facb	Comment
DW	11/6/80	8	Exacerbation 12 days earlier lasting 6 days. No evidence of disease activity on 11/6/80.
GH	29/1/80	7	Exacerbation 10 days earlier lasting 7 days. No evidence of disease activity on 29/1/80.
CG	11/12/80	17	Exacerbation 7 days earlier lasting 4 days. No evidence of clinical activity on 11/12/80.
HF	10/11/80	12	Clinical evidence of disease activity on 14/11/80.

since the base level of these cells varies considerably (normal range 0 - 5%), any information about the progression of the disease in one individual may only be gained by a serial study of %Facb R^+ cells.

SECTION 11

The effect of D - Penicillamine on %Facb R^+ cells

(A) Introduction

It has been shown that the %Facb R^+ cells varies with disease activity in early RA, It has not been established whether such a relationship exists in patients with aggressive, advanced disease. Such patients were invariably on second-line or steroidal therapy. To overcome this problem, a study was undertaken of patients with established RA who were maintained on D -Penicillamine (D - Pen) and from whom the drug was gradually being withdrawn. In such a study it was possible to examine the variation in %Facb R^+ cells when the patients suffered a 'flare-up' in disease activity owing to the reduction of their medication, Also, those patients who were taken of the drug completely provided an opportunity to examine these cells in advanced RA.

(B) The effect of withdrawal of D - Pen therapy on %Facb R^+ cells in RA.

Patients who had veen established on D-Pen for more than one year were assessed for disease activity (according to the Ritchie Index) at an out-patient clinic. Some of those patients whose disease was quiescent were chosen at random and were prescribed successively lower daily doses of D-Pen (500, 375, 250,125 or 0 mg) over a period of 6 months. Several patients were maintained on their initial dose of D-Pen and they were used as controls. If the patients suffered an exacerbation of disease activity, they were given higher doses of D-Pen until their clinical symptoms were settled. At each visit, the %Facb R^+ peripheral blood lymphocytes was determined, When the study was completed, the patients were classified as either sero-negative or sero-positive. The clinical side of this double-blind study was performed by Dr, M. Ahern and Mrs. M Maddison SRN.,

RNHRD, Bath. Four parameters were chosen to be studies in this results section: articular index (the number of joints showing evidence of synovitis), visual analogue scale (a subjective assessment of pain performed by the patient), plasma viscosity and %Facb R^+ cells,

The results were segregated into groups according to the concentration of D-Pen that the patient was taking at the time of examination. The results are shown in Figures 6.7, 6.8, 6.9 and 6.10 and were examined statistically using a single factor ANOVAR test. It was found that only the visual analogue scale (VA) altered significantly ($p < 0.05$) when the dosage of D-Pen was altered in the sero-positive test group. No such variation was observed in the sero-negative test patients. However, the control patients in this group showed significant variation in the mean VA ($p<0.01$), articular index (AI; $p < 0.05$) and plasma viscosity (PV; $p < 0.05$). The last two parameters both showed significant increases as the dose of the drug decreased.

Correlation coefficients were determined between VA, AI, PV and %Facb and between these parameters and the drug dose for each test or control group. The results are shown in Tables 6.3 and 6.4. The %Facb R^+ cells correlated only with the VA in the sero-negative control group ($p < 0.05$). The AI correlated with PV in the sero-negative control patients ($p < 0.02$) and the sero-positive test patients ($p < 0.001$). The latter correlation became less significant as the dose of the D-Pen was reduced. The visual analogue scale correlated with AI in both test and control sero-positive patients ($p < 0.001$) but only with the test patients in the sero-negative group ($p < 0.05$). When these parameters were analysed according to the dose of D-Pen, the correlation between AI and VA in the sero-positive test group became more significant as the dose of D-Pen was reduced to 0 mg / day.

Thus, the results suggest that in patients with sero-negative disease, decreasing the daily intake of D-Pen results in an increase

Figures 6.7, 6.8, 6.9 and 6.10. The effect of D-Penicillamine daily intake on various clinical parameters

Patients were grouped as either sero-negative or sero-positive and as either control or test. The results for various parameters of disease activity within these groups were assessed according to the concentration of D-Pen being taken at the time of examination.

Results

The results shown in Figures 6.7, 6.8, 6.9 and 6.10 are expressed as the means plus one standard deviation. The variance between the groups on different daily intakes of the drug was analysed by a single factor ANOVAR test.

	Sero-positive		Sero-negative	
	Control	Test	Control	Test
AI	F 0.50	F 5.17	F 1.47	
	p NS	p < 0.05	p NS	
VA	F 3.18	F 8.00	F 1.30	
	p < 0.05	p < 0.01	p NS	
PV	F 0.29	F 5.63	F 0.42	
	p NS	p < 0.05	p NS	
%Facb	F 0.23	F 2.86	F 0.19	
	p NS	p NS	p NS	

KEY

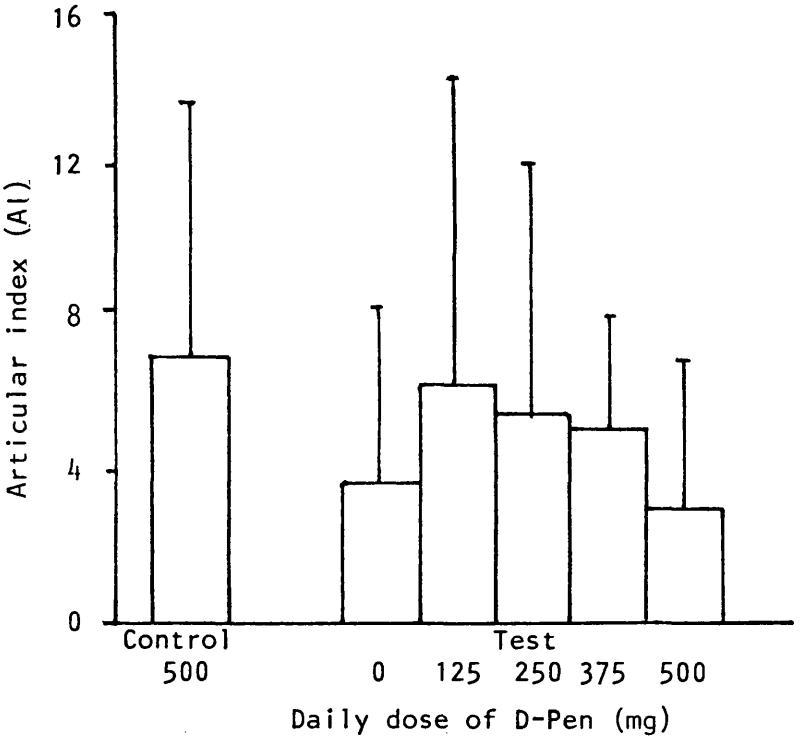
AI Articular index

VA Visual analogue scale

PV Plasma viscosity

F The F statistic for the single factor analysis of variance (ANOVAR) test.

