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Density distribution of peripheral blood lymphocytes in health and rheumatoid disease.

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DENSITY DISTRIBUTION OF PERIPHERAL BLOOD

LYMPHOCYTES IN HEALTH AND RHEUMATOID DISEASE

Submitted by,

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for the Degree of Doctor of Philosophy

at the University of Bath

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SUMMARY

The incidence, nature and significance of activated lymphocytes has been investigated in R.A. and healthy subjects.

The incidence of these cells using discontinuous Ficoll gradient analysis was studied in R.A. patients with different disease activity and in those undergoing gold therapy. The density gradient also provided a method for separating lymphocyte sub-populations for the further study of surface markers. These included the possession of surface Ig and receptors for sheep erythrocytes and IgG (Fc).
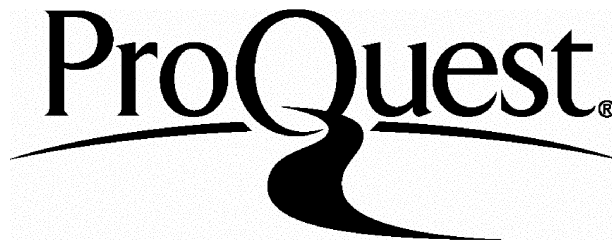
R.A. patients, particularly those with clinically active disease, had more circulatory activated lymphocytes (immunoblasts) and low density lymphocytes than healthy subjects. This increase in low density cells was not seen in patients receiving gold therapy.

Surface marker analysis showed that the population of low density (activated) lymphocytes in R.A. were enriched in surface Ig bearing cells and depleted of cells with sheep erythrocyte receptors, in comparison with the high density (small) lymphocytes. However, by comparison, high avidity Fc receptors were found predominantly on the dense lymphocytes whereas low avidity Fc receptors were more common on the low density cells.

The role of the activated cells in R.A. is considered with particular reference to the increased B cell activation in peripheral blood. The propagation of R.A. by activated lymphocytes is discussed in relation to their abnormal migratory patterns to inflammatory sites.

CONTENTS

CHAPTER ONE

INTRODUCTION

INTRODUCTION

This introduction will discuss the functions of leucocytes and their role in the pathogenesis of rheumatoid arthritis. Immunoblasts and lymphocyte activation in rheumatoid disease will be highlighted.

SECTION I   LEUCOCYTES

a) Introduction

William Hewson, more than 200 years ago, gave the first
description of the white blood cells (leucocytes). Under the
light microscope, the leucocytes were identifiable as colourless
refractile bodies, and because of the affinities of the nuclear
and cytoplasmic materials for certain dyes of the Romanovsky
type, a morphological distinction between two major cell
populations was possible. Cells possessing distinct cytoplamic
granules and a round to deeply lobulated nucleus were termed granulc-
cytes or polymorphonuclear cells; cells with sparse visible
granules and a larger rounded nucleus were classified as agranulo-
cytes or mononuclear cells.

On a histological and functional basis, the polymorphonuclear
cells can be further sub-divided into three sub-populations:
neutrophils, comprising 50-70% of the total leucocyte population,
and eosinophils and basophils, approximately 1.4% and 0.1%
respectively. Lymphocytes (20-40%) and monocytes (2-8%)
constitute the remaining mononuclear cell population of the leucocytes.

b) POLYMORPHONUCLEAR CELLS

There are three types of polymorphonuclear leucocytes. The
predominant type is the neutrophil, so called because of the neutral
nature of their cytoplasm. Less common are the basophils, which stain
with basic dyes and the eosinophils, which are acidophilic.

Granulocytes are produced from a common haematopoietic,
pluripotential, stem cell in the bone marrow. The process of
maturation and division requires 4-7 cell divisions (Craddock, 1972).

and a 9-10 day transit time before entering the peripheral blood.

During acute inflammation there is a loss of circulating

PMNs to a defence role at the sight of local demand. This is

rapidly adjusted by a compensatory discharge of reserve cells from

the marrow with a stimulated proliferation of their precursors

(Cartwright, Athens and Wintrobe, 1964). At the present time

the factors controlling maturation and granulocyte production

are poorly understood.

I. Life History

A dynamic perspective of the life history of granulocytes

emerges from kinetic studies using radio-isotope labelling to

monitor the rate of formation of new cells in the marrow and the

duration of their brief transit (6-10 hours) in the blood before

they emigrate into the extravascular tissues and body cavities.

The normal history of granulocytes is a progressive journey

through three compartments. i.e, from the bone marrow into the

circulating blood and thence out into the tissues with no

recirculation. In the steady state efflux of circulating granulocytes

is matched by influx of new cells from marrow reserves. The

marrow reserve of granulocytes is considerable, amounting to between

10 and 20 times the total population in blood and tissues. Granulocytes

survive 4-5days in tissues producing a total life span in the order of

10-14 days. Boggs (1967), using radiolabelling of granulocytes, calculated

their blood half life at 6.7 hours, implying a renewal of the total

blood population of these cells about two and a half times each day.

A minority of PMNs complete their life span in the circulation

and are removed from it by hepatic and splenic macrophages. The

majority of PMNs pass into the marginating pool in venules and

thence enter the extravascular tissues where, as free wandering

cells, they are able to engage in the phagocytic ingestion and

neutralisation of particulate antigens, such as bacteria. The

PMNs disintegrate soon after this and the remains are cleared by local

macrophages. Not all PMNs die in the tissues, a proportion must

emigrate across epithelia for they are a normal constituent of

the secretions of the lung and oral cavity.

II.Motility

Granulocytes are actively motile and the movement has been

compared to the amoeboid movement of free living protozoans

(Brewer, 1972). This has been tentatively explained as a result of

gel-sol transformation, aided by extremely fine contractile

microfilaments. It is aided by the firm adherence of granulocytes

to surfaces, as readily demonstrated on glass, and these cells have

been calculated to travel at 40-50 μm/minute (Dittrich, 1962).

III. Phagocytosis

The adherence of granulocytes has been regarded by some workers

as an extreme attempt to phagocytose (ingest) this surface, the cells

which most readily adhere are those which are the most actively

phagocytic. Phagocytosis by living granulocytes may be studied

under phase contrast when bacteria are mixed with the blood sample,

granulocytes extend as they move chemotactically, that is purposeful

directional movement towards strains of common bacteria. After pursuit

individual bacteria may be phagocytosed. In this glass slide system

granulocytes show no chemotactic interest in virulent strains of

bacteria and even after chance contact fail to ingest them;

adding specific antibody causes avid phagocytosis. Killing of these

bacteria occurs by degranulation of the granulocytes and the release

of antibacterial proteins (lyozyme and lactoferrin), alkaline

phosphatase and a range of acid hydrolase enzymes. Most of these

enzymes are released intracellularly, but an explosive degranulation

or frequent exposure to certain antigens may cause extracellular

enzyme release. Not only antigens are chemotactic, a number of serum

proteins under certain conditions are also effective, notably components

of the complement system.

Neither eosinophils or basophils are strongly motile or phagocytic

and neither are important in the phagocytosis of bacteria. However,

there is good evidence that eosinophils ingest and destroy antigen-

antibody complexes (Litt, 1964), possibly preferring IgE containing

complexes. Basophils are only believed to survive a few hours in tissues

(Parwaresch et al, 1973).

c)   MONOCYTES

Monocytes circulate for about 32 hours in the blood before emigrating

through the venules to populate the peritoneum, lungs and liver where

they can persist for 40-60 days (Van Furth, 1970), although they

invariably develop into fixed tissue macrophages (Vernon-Roberts, 1969;

Spector, 1969). Monocytes adhere most easily to glass, are actively

phagocytic and have lysosomal granules which store latent hydrolytic

enzymes; thus, monocytes can act in a similar manner to granulocytes

in phagocytosis and degranulation. Monocytes are also chemotactically

motile, but appear to have separate and distinct chemotactic factors

to polymorphonuclear cells (Spector, Walters and Willoughby, 1967).