Figure 6.7


16
12
8
4
0
Articular index (AI)
Control
Test
500
0 125 250 375 500
Daily dose of D-Pen (mg)

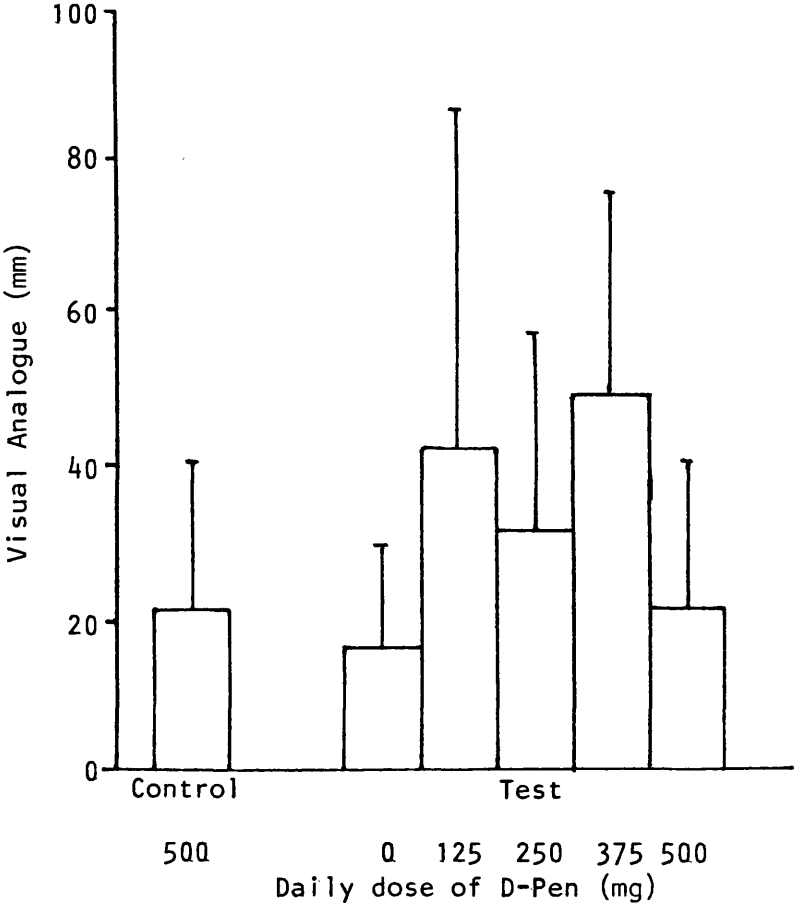
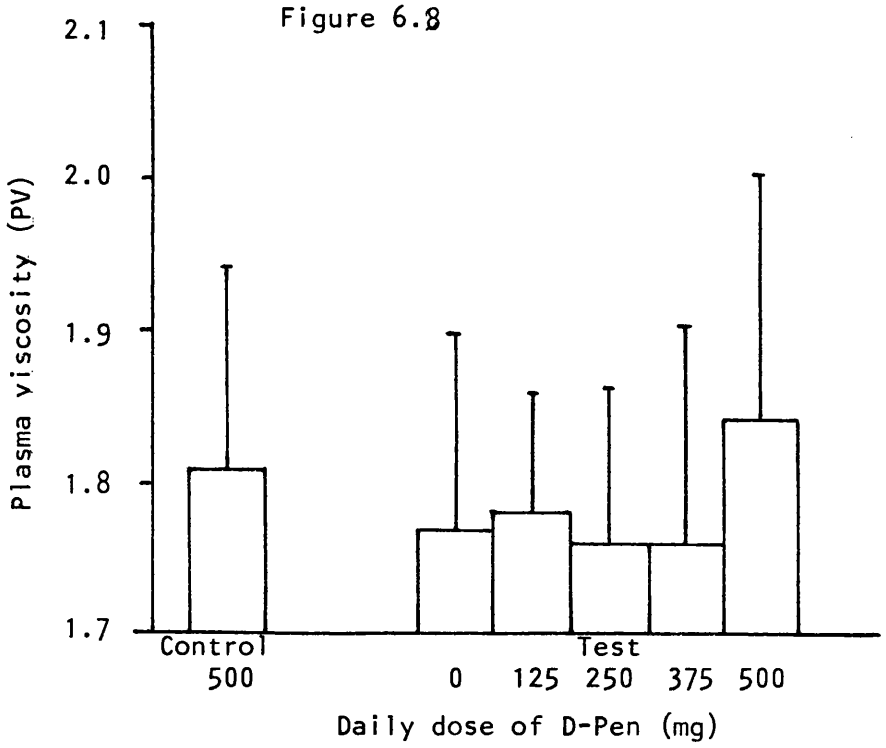

100
80
60
40
20
0
Visual Analogue (mm)
Control
Test
500
0 125 250 375 500
Daily dose of D-Pen (mg)

Figure 6.8


2.1
2.0
1.9
1.8
1.7
Plasma viscosity (PV)
Control
Test
500
0
125
250
375
500
Daily dose of D-Pen (mg)

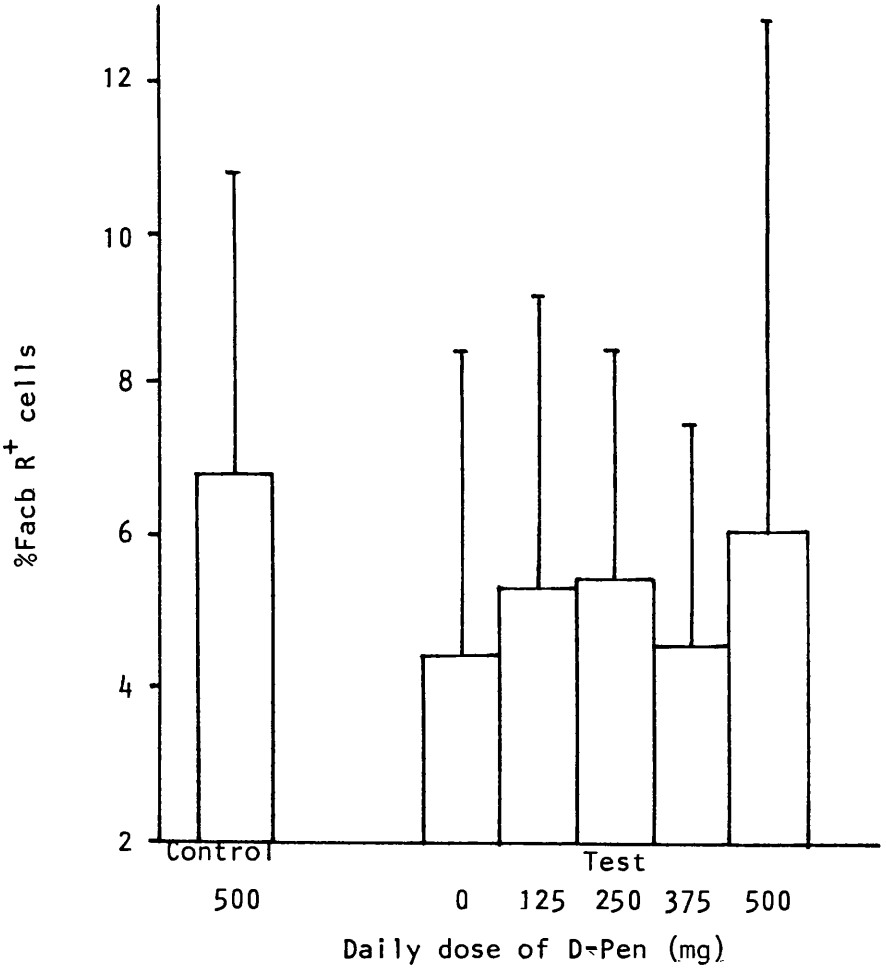
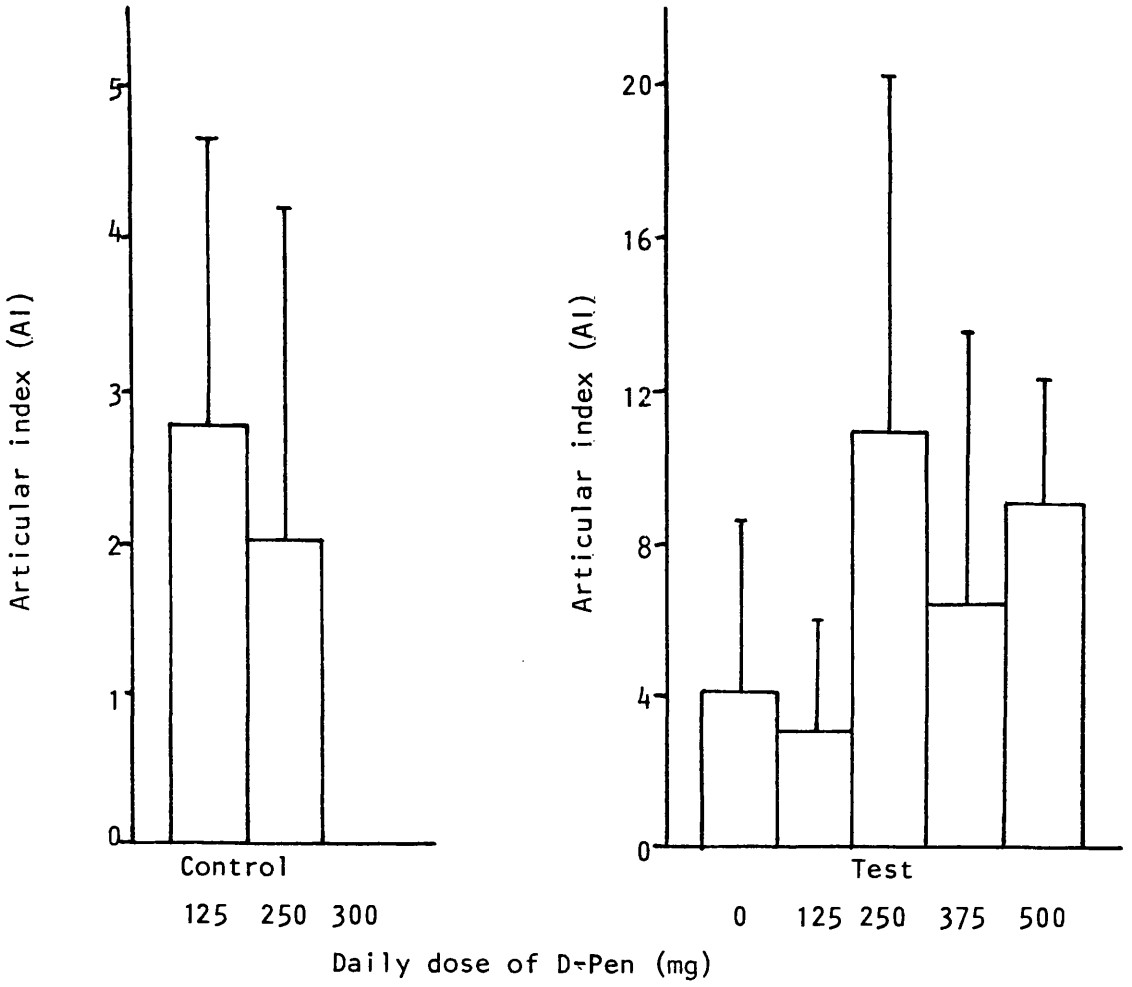