Lymphocytes stimulated by specific antigen may release a soluble

factor (lymphokine) which inhibits the migration of normal macrophages.

This migration inhibitory factor (MIF) is only produced in response

to the specific sensitizing antigen (David et al, 1964). Lymphocytes

also produce chemotactic factors which influence the behaviour

of macrophages, working in harmony with MIF.

SECTION 2   LYMPHOCYTES AND THE LYMPHATIC SYSTEM

Lymphocytes are named from their predominance in lymph, the

clear protein-rich fluid in the lymphatic system. Morphologically

the latter arises in the capillary beds as blind- ending terminal

lymphatics which converge and unite to form a tree-like system,

ending in the thoracic and right lymphatic ducts which open into

the vena cava near the heart. Functionally the lymphatic vessels

provide a unidirectional, low pressure drainage pathway through

which excess tissue fluid and leaked plasma protein molecules are

restored to the blood. But the walls of the terminal lymphatics

are more permeable than those of nearby small blood vessels,

so that many wandering cells, sub-cutaneously injected drugs and,

during infections, bacteria and soluble antigens also leave the

connective tissues by this route. However, the contents of the

peripheral lymph are monitored as it seeps through the regional lymph

nodes strategically sited at the convergence of lymphatics e.g.,

in the mesentery and near the root of the lung, or interposed

in the axis of lymphatic pathways e.g., the popliteal, anguinal

and iliac nodes in the leg.

Each lymph node may be regarded as a filter screen of macrophages

through which a changing population of small lymphocytes is
continually directed. In normal states these macrophages remove
various non-lymphoid cells e.g., senescent granulocytes, monocytes
and stray red blood cells and sometimes also particulate debris
e.g., carbon dust particles from pulmonary lymph. In disease
bacteria and soluble antigens leaving inflamed sites are also carried
into the nodes, where macrophages and sensitised lymphocytes react
with them. In the ensuing response large numbers of new lymphocytes
and sometimes also of antibody-synthesising cells, are generated
in the enlarged node and are dispersed via the efferent lymph.

Delivery of lymphocytes from the thoracic duct occurs on a
massive scale, sufficient in the rat to renew the blood lymphocyte
population between five and ten times a day. Gowans and Knight
(1964) showed that, within minutes of being infused into the blood
labelled lymphocytes localised in and around the walls of certain
post-capillary venules (PCVs) present in the deep cortex of lymph
nodes and in the Peyers patches of the intestine. Ultra-
structively, the walls of these PCVs contain numerous small
lymphocytes (Marchesi and Gowans, 1964). From this grew the hypothesis
that lymphocytes leaving the blood migrated through the tissues to enter
the lymphatic system and thence were redirected into the blood -
the concept of recirculation of lymphocytes.

a) DIFFERENCES BETWEEN LYMPHOCYTES AND OTHER WBC

In human blood, lymphocytes comprise 20-25% of the differential
WBC count. Most of these are small lymphocytes about 6-9μm in
diameter. A few are medium or large lymphocytes. The recirculating
small lymphocyte differs significantly from granulocytes and monocytes
in several respects.

I <u>MORPHOLOGY</u>

The small lymphocyte is the smallest WBC, since its dense round nucleus is enclosed by only a sparse amount of cytoplasm. This cytoplasm stains blue with Romanovsky dyes due to its slightly basophilic nature caused by the ribosomes present. Ultrastructurally, lysosomes are rare, the meagre development of the few organelles present e.g., scattered mitochondria, sparse rough endoplasmic reticulum and modest Golgi, conveys the impression of a cell which is functionally quiescent, but with proliferative potential.

II <u>ADHERENCE AND PHAGOCYTOSIS</u>

Small lymphocytes rarely adhere to glass and do not form pseudopodia. Movement is slow and there is no evidence of phagocytosis. Mouse lymphocytes have had directional movement measured at an average of 19μm/minute (Matthes, Ax and Fischer, 1971).

III <u>LIFE SPAN</u>

Some lymphocytes last only a few days, but others can survive for months or years in repeated recirculation.

IV <u>MITOTIC POTENTIAL</u>

Unlike the granulocytes the recirculating small lymphocyte is not a fully differentiated post-mitotic end cell. After recognition of antigen it divides and differentiates to mount an immune response against the antigen.

V <u>TRAFFIC PATHWAYS</u>

Granulocytes and monocytes have a unidirectional pathway from bone marrow through the blood to tissues. Small lymphocytes co-

exist with other WBC in these pools, but retain the ability

to recirculate through their fourth compartment, the lymphatic

system, and return to the blood.

b) LYMPHOCYTE SUB-POPULATIONS

Although the recirculating small lymphocytes appear morphologic-

ally identical, they are separable by difference in origin

in reaction to antigen, in subsequent differentiation potential

and in functional roles in the immune response.

It is generally accepted that there are two major different

small lymphocyte sub-populations (Raff, 1970):-

a) T-lymphocytes, processed by, or in some way

dependent on the thymus, and responsible for cell-mediated immunity.

b) B-lymphocytes, bursa-dependent and concerned

in the synthesis of circulating antibody (Roitt, 1974). The bursa,

discovered in chickens, has no equivalent mammalian organ, although

gut associated lymphoid tissues and the bone marrow itself have been

nominated as possible candidates.

T lymphocytes may be identified in vitro by anti-T antisera

(Aiuti and Wigzell, 1973; Williams et al, 1973), by the binding

of sheep erythrocytes in the cold (Wybran, Carr and Fudenberg,

1972; Jondal, Holm and Wigzell, 1972) and their lack of B cell

markers.

The presence of integral surface immunoglobulin is often

used to identify B cells (Van Furth et al, 1965; Lawton et al,

1972; Vossen and Hijams, 1975). Other markers used are surface

receptors for the C3 component of complement (Ross et al, 1973).

Receptors for the Fc portion of immunoglobulin (Dickler, 1976)

are possessed by B cells but some other cell types also

possess them.

Recently cells with neither T or B cell markers have been

described. Perlmann and Holm (1969) described K cells and Horwitz

and Lobo (1975) defined L cells. ,

C) LYMPHOCYTE-ANTIGEN INTERACTION

Immune responses depend on the interaction of antigens

with cells of both the mononuclear phagocytic system and the lymph-

oid system (Schrader and Feldmann, 1973; Askonas and Roelants,

1974). This often happens in the regional lymph node, the antigen

arriving via the afferent lymph. There is a sudden fall in

the number of lymphocytes that leave the node in the efferent

lymph (Hall and Morris, 1965). Ford and Gowans (1969) suggested

that this 'lymphocyte trapping' was part of the role of lymphocyte

recirculation to offer to the lymph nodes a large diverse

population of lymphocytes from which those with appropriate

immunological potentials could be selected and retained in order

to react with antigens that had been fixed by the phagocytic cells.

From this general concept it is a short step to view this trapping

as a specific immunological selection'recruitment'. (Strober and Dilley

1973).