12
10
8
6
4
2
%Facb R+ cells
Control
Test
500
0
125
250
375
500
Daily dose of D-Pen (mg)

Figure 6.9


Articular index (AI)
0
1
2
3
4
5
Control
125 250 300
Articular index (AI)
0
4
8
12
16
20
Test
0 125 250 375 500
Daily dose of D-Pen (mg)

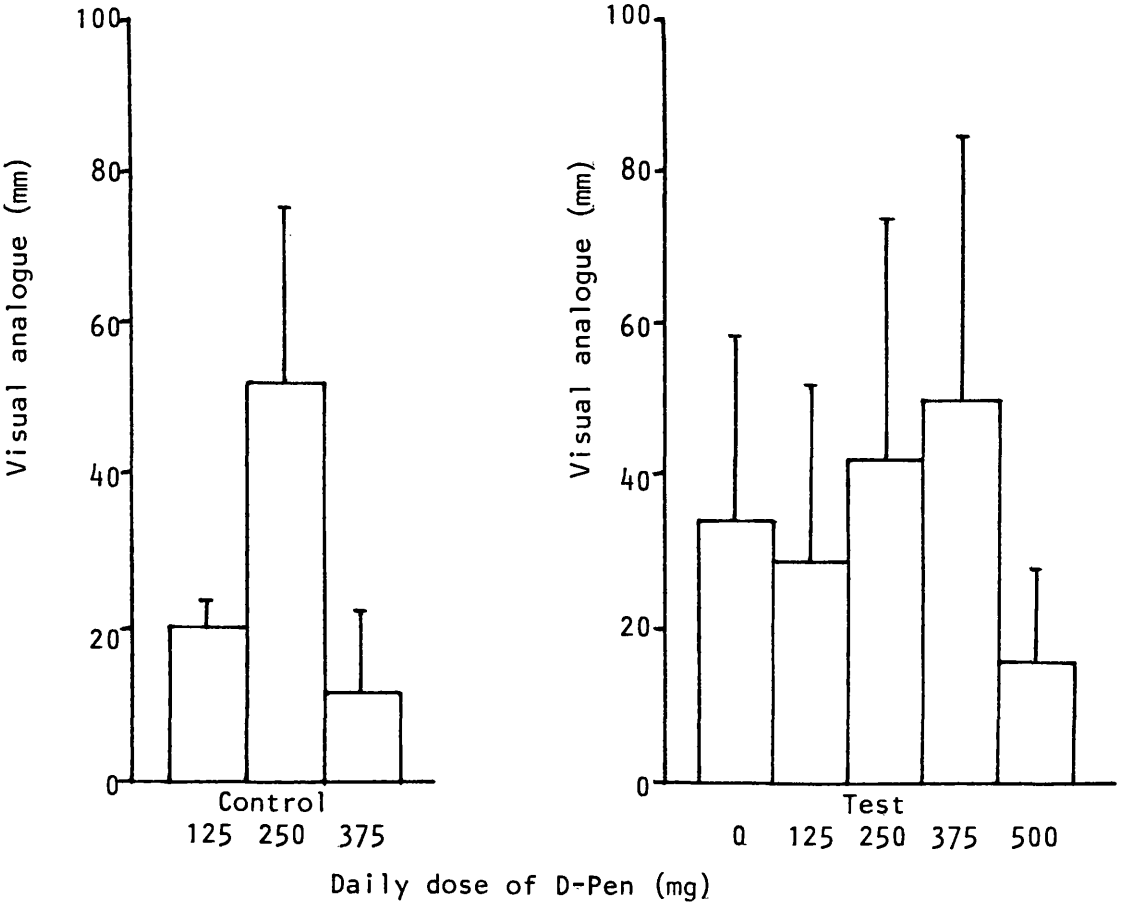
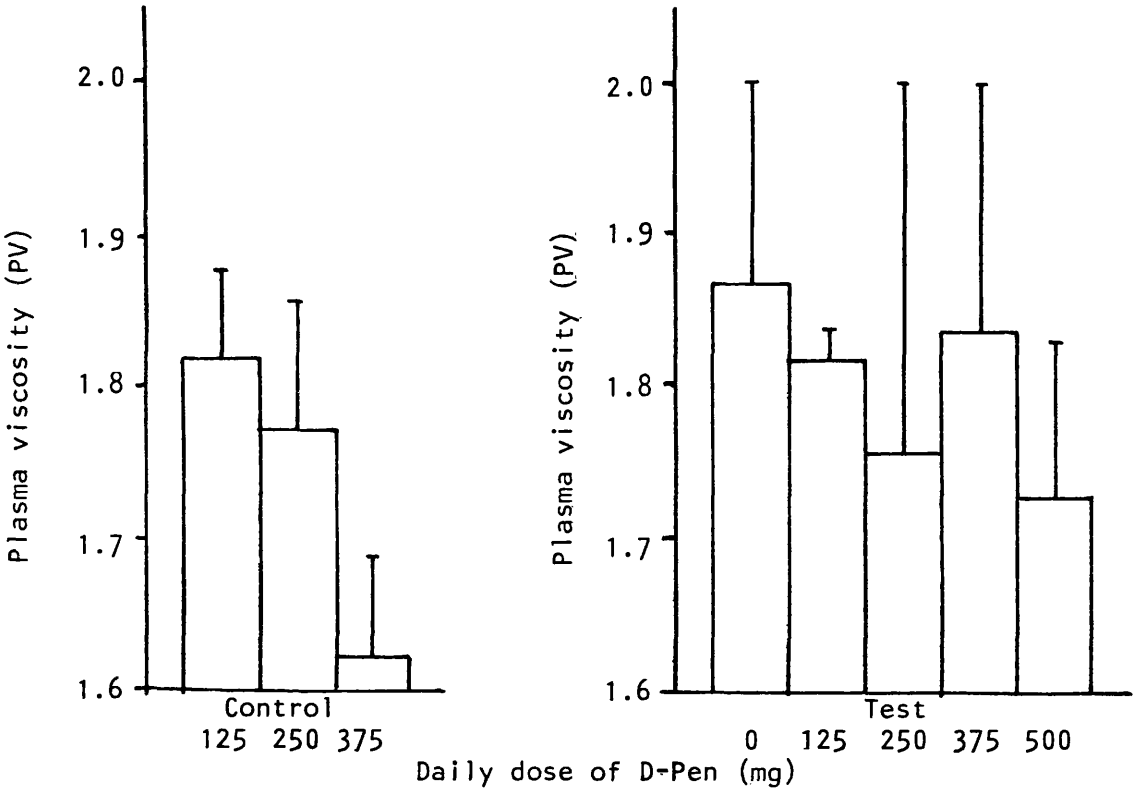