Immediately after this shut-down period, which lasts about

24 hours, lymphocytes reappear in the lymph in increased numbers

so that the extent of lymphocyte recirculation may be double or

treble the basal level (Hall et al, 1967). The characteristic

feature of the lymph cells during this time is the presence of

large numbers of immunoblasts (Hall and Morris, 1963). These

are a mixture of both T and B cells, like other lymphoid

populations, depending upon the stimulus. In many experiments

B cell responses predominate so it is easy to demonstrate that

these lymph-borne blasts contain and secrete antibody and that

many become plasma cells (Hall and Morris, 1963; Hall et al,

1967; Birbeck and Hall, 1967; Hall, 1970; Hall, Parry and

Smith, 1971; Murphy et al, 1972), which are the major antibody-

producing cells.

Unlike small lymphocytes the immunoblasts incorporate

DNA precursors rapidly and some may be specifically cytotoxic

in vitro (Denham et al, 1969). Immunoblasts produced by the

caudal lymph nodes of rats can be obtained by collecting the

thoracic duct lymph 3-6 days after antigenic stimulation of the

hindquarter regions (Delorme et al, 1969). Because immunoblasts

are released in large numbers before antibodies appear in the blood

it has been suggested that their most important function is to

extravasate into the tissues and so provide, by local synthesis,

the antibody that the blood is unable to supply (Hall, 1969). This

is supported by evidence that only a small percentage of immunoblasts

(less than 5%) re-enter the lymph in rats (Hall, 1974). Some

colonize lymphoid tissue in the spleen and nodes remote from the

site of the localisation of antigen, and many go to the gut.

Gowans and Knight (1964) first showed that the small number

of immunoblasts present in normal thoracic duct lymph 'homed'

preferentially to the lamina propria of the small gut after I.V.

injection into syngeneic recipients. This 'homing' is not influenced

by gut contents; it is found in the small gut of Caesarian-delivered

neonates where the recipient is agammaglobulinaemic and the gut is

sterile (Halstead and Hall, 1972). Parrott and Ferguson (1974) showed

that a foetal gut graft under the kidney capsule, away from the

influence of normally situated gut attracted a normal share of

immunoblasts.

There can be little doubt that most immune responses bring

about an investment of activated cells in the gut; the B blasts

go to the lamina propria and become IgA-secreting plasma cells

(Hall and Smith, 1970), the T blasts go to the Peyers patches

(Ford, 1974). Recent evidence (Hopkins and Hall, 1976) suggests

that this applies more so to blasts from gut associated lymphoid

tissue (GALT).

Evidence for the localization of activated lymphoid cells

in GALT has been reviewed by Hall (1974); unfortunately, attempts

to demonstrate selective 'homing' of sensitized lymphocytes into

delayed-type hypersensitivity (DTH) lesions, allografts or tumours

have not met with conspicuous success (McCluskey, Benacerraf and

McCluskey, 1963; Hall, 1967; Moore and Hall, 1973).

In humans the increased levels of immunoblasts from lymph

nodes caused by antigen activation is reflected in an increase

in the numbers found in peripheral blood (Crowther, Hamilton-Fairley

and Sewell, 1969a).

d) IMMUNOBLASTS

    I MORPHOLOGY

    The first description of an atypical lymphocyte was given by Turk

in 1907; greater detail was provided by Downey and McKinlay (1923). The term immunoblast was first suggested by Dameshak (1963) and the morphological characteristics of these cells from various sources reviewed by Wood and Frenkel (1967) and Chalmers et al (1966). Stimulation of lymphocytes in vitro with both antigens and mitogens has been used to study the various stages of activation. It is apparent that both the morphology and time course of activation by antigens, mitogens and mixed lymphocytes culture are very similar in vitro (Inman and Cooper, 1965), the main differences being in the T or B cell origin of the activated cell (Asherson, Allwood and Mayhew, 1973).

The small lymphocyte measures 6-9μm in diameter and is characterised by its dense nucleus which occupies the large part of the cell, leaving a thin rim of clear cytoplasm. Intermediate lymphocytes measure 9-12μm in diameter, also have a dense nucleus sometimes with a slight nuclearcleft, and a greater amount of cytoplasm, in relation to the nucleus, than seen in small lymphocytes. Large lymphocytes (immunoblasts) range from 12-25um in diameter and have a strongly basophilic cytoplasm with an eccentrically placed nucleus. The chromatin of these cells is in interphase and appears to have a combination of a stippled and a fine reticular pattern. The nucleus contains two or more nucleoli and some cells may have azurophilic granules in the cytoplasm. These characteristics are all easily seen on dry smears stained with a Romanovsky dye.

Electron microscopical examination emphasises the difference between resting and transformed cells.

The cytoplasm of the small cells contains a granular matrix which is thought to be mainly structural protein. There are a few regular-shaped mitochondria and a relatively undeveloped Golgi zone. Lysosome-like bodies are occasionally seen. The cytoplasm of the transformed cells has a similar granular matrix, but also contains large numbers of free ribosomes; high magnification has shown that polyribosomes are present in their cytoplasm. The Golgi zone is highly elaborate and irregularly-shaped mitochondria and lysosomes lie within this region. Other features seen in a number of cells include a high degree of irregularity of the outer nuclear membrane with marked extensions of this membrane into the cytoplasm and a prominent nuclear Hof .

Smetana and Potmesil (1970) have shown that resting, small lymphocytes may be characterised by the presence of ring-shaped nucleoli whilst transformed lymphocytes have larger dense nucleoli with a greater content and uniform distribution of ribo-nucleic basophil structures. This has been confirmed in infectious diseases (Skramkova and Fortova, 1972) and in experimental allergic encephalomyelitis in the guinea pig (Pekarek et al, 1972). Regions of the cytoplasm of some transformed cells contains fibrillar material and stain deeply with pyronin.

These characteristics are typical of the immunoblast, the largest lymphoid cell type. Other authors have disagreed as to the morphological criteria for the various atypical types and a large number of names have been used, presumably for cells in different parts of the cell cycle. These include lymphoblast, plasmablast and lymphoplasmacyte (Avrameas and Leduc, 1970).

However, to a haematologist a lymphoblast is a bone marrow-derived small lymphocyte precursor. The terms plasmablast and lymphoplasm-ocyte assume that the blasts are precursors of antibody forming cells.

Evidence has suggested that immunoblasts with a B cell origin have a rough endoplasmic reticulum (ER) whilst the T cell has a smooth E.R., The B cell E.R having attached clusters of polyribosomes. During the development of B cells to plasma cells, there is a more extensive E.R. with electron-dense material in the distended sacs which are arranged concentrically around the nucleus. (Birbeck and Hall, 1967).

Polliack et al (1973) and Lin et al (1973) reported that, by scanning electron microscope investigation, B cells had a villous (hairy) surface and T cells were smooth. However, it now appears that the development of a villous surface is either part of both T and B cell differentiation (Baur et al, 1975) or a reflection of lymphocyte activation. Criswell et al (1975) stimulated mouse lymphocytes with various T and B cell mitogens and found increased numbers of highly villous cells. Examination of the surface morphology of human T and B cells showed no difference between these two subpopulations and was dramatically affected by the experimental parameters and preparative techniques for SEM (Alexander and Wetzel, 1975). To date, the surface morphology of resting and activated human lymphocytes has not been investigated. However, the formation of spontaneous rosettes between human T cells and sheep erythrocytes stimulates formation of villi (Polliack et al, 1974; Lin et al, 1973; Wetzel et al, 1974). Van Ewijk et al

(1975) suggest that the presence of microvilli is more related

to activation at the lymphocyte-endothelial contact point in

post-capilliary venules (PCVs) during lymphocyte recirculation.

Microvilli also depend upon the method of cell fixation

(Scott-Linthicum and Sell, 1974). Reducing the temperature of

cell culture media for the bovine lymphocyte line, NBC-6

affected the size and number of microvilli. At $25^{\circ}C$ there were

a large number and at $4^{\circ}C$ there. were only a few of increased

diameter (Lin, Wallach and Tsai, 1973).

II LIFE SPAN

Immunoblasts do not last for many hours before further

development. Cell division follows and results in smaller

lymphocytes (and possibly plasma cells, if of B cell origin).

After antigenic stimulation of small lymphocytes in a lymph

node the peak number of immunoblasts in efferent lymph is reached

within 80-120 hours (Hall, Parry and Smith,.1971). At this stage

15-30% of the lymph cells are immunoblasts. Most of these

appear to extravasate into the tissues (Hall, 1969; Hall and

Smith, 1970). Certainly, very few, if any, recirculate from

blood to lymph. This has been related to putative complementary

receptors on the immunoblasts and vascular endothelia which allow

their extravasation into the tissues. On the other hand the

small lymphocytes may possess a receptor for the endothelia

of PCVs, which allows their passage from blood to lymph (Gesner

and Ginsburg, 1964).

Apart from extravasation, these immunoblasts may be removed

from the circulation and destroyed by the monocyte/macrophage

system (Wood and Frenkel, 1967).

A resting lymphocyte (at phase Go of the cell cycle) takes up to 12 hours after antigenic stimulation to enter DNA synthesis in vitro (G phase). The next stage is of increased DNA synthesis, during which the cell enlarges and is easily detected by increased uptake of DNA precursors, such as ^{3}H-thymidine (S-phase). The increase in cell size is more related to the presence of cells in the S and G_2 + M phases in the cell cycle, rather than to blast transformation per se (Steinman et al, 1978). Gunthe et al (1974) obtained information that stimulated cells in G_1 are irreversibly committed to entering S phase many hours before they actually synthesize DNA.

Reports on the duration of S-phase have been wildly different, probably due to the source of lymphocytes and the antigen used. Yoshida and Osmond (1971) reported that the S-phase lasted 13-14 hours whereas Steinman et al (1978) recorded a period of 18-24 hours using flow microfluorometry. However, maximal incorporation of tritiated thymidine takes place after 60-70 hours of mitogen stimulation (Ling, 1968), almost certainly reflecting in vitro recruitment.

Correlation of these numerous in vitro findings with in vivo events is difficult. The role of cell-cell co-operation is very important. The role of macrophages and regulator T cells in vivo has not been accurately determined in man. Polyclonal stimulation by mitogens is not always comparable to antigen stimulation, for example, maximum ^{3}H-thymidine uptake with mitogens is after 60-70 hours of culture whereas it is 110-120 hours with antigens.

The failure of lymphocytes to proliferate continuously in vitro has often been assumed to be a reflection of poor tissue culture conditions. However, it may be that rapid maturation of the blast to an end stage cell limits proliferation. For example, Schooley's data (1961) indicate that at least 80% of mature plasma cells in situ may live less than 12 hours.

Feldbush and Stewart (1978) have also shown that B blasts do not recirculate in the rat. Large memory cells were shown to be formed in antigen draining lymph nodes and able to circulate, but not recirculate in the normal way by passing from blood to lymph.

Steinman et al (1978) suggest that not all blast cells divide to produce viable, haploid cells. Some blasts die, presumably effector cells e.g., plasma cells and cytotoxic cells, even those which divide do not produce all viable progeny.

Gowans et al (1962) and Birbeck and Hall (1967) have attempted to maintain in vivo generated immunoblasts in tissue culture and been uniformly unsuccessful, concluding that the requirements of these cells for survival and development are fairly critical and best provided by their normal physiological environment, inside a lymph node. This may be true, to some extent, but allied to the rapid maturation and the spontaneous death of blasts, the reasons for the rapid loss of these cells from the circulation are complex. Their life span in tissues has not been measured.

The life span of small lymphocytes is relatively well documented (Gowans, 1966; Parrott and de Sousa, 1971).

There are two populations of lymphocytes called short lived and long lived; the short lived consist of a population of cells, the majority of which will incorporate ^{3}H-thymidine within 4-5days

whereas the long lived population consists of cells, many of

which remain unlabelled over weeks or months. Rieke and

Schwarz (1967) related the long lived population to T cells

and the short lived population to B cells. This postulate

has enjoyed general support, with some reservations (Parrott

and de Sousa, 1971). Asherson, Allwood and Mayhew (1973)

have shown that T blasts, produced by contact sensitivity

to oxazolone, in the mouse, arise in the draining lymph nodes

with the same time course as B blasts produced by antigenic

challenge at lymph nodes. This confirms the work of Pritchard

and Micklem (1972) and Asherson and Barnes (1973), who analysed

the time course of DNA synthesis.

III FUNCTION

Immunoblasts are believed to be the effector cells of the

immune response and to be the precursors of immunological

memory. B blasts invariably become plasma cells and memory

cells. T blasts become T effector cells and are involved in

cell mediated immunity and the immunoregulation of T and B

cells. Memory cells are also produced by T blasts.

Antigenically stimulated lymph nodes release immunoblasts

in the efferent lymph which have been shown to contain (Hall

and Morris, 1963) and to secrete (Cunningham, Smith and Mercer,

1966; English, Morris and Adams, 1976) specific antibody even

though they display little organised endoplasmic reticulum

(Hall et al, 1967). Immunoblasts normally extravasate into

the tissues (Hall, 1969) and, certainly, some such process is

necessary if effective titres of antibody are to be established

in the extravascular tissue spaces (Hall et al, 1967).

These antibodies may sensitize macrophages and mast cells in the

tissues rather than simply produce conventional circulating

immunoglobulins. B blasts which enter the gut secrete IgA

as the major immunoglobulin (Hopkins and Hall, 1976) and

surface IgA before release from mesenteric lymph nodes (McWilliams

et al, 1977). The immunoblasts which develop into plasma cells in the

lymph or blood are believed to produce the circulating antibodies.

Plasma cells, themselves do not extravasate into tissues. The

nature of the lymph may have a suppressive action on the production

of antibody from B blasts, but the nature of this inhibitor is so far

unknown (English, Morris and Adams, 1977). Immunoblasts from a

primary response undergo a fundamental physiological change to

produce recirculating long lived cells involved in the secondary

antibody response (Strober, 1972). Memory cells from draining

lymph nodes are blast cells soon after antigenic challenge, but

after many days the memory cells are all small lymphocytes. This

supports the views that immunoblasts do not recirculate, that

small lymphocytes do recirculate and that some B blasts

divide in the circulation to provide B memory cells (Feldbush

and Stewart, 1978).

The generation of T blasts heralds a more diverse range of

potential effects and function being performed by a particular

lymphocyte sub-set, conforming with the doctrine of clonal

selection. T-immunoblasts have been demonstrated to be cytotoxic

in vitro to   alloantigens (Cerottini et al, 1970;

Roberts and Hayry, 1977). T blasts also give rise to memory cells

to alloantigens (Wilson and Nowell, 1971; Hollander et al, 1974).

T blasts can also modulate the effectiveness of antibody production

by B blasts and plasma cells. (Strelkauskas et al, 1977) This

modulation is demonstrated in vitro by the generation of T

cells with either helper or suppressor functions in cultures,

containing low or high levels of protein antigen, respectively.

(Kontainen and Feldman, 1975, 1976). The first helper and

suppressor cells to be detected are T blasts whereas later both help

and suppression is mediated by medium and small sized lymphocytes.

The blasts are proliferating to increase the number of small

effector cells, but are still themselves active, particularly

as suppressors   (Andersson et al, 1977).T blasts may produce

small lymphocytes which carry specific memory and have the

ability to recirculate. (Sprent and Miller, 1972 a, b, c.).