Visual analogue (mm)
0
20
40
60
80
100
Control
125 250 375
Visual analogue (mm)
0
20
40
60
80
100
Test
0 125 250 375 500
Daily dose of D-Pen (mg)

Figure 6.10


Plasma viscosity (PV)
2.0
1.9
1.8
1.7
1.6
Control
125 250 375
Test
0 125 250 375 500
Daily dose of D-Pen (mg)

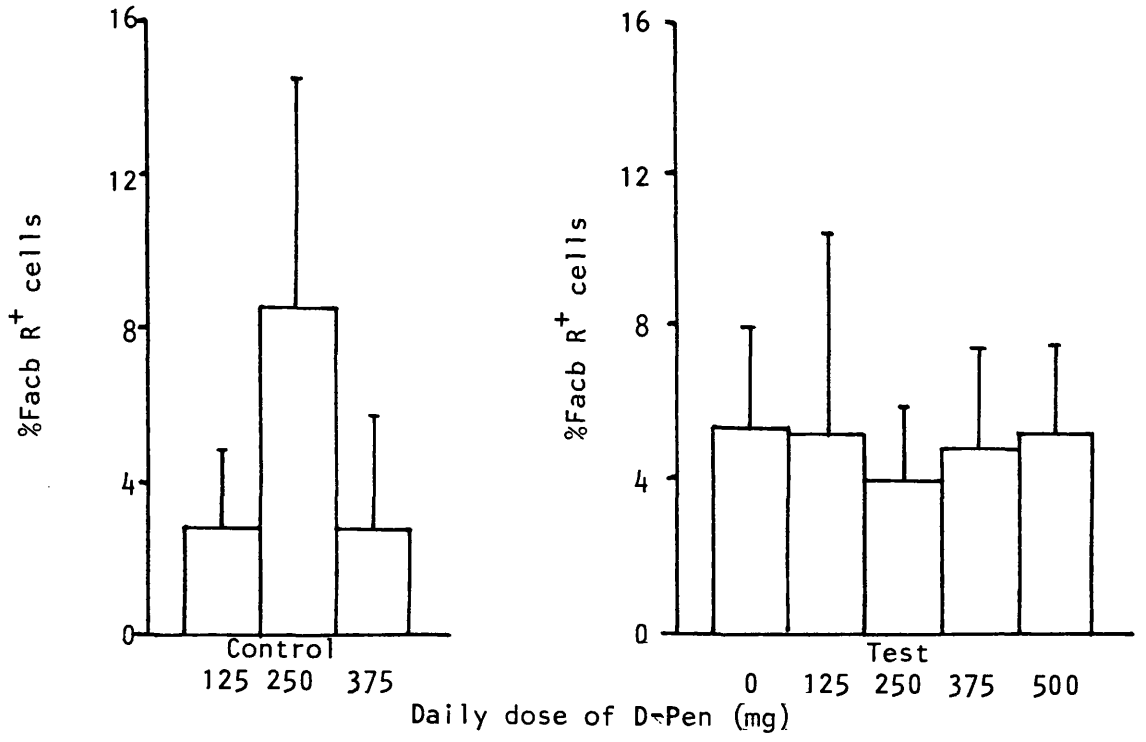

%Facb R+ cells
16
12
8
4
0
Control
125 250 375
Test
0 125 250 375 500
Daily dose of D-Pen (mg)

Table 6.3 The correlation between various parameters of disease activity in patients taking the same daily dose of D-Penicillamine

Correlation between:	Daily dose of D-Penicillamine 500	375	250	125	0
Sero-negative test					
AI v Facb	NS	NS	NS	-	NS
VA v Facb	NS	NS	NS	-	NS
PV v Facb	NS	NS	NS	-	NS
AI v VA	NS	NS	NS	-	NS
AI v PV	NS	NS	NS	-	NS
VA v PV	NS	NS	NS	-	<0.02
Sero-negative control					
AI v Facb	-	-	NS	NS	-
VA v Facb	-	NS	NS	NS	-
PV v Facb	-	NS	NS	NS	-
AI v VA	-	-	NS	NS	-
AI v PV	-	-	NS	NS	-
VA v PV	-	NS	NS	NS	-
Sero-positive test					
AI v Facb	NS	NS	NS	-	NS
VA v Facb	NS	NS	NS	-	NS
PV v Facb	NS	NS	NS	-	NS
AI v VA	<0.05	<0.02	<0,01	-	<0.001
AI v PV	<0.01	<0.01	<0.01	-	<0.05
VA v PV	<0.01	NS	<0.01	-	NS

KEY

NS The correlation coefficients were not statistically significant. The results are expressed as probabilities indicating the significance of the correlation coefficients, and were calculated from the equation shown in Table 6.1.

Table 6.4 The correlation between various parameters of disease activity in rheumatoid patients on D-Penicillamine

	AI	VA	PV	%Facb	(Drug)
AI		r 0.45 p NS	r 0.59 p < 0.02	r 0.23 p NS	r -0.70 p < 0.01
VA	r 0.35 p < 0.05		r 0.29 p NS	r 0.58 p < 0.05	r -0.15 p NS
PV	r 0.24 p NS	r 0.04 p NS		r 0.45 p NS	r -0.72 p < 0.01
%Facb	r -0.15 p NS	r 0.17 p NS	r -0.04 p NS		r -0.12 p NS
(Drug)	r 0.28 p NS	r -0.04 p NS	r -0.19 p NS	r -0.01 p NS	

CONTROL

TEST

SERO-POSITIVE

	AI	VA	PV	%Facb	(Drug)
AI		r 0.94 p < 0.001	r -0.27 p NS	r -0.20 p NS	ND
VA	r 0.77 p < 0.001		r -0.29 p NS	r 0.03 p NS	ND
PV	r 0.60 p < 0.001	r 0.54 p < 0.001		r 0.21 p NS	ND
%Facb	r 0.05 p NS	r -0.07 p NS	r -0.09 p NS		ND
(Drug)	r 0.00 p NS	r 0.24 p NS	r 0.14 p NS	r 0.10 p NS	

CONTROL

TEST

SERO-NEGATIVE

in clinical activity (as assessed by the articular index). This change is not reflected by a significant increase in %Facb R^+ cells as might be expected from the results of the early RA study. In contrast, the articular index of patients with sero-positive RA was unaffected by reducing the daily dose of D-Pen. Also, plasma viscosity correlated with disease activity in those sero-positive patients who were being taken off D-Pen ($p < 0.001$) but not in the corresponding sero-negative group. The total lack of correlation between %Facb R^+ cells and disease activity (as assessed by the articular index) may result from a number of factors. The simplest of these is probably that Facb R^+ cells are not involved in late rheumatoid disease. However, this is unlikely since the levels of %Facb R^+ cells in both sero-negative and sero-positive groups were significantly higher than the normal mean for healthy controls ($p < 0.01$). This may indicate that D-Pen interferes with the inflammatory process at a stage beyond that where Facb R^+ cells are involved or that D-Pen affects the expression of Facb R. In order to investigate this further, in vitro experiments were performed with the thiol containing compound - 2-mercaptoethanol.

(C) The in vitro effect of 2-mercaptoethanol on Facb R expression

Lymphocytes were obtained from patients with established RA who were in remission and were taking non-steroidal anti-inflammatory drugs (Chap.11 Sec. 11 C ii). These cells were incubated at 37°C for 30 minutes with various concentrations of 2-mercaptoethanol (2-ME). After incubation, the cells were washed and Facb rosettes were performed as described previously (Chap. 11 Sec. 11 D ii).

The results were analysed using a single factor analysis of variance (ANOVAR) test. This showed that the %Facb R^+ cells did not vary significantly in those cultures which contained 2-ME. However,

when compared to those cells which had not been incubated with 2-ME, the percentage of these cells was raised; this increase reaching statistical significance in those cultures containing 5×10^{-4} M 2-ME ($p < 0.05$; Table 6.5). This seems to indicate that thiol containing compounds may affect the expression of the Facb receptor, If this phenomenon is real, it may explain the lack of correlation between %Facb R^+ cells and disease activity in patients receiving D-Penicillamine therapy.

Table 6.5 The effect of 2-mercaptoethanol on Facb R expression

	Concentration of 2-mercaptoethanol (M)					
	$5x10^{-4}$	$2x10^{-4}$	$1x10^{-4}$	$2x10^{-5}$	$1x10^{-5}$	0
n	5	5	4	4	5	5
$\bar{x}$	7.8*	6.8	5.0	6.0	5.0	2.8
sd	3.1	3.0	1.4	2.5	2.5	2.4

Statistical analysis of the results

* $p < 0.05$ Determined by Student's t-test. The marked value is significant with respect to the value obtained for those cultures incubated without mercaptoethanol.

F 2.65 An ANOVAR test performed on those cultures containing 2-mercaptoethanol gave an F statistic of 2,65 which is not significant at the 5% level with 3 and 18 degrees of freedom.

CHAPTER VII

DISCUSSION

Facb rosette forming (Facb R^+) cells have been shown to be lymphocytes which lack surface Ig (SIg^-) and receptors for sheep RBC (E^-) and the third component of complement (C_3R^-; Winrow, 1982). Thus, these cells appear to belong to the null cell population discussed in Chapter 1 Sections 1 B iii and 1 C iii. Winrow (1982) also has shown that Facb R^+ cells are significantly raised in patients with rheumatoid arthritis (RA). These observations form the basis of the work in this thesis. A morphological comparison was made between Facb R^+ cells in patients with RA and healthy controls. The results of this study prompted the performance of experiments to investigate the function of Facb R^+ cells in an immune response. Clinical studies were also performed to investigate any possible involvement of Facb R^+ cells in the pathogenesis of RA.

Electron micrographs of both rheumatoid and control Facb R^+ cells showed that they possess deep nuclear clefts (Chapter 111). In patients with RA these cells were shown to be much larger than those from healthy controls. They also show evidence of activation since they possess many mitochondria and ribosomes, some polysomes and rough endoplasmic reticulum and several Golgi complexes. Horwitz et al., (1978) demonstrated that L cells possess very few cytoplasmic organelles but have a prominent nuclear cleft. This corresponds with the observations made of control Facb R^+ cells. Further evidence of a physical difference between rheumatoid and control Facb R^+ cells came from the study of density gradient separation of mononuclear cells. It was found that Facb R^+ cells from patients with active RA (with levels of Facb R^+ cells above the normal range) do not sediment through 52% v/v Percoll, whilst those from patients with levels of Facb R^+ cells within the normal range