Specfic antigen stimulation of T cells causes the release

of soluble, non-antibody mediators - lymphokines (Dumonde et al,

1969). To date, a large number have been reported with widely

differing effects on various cells and tissues. Some even

activate other lymphocytes in vitro. Immunoblasts can produce

lymphokines before cell division occurs (Rocklin, 1973). Thus,

antigen-activation of T cells can trigger off a whole sequence of

events.

After immunization with the contact sensitizing agent

Oxazolone the cells that move to sites of inflammation are

predominantly T blasts and have the properties of non-specific

cytotoxicity and the capacity to passively transfer cellular

immunity (Asherson et al, 1973).

IV METABOLISM, MARKERS AND MOTILITY

As proliferating cells, immunoblasts have increased rates of

metabolism measurable by several parameters. The more easily

detectable change are increases in newly formed DNA and RNA,

both easily shown with Romanovsky stains or methyl green

pyronin (Elves et al, 1963).

Increases in DNA content have been demonstrated by increased

incorporation of radiolabelled thymidine in lymphocytes stimulated in

vitro with the mitogens phytohaemagglutinin (PHA) (Waithe et

al, 1971), Concanavalin A (Con A) (Stobo et al, 1972), Pokeweed

(PWM) (Froebel et al, 1975), with numerous antigens including

bacterial products (Blomgren, 1975) and viral antigens

(Mochizuki et al, 1977). Lymphocytes activated in vivo also

incorporate ^{3}H-thymidine in vitro (Carter, 1965; Yoshida and

Osmond, 1971). The proliferative response has been demonstrated

with ^{3}H-thymidine and autoradiography (Schrek, 1963)

or ^{14}C-thymidine and scintillation (Dutton and Eady, 1964).

Flow microfluometric analysis of DNA content has been used to

measure blastogenic responses (Cassidy et al, 1976).

The increased synthesis of RNA is detectable using

radiolabelled uridine (Epstein and Brecher, 1965) and flow

cytofluorometry (Braunstein et al, 1976).

Biochemical studies of membrane receptor sites for

various activators and kinetic and inhibition studies of the

early events indicate that the initial events of activation occur

at the level of the lymphocyte membrane (Fischer and Mueller, 1969;

Coulson, 1969). Increased turnover of membrane phospholipids

(Fischer and Mueller, 1969; Lucas et al, 1971),induction of pinocytosis

and labilization of lysosomes (Hirschhorn and Hirschhorn, 1965;

Hirschhorn et al, 1968), activation of an adenyl cyclase

'message' (Smith et al, 1971; Watson, 1975), increases in

carbohydrate metabolism (Roos and Loos, 1973; Wang, Marquardt

and Foker, 1976) and other biochemical changes, all occurring

within the first hour or two of activation, are followed by

events associated with derepression of eukaryote genomes

(Hirschhorn et al, 1969). Ling (1968) has reviewed lymphocyte

stimulation in greater detail than given here. ·

Apart from the internal metabolic changes there are a

number of changes which are detectable on the lymphocyte surface,

thus allowing analysis of individual cells and the use of

definitive markers for lymphocyte activation in vivo, often before

the appearance of immunoblasts.

A number of workers have analysed the T and B cell markers

on blast transformed cells and found some striking differences

by comparison with resting lymphocytes. Blast cells produced by

PHA, Con A, pokeweed and allogeneic cell culture all had either

E-rosette markers or surface Ig. B blasts lost their C_3 and Fc

receptors (Jondal, 1974 a, b). However, Svedmyr, Wigzell and

Jondal (1974) found that T blasts produced by sensitization to

allogeneic or autologous lymphoblastoid cell lines were 'null'

cells i.e., had neither surface Ig, SRBC receptors, C_3 receptors

or Fc receptors, suggesting that the nature of the antigen can

affect the marker expressed on the T blast. Whiteside and Rabin

(1976) found that the predominantly T cell mitogens (PHA and Con A)

produced E-rosetting blasts which had surface IgM. IgG and IgA

and light chain determinants were only detected in PWM-induced

blasts. Hellstrom et al (1971) found that mitogenic stimulation

of human T cells lead to the presence of easily detectable

concentrations of a light chain. Vitetta, Forman and Kettman

(1976) showed that B lymphocytes with different surface Ig

markers gave rise to different responses to bacterial

lipopolysaccharide (LPS), those with IgM elaborate an IgM

response, those with IgD only undergo proliferation to produce

numbers of memory cells.

Immature bone marrow B cells have a reduction in the number

of cells with receptors for C_3 or Fc and a large number of bone

marrow cells (60% $\pm$ 13%) have been classified as null cells

(Abdou, Alavi and Abdou, 1976).

Cytochemical methods have been used by Catovsky et al (1974)

to differentiate T and B blasts in lymphoproliferative disorders.

B leukaemias were PAS positive and T leukaemias were PAS negative

whilst T leukaemias were acid phosphatase positive and the B

leukaemias were negative. The PAS (Periodic acid Schiff) positivity

is diastase - sensitive and therefore represents cytoplasmic

glycogen content. Chronic lymphocytic leukaemias have been

divided into a B or T cell origin by the presence of surface Ig on the

neoplastic cell with reservations as to its use for functional studies

(Knapp et al, 1974; Sagone and Murphy, 1975).

A certain proportion of T cells form E-rosettes easily -

called 'active' or 'early' rosettes (Wybran and Fudenberg, 1973).

Immature tonsillar T cells and MLC blasts form mainly, if not

exclusively, early rosettes. Mitogens stimulate 'late' rosetting

T cells to become early rosetting T cells. (Gergely, Vanky and

Klein, 1976). Felsburg and Edelman (1977) showed that sensitized

lymphocytes incubated with the testing antigen increasingly formed early rosettes, thus showing another in vitro correlate for DTH as well as confirming this system as a marker for activated T cells.

Peripheral blood lymphocytes form low numbers of rosettes with autologous erythrocytes - auto-rosettes. This rosetting is a property of a high percentage of human thymocytes, and of lymphocytes treated with neuraminidase or stimulated to blast transformation by mitogens and is probably related to the low content of cell surface sialic acid (Sandilands et al, 1975).

The expression of Fc receptors (FcR) has been related to the activation of T lymphocytes, on which FcR are not usually found (Yoshida and Andersson, 1972). Cell maturation may also effect FcR expression. Miyama et al (1978) showed that precursor B cells do not express FcR. However, these are probably blasts which are plasma cell precursors. Recently, attention has centred on the function of FcR + and FcR- T blasts. Stout and Herzenberg (1975 a, b) found that helper T cells were FcR-, Van Boxel and Rosenstreich,(1974)demonstrated that FcR + cells were found as a result of antigen activation. T Blasts that become cytotoxic T cells are also FcR+(Stout et al, 1976), but the presence of FcR on cytotoxic effector cells is equivocal. Suppressor T blasts are FcR+ (Moretta et al, 1977) and modulate the immune response by synergistic interraction with FcR - helper cells (Miyama et al, 1978). Helper T cells do not develop FcR during any stage of cooperation (Yodoi, Miyama and Masuda,, 1978).

Electrophoresis has been used to estimate lymphocyte surface charge differences. Andersson et al (1973) demonstrated that T and

B cells could be electrophoretically separated, T cells having a

greater negative charge than B cells. Neuraminidase treatment

of lymphocytes resulted in a disappearance of the normal

biphasic distribution, suggesting that sialic acid content exerts

a strong influence on the surface charge (Nordling, Andersson

and Hayry, 1972). Also, the presence of membrane Ig reduces the

negative charge of lymphocytes, explaining the lower electrophoretic

mobility (EPM) of B cells. This surface Ig is lost by B cells

triggered to antibody production (Takahasi et al, 1971) and thus

these have an increased EPM. Von Boehmer et al (1974) confirmed

this finding and showed that null cells also have an EPM

intermediate between that of T and B cells, and these cells are

FcR+. T blasts have a slower EPM than small T lymphocytes (Wiig,

1973). Some of the cytotoxic activity in an allograft is

mediated by T cells that are electrokinetically blasts (Roberts

and Hayry, 1977). The comparison of EPMs of activated T and B

blasts has not yet been made, and although B cell activation causes

an increased EPM, T cell activation has only been studied with

mitogens, which decreased the EPM (Sato and Kojima, 1976).

Sensitized B cells have been shown to have specific

endogenously synthesized surface immunoglobulin as antigen

receptors (Julius and Herzenberg, 1974). However, the identifica-

tion of specific antigen receptors on the various T cell subsets

has not been successful (Elliott and Haskill, 1975; Elliott,

Haskill and Axelrad, 1975), except on T blasts in mixed

leucocyte cultures (Nagy et al, 1976; Krammer, 1978) suggesting

that either blasts have more receptors or that in the development

to memory cells the antigen receptors are masked. The theta (Θ)

antigen of mouse thymocytes (Thy 1) is also believed to vary in

distribution during differentiation and maturation, the populations

being separable by cell density (Shortman and Jackson, 1974). Further

work to characterize the cell surface changes of T blasts has

shown differences in ^{125}I-labelling patterns from those of a starting T

cell population (Dunlap, Bach and Bach, 1978).

Antibody-forming immunoblasts degenerate quickly in culture,

but Hall (1970) has developed a way to assess specific antibody

production using bacterial adherence. Human immunoblasts produced

in culture with mitogen have been used to provide evidence for

membrane antigens specific for human B blasts in the 'S' phase

of the cellcycle (Thomas and Phillips, 1973).

Subsets of mouse T cells have been defined by the presence

or absence of surface antigens Ly 1, 2 and 3. T cells with Lyl+

Ly23- are programmed for helper function and those with Lyl¯

Ly23+ for killer/suppressor function (Huber et al, 1976). Antigen

stimulation of some Ly 123+ cells gives rise to Lyl-Ly23+ cells

(Cantor, Shen and Boyse, 1976).

Thus, there are a number of ways of investigating the derivation

of immunoblasts, firstly to identify a T or B cell origin, and,

if T cell, to assess which subset has been stimulated.

Lymphocytes are relatively non-motile until they are activated

(Wilkinson et al, 1976, 1977). The invasiveness of cancer tissue

has been related to an increase of intracellular contractile proteins

(Gabbiani, Trenchev and Holborow, 1975). Immunoblasts have

orientated motility - chemotaxis, which is shown more by B blasts

than T blasts. However, B blasts have a lower unstimulated motility

than T blasts (Russell et al, 1975). This chemotaxis has been shown

to be increased towards the specific priming antigen

(Wilkinson et al, 1977). The lymphocytes which can be shown

most clearly to migrate into inflammatory lesions in vivo are

blast- transformed cells (Moore and Hall, 1973; Asherson, All-

wood and Mayhew, 1973).

SECTION .   RHEUMATOID ARTHRITIS

a) DEFINITION

Rheumatoid arthritis (R.A.) is a systemic disease in which
an inflammatory polyarthritis in the diathrodial joints is the
predominant feature. Characteristically it involves peripheral
joints (often symmetrically) and runs a sub-acute to chronic
course of exacerbation and remission, depending on the degree
of synovial involvement.

That joint involvement is the predominant feature is
exemplified by the American Rheumatism Associations Diagnostic
Criteria, in which nine of the 11 criteria refer to joint
involvement (Rodnan, 1973).

The aetiology of R.A. is unknown. Glynn (1969), Bland
and Phillips (1972), Barland (1973) and Denman (1975) have
all put forward the hypothesis that R.A. is a disease of viral
origin which manifests itself only in genetically pre-disposed
people. The pathologically damaged joint which is characteristic
of R.A. is due to an on-going immune response, either to the
persisting virus or to antigens evoked during the initial reaction
to the infective agent.

b) THE PATHOLOGY OF JOINT INFLAMMATION IN R.A.

The pathdogy of joint inflammation in the chronic lesion
has been extensively reviewed (Gardner, 1972; Zvaifler, 1973).
The synovitis of R.A. is a predominantly polymorphonuclear (PMN)
exudation of the synovial fluid, and a mononuclear cell infiltration
of the deep synovium. Weissman (1971) characterised the lesions in
in R.A. joints as follows:

a) Margination of leucocytes and their appearance in synovial fluid.

b) Hypertrophy and hyperplasia of synovial lining cells.

c) Infiltration of the synovium by many lymphocytes.

d) The transformation of synovium to granulation tissue which, as pannus, overgrows and invades cartilage.

e) Erosion of cartilage; initially protoglycon, followed by collagen breakdown, accompanied by chrondrocyte death and attempts at regeneration.

I SYNOVIAL FLUID

In chronic R.A. the volume of synovial fluid increases from 0.1 - 0.4ml to as much as 200 - 400ml. When seen at arthroscopy the fluid appears turbid, containing fibrinous clots or strands of fibrin which are most conspicuous on the synovial lining cells. The protein content increases to levels obtained for serum. Many agents capable of influencing the coagulation mechanisms are found, e.g., the Hageman Factor (Kellermeyer and Brekennage, 1966) and fibrin. Complement levels are characteristically low when compared with other joint diseases. Patients seropositive for Rheumatoid Factor (R.F) usually display high synovial fluid levels of R.F. The fluid also contains immunoglobulin (Ig) of other specificities. Many enzymes are found including proteases, lipases and collagenases. The predominant nucleated cells are PMNs, accounting for 75 - 90% of the population; lymphocytes, monocytes and occasional macrophages constituting the remainder. Cell counts vary from

2,500 to 25,000 per cubic millilitre in an exudation which shows

remarkable population stability over a long time period even

though the PMNs half life in synovial fluid is only four hours

(Zvaifler, 1972). Hence, the joint cell turnover is very high

and Hollingworth, Siegel and Creasey (1967) estimated that

the daily joint cell turnover may exceed 1,000 million cells.

II SYNOVIAL MEMBRANE

Rheumatoid synovitis is characterized by a large number of

histologic changes which are characteristic but not pathogno-

monic of this disease (Hamerman et al, 1969).

The synovial lining layer proliferates and the synovium

appears oedematous and inflamed and protrudes into the joint

space as slender villous projections. The lining cells are

multilayered, reaching to a depth of 6-10 cells, as compared

to the normal 1-3 layers. Multinucleated giant cells are common

and located in areas of lining cell hyperplasia or in the

sub-intimal layer. These are not unique to R.A. and have been

observed in other forms of chronic synovitis (Bhan and Roy,

1971).

Oedema of the connective tissue stroma beneath the lining

cells is a conspicuous feature of the early rheumatoid lesion.

This contains fibrin or fibrinoid masses, occasionally interrupted

by cytoplasmic processes from the synovial lining cells

(Hamerman et al, 1969). The small blood vessels immediately beneath

the lining cells appear to be increased in number and their lumens

may be occluded by hypertrophied endothelial cells (Kulka, 1966).

Schumacher and Kitridou (1972) found that the prominent vascular

changes mainly affected small venules which showed congestion and obliteration of the lumen by inflammatory cells, fibrin, platelets and enlarged endothelial cells.