accumulated below the Percoll. Thus, Facb R^+ cells from patients with active RA are less dense than those from rheumatoids with inactive disease. These results suggest that Facb R^+ cells are activated in RA patients. Recently, it has been shown that T cells express HLA-DR related antigens as a result of activation by mitogens (Evans et al., 1978) and that such DR^+ cells are significantly raised in patients with RA (Yu et al., 1980). Thus, since Facb R^+ cells are raised in RA and appear to be activated, it was decided to look for the presence of HLA-DR related antigens on them. It was found that Facb R^+ cells express HLA-DR related antigens in both rheumatoid patients and control subjects. Whether or not there was a quantitative difference in expression of these antigens between the two groups was not examined. Some workers have reported that the majority of null lymphocytes express these antigens (Horwitz et al., 1978; Niaudet, Greaves and Horwitz, 1978) whilst others have demonstrated their absence on these cells (Lobo, 1981; Despont, Steimer and Banderet, 1981). Clearly, the results described in this thesis agree with those of the former group of authors. Thus, although rheumatoid and control Facb R^+ cells show no differential expression of HLA-DR related antigens, they appear to be activated in patients with RA. In order to investigate this possibility further, the steroid sensitivity of these cells was examined. Although the mechanisms of corticosteroid action on mononuclear cells are not completely understood, the observed alterations in cell recirculation patterns are thought to result from a change in membrane characteristics caused by corticosteroid dependent alterations of cellular metabolism (reviewed by Pearson, Clements and Yu, 1979). Thus, these effects would appear to depend on the ability to synthesize various macromolecules and would thus require the presence of subcellular organelles and an active metabolism. It was found that Facb R^+ cells from patients with RA were steroid sensitive whilst those from control subjects were not. It is of interest to note

that in two control subjects Facb R^+ cells were steroid sensitive when tested at the time of their peak response to a Tine test (day 3 after skin test). Thus, the observations discussed so far appear to complement the data obtained from the electron micrographs. It was noted that Facb R^+ cells in patients with RA show a similar pattern of steroid sensitivity to monocytes. Lobo (1981) has shown that a large proportion of null cells express an antigen reactive with the monoclonal antibody OKM1 but that none of these cells reacts with a monoclonal anti-monocyte antibody produced by Bethesda Research Laboratories (BRL). Roberts and Greaves (1978) showed that 7% of peripheral blood lymphocytes are Sig^-, E^-, do not ingest latex but possess an antigen detected by a myelomonocyte - specific antiserum. These results were confirmed by Niaudet, Greaves and Horwitz (1979). In contrast to the work of Lobo (1981) it was found that Facb R^+ cells react with the BRL anti-monocyte antibody. These results, together with the observations that the myelo-monocytic cell line - K562 bears receptors which bind Facb, suggested that the Facb R^+ cell may be of the monocytic lineage.

Thus, Facb R^+ cells exhibit both monocyte derived and HLA-DR related antigens in control subjects and in patients with RA. Despite this phenotypic similarity, these cells are morphologically distinct in health and disease. Facb R^+ cells are larger in patients with active RA compared to healthy controls and appear to be metabolically active.

The data discussed above indicates that the increased percentages of Facb R^+ cells observed in patients with RA (Hall, Winrow and Bacon, 1980; Hall and Bacon, 1981; Winrow, 1982) may result from activation of these cells. The stimulus required for this activation was examined in control subjects and in mice. It was shown that the %Facb R^+ cells could be enhanced in sensitized individuals by challenge with a specific antigen (purified protein derivative of tuberculin, in humans; erythrocyte antigens, in mice; Chapters 4 and 5). This is in contrast

to the results of Horwitz and Garrett (1977) and Caraux et al., (1982) who showed that null (L) cells are not stimulated by soluble or cell surface antigens. These workers also showed that null cells are not stimulated by mitogens but that they do enhance the blastogenesis of other lymphocyte subpopulations in the mitogen stimulated cultures. This function has also been reported to be performed by the P cell in mice (described by Schrader and Nossal, 1980). The results presented in this thesis partly concur with these observations in that Facb R^+ cells from humans do not proliferate in response to mitogenic stimulation (Chapter 5). However, they do not enhance the mitogen induced blastogenesis of other lymphocyte subpopulations. Studies in mice showed that Facb R^+ cells could be increased by a single injection of lipopolysaccharide (LPS), a polyclonal B cell activator which stimulates B cells to secrete IgG (Anderson and Moller, 1972). This suggested that Facb R^+ cells may be involved (either directly or indirectly) in the production of IgG. Lobo (1981) showed that null cells could enhance IgG synthesis by B cells in a PWM stimulated system. The same cells could be made to suppress this production of IgG by pre-incubating them with immune complexes. The adoptive transfer into naive mice of erythrocyte primed, Facb R^+ cell depleted mononuclear cell suspensions resulted in an increase in the number of cells producting erythrocyte specific antibody (PFC) when compared with the effect of sham depleted cells. Also, when antigen specific Facb was injected into mice concomitantly with a challenge dose of antigen, the resulting number of antigen specific PFC was significanltly lower than when antigen alone was administered. This latter observation agrees with the findings of Lobo (1981) although the results of the adoptive transfer do not. This anomaly may be explained if those Facb R^+ cells adoptively transferred to naive mice from primed mice (challenged 3 days before transfer) were already activated to express suppressor function. Grantham and Fitch (1975) have shown that mice (recently

primed with high doses of antigen), produced lower levels of specific antibody after challenge than those animals primed with low doses of antigen. The authors suggest that the former group still have low levels of circulating antigen specific antibody at the time of challenge. This combines with antigen and by interaction with high avidity Fc R, prevents B cell stimulation. It is feasible that such a mechanism might have occurred in the host mice and that the Facb R^+ cells were activated in the host and hence, on transfer, suppressed the B cell response to the antigen. This effect would appear to require an high avidity Fc R. Winrow (1982) showed that the Facb R has similar characteristics to high avidity receptors found on other cells. Also, the results reported in Chapter 3 concerning the stability of the Facb R in response to temperature shock correspond to those of high avidity Fc receptors (described by Sandor et al., 1978; 1979; Sarmay, Istvan and Gergely, 1978). When human lymphocytes were cultured in vitro with PWM, addition of Facb (but not $F(ab')_2$) at the start of the culture significantly decreased the production of IgG. Thus, these results concur with those of Lobo (1981) despite the differences in experimental procedures. It appears therefore, that Facb R^+ cells are involved in the process of antibody mediated immune suppression. The experimental evidence supporting the existence of this phenomenon was discussed in Chapter 1 Section 11 D ii. Although all the data shows that this effect is mediated by an Fc R, the identity of the cells involved remains controvertial since T cells (Setcavage and Kim, 1980) and B cells (Miyama-Inaba et al., 1982) have been implicated. However, in both of these studies, subpopulations were isolated by negative selection (ie. by removal of the unwanted population from the mononuclear preparation) and hence null cells were not eliminated. Thus, whilst it is not immpossible that antibody dependent immune suppression is mediated by cells from all 3 major lymphocyte subpopulations, it is

feasible that these studies are demonstrating the activities of null cells alone.

Although the results indicate that Facb R^+ cells mediate suppression of IgG synthesis after interaction with immune complexes, the precise stimulus required for the activation of these cells is unclear. It cannot result merely from the interaction of IgG with the Facb R since Winrow (1982) has shown that almost 50% of the Facb R^+ cells in the peripheral blood of healthy controls carry passively absorbed IgG. This blocks the Facb R but does not result in the activation of these cells. It is of considerable note that the time course of the response of Facb R^+ cells to an antigenic stimulus mimics that of C-reactive protein (CRP). Synthesis of the latter is known to be stimulated by interleukin 1 (IL 1; Kampschmidt et al., 1980). Thus, it is possible that IL 1 activates Facb R^+ cells which may then suppress Ig production after interaction with immune complexes.

Therefore, the role of the Facb R^+ cell in an immune response may be to limit the reaction to low concentrations of foreign antigen to which the host has previously been exposed. The formation of immune complexes (by interaction of the antigen with the low levels of circulating antigen-specific antibody) and their subsequent sequestration by Facb R^+ cells would prevent a vigorous (and wasteful) secondary response. At higher concentrations of antigen, sequestration would not be sufficiently effective to prevent free antigen from stimulating reactive B cells. This would suggest that Facb R^+ cells are involved at an early stage in the immune response. Credence is given to this idea by the observations of Andersson, Hayry and Kontiainen (1977) which showed that early appearing suppressor cells are large lymphocytes whilst those appearing at a later stage are small to medium size. Electron microscope studies of Facb R^+ cells in patients with rheumatoid anthritis showed that these cells are significantly larger

in the disease state than in normal controls.

The observations of Hall, Winrow and Bacon (1980), Hall and Bacon (1981) and Winrow (1982) that Facb R^+ cells are raised in patients with rheumatoid arthritis, together with the results discussed above, suggest that the %Facb R^+ cells may give an indication of active synovitis in RA. A longitudinal survey of patients with early rheumatic diseases showed that the %Facb R^+ cells correlated with clinical assessment of disease activity in all inflammatory forms of the disease studied, except in patients with palindromic RA (Chapter 6). This lack of correlation probably results from the peculiar nature of the disease and it is possible that measurements of the %Facb R^+ cells in patients with palindromic RA would give a better indication of active synovitis than the more usual clinical methods. In contrast to this work, a study of patients with established RA who were in remission and were receiving D-Penicillamine therapy, appeared to contradict the findings of the early RA study. In these patients there was no correlation between their disease activity and the %Facb R^+ peripheral blood lymphocytes. However, the observation that a thiol containing compound (2-mercaptoethanol) could increase the %Facb R^+ cells in vitro, suggests that a similar mechanism may be occurring in patients receiving D-Penicillamine therapy. This would abolish the correlation between this parameter and disease activity observed in the early RA study. Thus, it is possible to postulate that D-Penicillamine enhances Facb R expression and thus enhances the suppressive activity of these cells.

The results of these clinical studies give rise to some interesting speculations concerning the role of Facb R^+ cells in the pathogenesis of RA. The in vitro and in vivo results discussed above indicate that Facb R^+ cells are increased in number after exposure to an antigen to which the host has previously been sensitized. If a similar mechanism is

occurring in RA, it would suggest that specific antigens initiate the inflammatory reactions observed in this disease. This has been shown to be true in patients with reactive arthritis. Chemotherapy designed to eliminate the precipitating micro-organism, results in the limitation of the inflammatory response. In such patients, the %Facb R^+ cells correlates strongly with disease activity. Causative organisms have not been identified in other forms of rheumatic disease (discussed in Chapter 1 Section 111). The autoimmune nature of the disease may reflect a loss of tolerance to self antigens initiated by a foreign, cross-reacting antigen. The role of Facb R^+ cells in such a reaction could be investigated using the autoimmune haemolytic anaemia model in mice. Another explanation for the presence of autoantibodies in RA was proposed by Moller, Strom and Al-Balaghi (1980). These workers suggested that autoantibody formation may be the result of polyclonal B cell activation induced by ligands acting non-specifically on T and / or B cells. The observations concerning the response of Facb R^+ cells in mice injected with LPS (a polyclonal B cell activator) would imply that Facb R^+ cells could participate in such a phenomenon. Thus it appears that Facb R^+ cells may play an important role in the pathogenesis of RA.This therefore implies, that the observations made concerning the effect of thiols on Facb R expression may reflect part of the mechanism involved in D-Penicillamine induced remission of RA.

Thus, the general conclusions to be drawn from the work presented in this thesis are :

i) that Facb R^+ cells appear to be activated in patients with RA

ii) that these cells may be increased in number as a direct result of stimulation (both in vivo and in vitro) by an antigen to which the host

has been previously immunized.

iii) that these cells mediate suppression of Ig production via the interaction of immune complexes with the Facb receptor.

Although Facb R^+ cells have been shown to be similar to L cells, the work presented in this thesis indicates that these two populations are not identical but that Facb R^+ cells may form a part of the L cell population.

Clearly, there are many directions in which this project could progress. Further in vitro work with human cells is required to confirm the findings that Facb R^+ cells suppress Ig synthesis. Ideally, it would be better to examine the phenomenon in an antigen stimulated system using both specific and non-specific Facb. The mechanism of suppression should be examined. The presence of soluble suppressor factors could be demonstrated by the effect of supernatants from cultures of stimulated Facb R^+ cells on fresh, antigen stimulated cells. The kinetics of this phenomenon could be examined using the same system. The antigen specificity of these cells has not been conclusively demonstrated. This could be done by adoptively transfering cells primed with two non cross-reacting antigens and challenging the hosts with both antigens but with Facb to only one of them. Mice could also be used to investigate the role of Facb R^+ cells in autoimmune phenomena as discussed previously. Thus, although the identity of these cells is relatively well established, much more work is required in order to fully elucidate the precise function of the Facb R^+ cell.

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