Synovial cells speed up their metabolism with the onset of inflammation and Lindner (1971) suggests that this may contribute to the self-perpetuating lesion of R.A. This characteristic of involved synovial cells is one of a number of abnormalities which may persist through several generations as shown in cell culture (Gardner, 1972).

Synovial and subsynovial tissues are found to be infiltrated by many mononuclear cells. Generally, the lymphocytic infiltrate is diffuse and varies from sparse to prolific. They are thought to originate from blood and to leave through the endothelial cells of the post-capillary venules (Ziff, 1974). Within the hyperaemic, swollen synovial tissue the diffuse lymphocyte infiltrate is sometimes accompanied by a follicular arrangement and occasionally secondary germinal centres are seen. Investigations of the early lesions of the active synovitis have failed to establish the sequential order of the cellular exudation. A mixture of cell types is found in the extravascular region: small lymphocytes, undifferentiated blast cells, plasma blasts, plasma cells and macrophages, all in significant numbers. Close association of these cell types is suggestive of cell-cell inter action having a role in the prominent feature of chronic synovitis. Macrophages are found in both superficial and deep layers of the synovium (Muirden, 1966).

Granulation tissue formation at the margin of joints affected

by progressive rheumatoid arthritis is invariable and is generally
regarded as a key to the peripheral, progressive destruction of
articular cartilage that accounts for so many of the principal
pathological features of the disease. This tissue , pannus,
consists of proliferating fibroblasts, numerous small blood
vessels, various numbers of inflammatory cells and occasional
collagen fibres. It extends over, and takes the place of,
peripheral cartilage; although invariably associated with pannus
cartilage destruction can occur where there is no overlying
pannus (Hamerman et al, 1967).

The prevailing view is that the pannus is responsible
for the observed articular cartilage injury, although there is
no general agreement how this comes about. Many workers support
the theory that pannus is responsible for enzymatic digestion of
constituents of cartilage (Hamerman, et al, 1967; Fell and Thomas, 1960;
Fell and Dingle, 1963). However, other authors (Hamerman et al, 1969;
Tateishi et al, 1971) believe the release of enzymes from PMNs
in the synovial fluid is of greater importance, suggesting that
pannus is the result rather than the cause of cartilage injury,
pannus formation following. Enzyme production has been shown from
both chondrocytes and synovial fluid PMNs, including acid phosphatase,
B- glucuronidase and collagenases (Page-Thomas, 1967, 1969; Chayen and
Bitensky, 1971). These enzymes are believed to attack the cartilage
proteoglycans which are leached out, making the remaining collagen
fibres susceptible to mechanical stress (Holt, 1971).

c) IMMUNOPATHOGENESIS

Despite the lack of progress in identifying the cause of rheumatoid
arthritis, there is considerable evidence implicating immune mechanisms.

In the early 1960's the discovery, by Hedberg (1903)

and Pekin and Zvaifler (1964), that synovial fluid haemolytic

complement activity was reduced in comparison with simultaneously

determined levels in the serum suggested that immune mechanisms

were involved in rheumatoid synovitis. Vaughan Barnett and Sobel

(1968) showed that PMNs in synovial fluid had ingested

immunoglobulins which were associated with the C_3 component

of complement. The association of low synovial fluid

complement and seropositivity for 19S antiglobulin (R.F.)

has implicated these rheumatoid factors in the immunopathogenesis

of rheumatoid synovitis (Vaughan Jacox and Noell, 1968). Patients

with the highest titres of serum R.F. are more efficient in

utilizing complement and are the patients most likely to have

vasculitis and depressed serum haemolytic complement titres (Franco

and Schur, 1971). IgG antiglobulins have been noted and the resulting

complexes also reduce complement levels (Britton and Schur,

1971).

The inter action of antigen and antibody initiates the

complement sequence in the synovial fluid, generating a number

of biologically active products. Some of those cause increased

vascular permeability, allowing an influx of serum proteins

and cellular blood elements into the site where the complexes

reside. PMNs are attracted by complement-derived chemotactic

factors and the complexes are attached to their cell surfaces

by receptors for IgG and C_3. Engulfment follows, with a con-

comitant release of large quantities of hydrolytic enzymes.

It is these lysosomal enzymes that are responsible for much

of the inflammation and some of the tissue damage. Immune

complexes alone cannot explain all the articular changes in

R.A.. Of late there has been a greater appreciation of the

role of cellular hypersensitivity and granulation tissues

in the proliferative and destructive aspects of rheumatoid

joint disease. Any theoretical explanation of rheumatoid

joint disease must consider these findings.

Cryoprecipitable complement fixing immunoreactants

have also been found in rheumatoid synovial fluid by Marcus

and Townes (1971). Antibodies to IgG, IgM, fibrinogen, cartilage

and DNA have been identified in these cryoprecipitates.

The earliest and most severe cartilage damage, however, does

not occur over the broad, exposed articular surface, as one might

expect, from the potentially damaging events of lysosomal enzyme

release caused by immune complex ingestion occurring in the

free synovial fluid, but in those areas where synovial membranes

and cartilage are in close apposition. The synovial membrane

contains large numbers of lymphocytes, frequently collected into

aggregates or follicles, particularly around the small blood vessels,

but true germinal centres are rarely seen (Gardner, 1972). The

predominant cell in these nodules is the small lymphocyte, but

about the periphery are typical plasma cells that, by

immunofluorescent studies are found to contain immunoglobulin

deposits (Fish et al, 1966).

What kind of lymphocytes infiltrate rheumatoid synovial

membrane? Electron micrographs of areas rich in lymphocytes

reveal predominantly small lymphocytes with less than five per cent

transformed blast-like cells and variable numbers of plasma cells (Kobayashi and Ziff, 1973). Fluoresceinated rabbit anti-human T cell antiserum applied to frozen sections of human rheumatoid synovium stains the majority of the cells within the lymphoid infiltrates, suggesting that the predominant cell is a T lymphocyte (Williams, 1975). The technique of cytoadherence, applied to the study of rheumatoid synovial membranes, has yielded conflicting data. When suspensions of sheep erythrocytes (E) or erythrocytes coated with antibody and the C_3 component of complement (EAC) are layered over frozen serial sections of rheumatoid synovium, the respective T and B markers adhere to the relevant cell in the lymphoid infiltration. Van Boxel and Paget (1975) claim that the major lymphocyte is a T cell, whereas Sheldon and Holborow (1975) concluded that B lymphocytes predominate. There is general agreement, however, that the majority of viable lymphocytes extracted from rheumatoid synovial membrane are T cells and the proportion of B cells (identified by membrane bound immunoglobulin or C_3 receptors) is usually below that of levels normally found in the blood (Van Boxel and Paget, 1975; Abrahamsen et al, 1975; Wangel and Klockars, 1977).

There is ample evidence that the rheumatoid synovium contains large amounts of immunoglobulin, much of it synthesized locally. (Smiley, Sachs and Ziff, 1968; Sliwinski and Zvaifler, 1970). Immunoglobulin G, generally found in abundance is in areas that also stain for complement components (C_3 and C_4); whereas the IgM is in lesser amounts and not identified with complement.

IgG is the predominant immunoglobulin in the synovial membrane

plasma cells of both seropositive and seronegative patients

and possesses in many cells, antiglobulin (IgGRF) activity

(Munthe and Natvig, 1972). The authors interpret their findings

to indicate that many of the plasma cells in the rheumatoid

synovium make an IgG R.F. which combines with similar IgG

molecules ('Self associating IgG') within the cell. Although

these complexes do not appear to activate or bind complement

within the plasma cell cytoplasm, they might have important

complement fixing activities after secretion from the cells.

II. LYMPHOCYTE SUB-POPULATIONS

A number of investigations have enumerated the T and B cells

in the blood of patients with R.A., attempting to provide

insights into the pathogenesis of the disease, and to find

correlates of disease activity. For a variety of reasons,

these studies have yielded conflicting data.

Several reports have cited normal percentages of T cells

in the blood of patients with R.A.(Micheli and Bron, 1974;

Tannenbaum and Schur, 1974; Zeiders et al, 1974; Brenner, Scheinberg

and Cathcart, 1975; Clements et al, 1974). Williams et al (1973)

noted a tendency for the proportion of T cells to decrease below

normal with the development of severe disease or vasculitis.

Utsinger and Bluestein (1974) demonstrated an overall decrease in the

absolute number of lymphocytes, as well as a preferential decrease in

T cells, associated with increases in disease activity. Froland et al

(1975) found an increased percent of E rosette-forming cells in R.A.

patients. Hoyeraal, Froland and Wisloff (1975) found both normal propor-

tions and normal absolute

numbers of T lymphocytes in juvenile R.A. in a comparison
with age and sex matched controls.

Most investigators have used anti-immunoglobulin antisera
to assess the percent of B cells in peripheral blood. R.A.
patients are generally equivalent to normal controls (Williams,
et al, 1973; Zeiders et al, 1973; Froland, Natvig and Wisloff,
1975; Micheli and Bron, 1974; Tennenbaum and Schur, 1974;
Utsinger and Bluestein, 1971) but an increased (Papamichael,
Brown and Holborow, 1971) or decreased (Mellbye et al, 1972)
percent of B cells in peripheral blood has been reported.
In addition, several studies have noted increased numbers of
null cells in patients with R.A. (Williams et al, 1973; Zeiders
et al, 1973; Tannenbaum and Schur, 1974; Brenner, Scheinberg
and Cathcart, 1975).

Since data from peripheral blood analysis have been of
only limited usefulness, some investigators have attempted to
classify lymphocytes present in synovial effusions. Again the
results are conflicting. The percent of T cells was increased
in some studies (Froland et al, 1975; Vernon-Roberts, Currey
and Perrin, 1974). Utsinger (1975) and Brenner et al (1975) have
found normal levels of T cells, a result confirming the work of
Winchester et al (1973) who also found that the joints with a
marked diminution of complement had increased numbers of null
cells. The percent B cells has been variably reported to be increased
when measured by EAC rosettes and surface Ig (Mellbye et al, 1972),
normal by surface Ig (Vernon-Roberts et al, 1974) and decreased
using surface Ig (Winchester et al, 1973; Brenner et al, 1975).

Lymphocytes bearing FcR are of particular interest in R.A.
These have usually been detected by using erythrocytes coated with
specific IgG antibody and have been called 'rheumatoid rosettes',

suggested to be characteristic of R.A. (Bach, Delrieu and Delbarre,

1970; Sany et al, 1975). However, these cells are found in normal

peripheral blood (Brain and Marston, 1973; Froland, Wisloff and

Michaelson, 1974). Although most B cells have FcR some FcR +ve

cells do not have surface membrane immunoglobulins, complement

C_3 receptors or bind to nylon fibre columns (Froland et al, 1974) and

hence usually form part of the null cell fraction. Sharpin and

Wilson (1977 a, b) have investigated the nature of EA- rosettes

formation in R.A. and found that varying the titre of erythrocyte

coating antibody showed up big differences of percent rosettes

between R.A. and control.

R.A. patients formed more rosettes than healthy subjects

at all concentrations of erythrocyte-coating antibody, particularly

at the low concentrations. The authors explained this as being due

to a substantial increase in the density or avidity of FcR

on the lymphocyte surface. The binding of both antigen-antibody

complexes and aggregated IgG has been extensively studied (Dickler,

1976) and increased uptake of immune complexes, as found in R.A.

joints and peripheral blood, may affect their clearance or the activity

of this cell population. Also, this population seems to contain the

effector cells of antibody dependent cell mediated cytotoxicity

(ADCMC) - the K cells which may be cytotoxic for the numerous tissues

for which specific antibodies are found in R.A. FcR may be important

for B cell recognition of immune complexes and be required for

complete antigenic stimulation and antiglobulin production

(Johnson, Watkins and Holborow, 1975).

III ABNORMAL LYMPHOCYTE RESPONSES IN VITRO

In vivo cellular immunity has been evaluated using skin-test

reactions to a panel of common antigens (i.e. PPD, SK-SD, mumps,

Candida albicans) and immunization with compounds such as DNCB

or KLH. Although not unanimous, most studies of responses to memory

antigens suggest an impairment of delayed hypersensitivity (Waxman

et al, 1973; Bitter, 1971; Whaley et al, 1971). The reason for this

disturbance is unclear. However, it has been observed that prolonged

stimulation by a single antigen (i.e., in this instance the putative

antigen of R.A.) can lead to poor responses to other antigens,

resulting from antigenic competition (Adler, 1964).

In vitro function of rheumatoid T cells has been evaluated

by mitogen - and antigen-induced blastogenesis and mediator production.

Some workers have found that R.A. lymphocytes respond normally to PHA

(Reynolds and Abdou, 1973; Paty et al, 1974; Georgescu, Gheorgiu and

Gociu, 1975), but the majority of evidence seems to support diminished

responses to PHA (Ivanyi, Lehner and Burry, 1973; Rawson and Huang,

1974 a; Froebel et al, 1975; Lockshin et al, 1975; Rawson, 1975;

Clot et al, 1975; Hepburn, McDuffie and Ritts, 1976; Silverman

et al, 1976; Menard, Dion and Richard, 1977). Responses to Con A

have been reported as being both unchanged (Rawson and Huang, 1974b)

and diminished (Clot et al, 1975; Lockshin et al, 1975; Silverman

et al, 1976) in R.A. Responses to PWM (transforms predominantly B

cells) are usually reported as being the same in R.A. and healthy

controls (Ivanyi et al, 1973; Silverman et al, 1976).

The MLC test was developed as an in vitro model for the

recognition phase of the homograft reaction. R.A. lymphocytes

usually respond and stimulate appropriately in an MLC reaction with

normal lymphocytes (Astorga and Williams, 1969; Stastny, 1974).

The lymphocyte surface antigens are coded for by genes in the major

histocompatability complex (MHC) which also code for HLA antigens.

A number of human diseases are correlated with an increased

frequency of HLA antigens; the most notable in rheumatic diseases

being the association between ankylosing spondylitis and HLA

B-27. Recently, an association has been found between R.A.

and HLA-Dw4 (Stastny, 1978) and related to patients with a

family history of R.A. (Wooley, Panayi and Batchelor, 1978) and

serum RF levels (Roitt et al, 1978).

Due to the difficulty of assaying immunoglobulin production

by antigenic stimulation of human B cells in vitro, attempts to identify

B cell defects can only be explored indirectly. Most studies

are concerned with circulating immunoglobulin levels or antibody

responses to immunization. However, deficiences or excesses may

be due to T cell abnormalities in the modulation of antibody

production. Total serum Ig levels in patients with R.A. are

increased (Barden, Mullina and Waller, 1967; Bandilla,

Pitto and McDuffie, 1970), but this may well represent the non-

specific stimulatory effect of chronic inflammation, rather than

specific hyperreactivity of the humoral immune system. Naturally

occurring antibodies, such as the isohaemagglutinins (anti-A and

anti-B) are not elevated in rheumatoids, but possibly decreased

(Rawson, Abelson and McCarty, 1961). Antibody responses to

exogenous antigens, such as Brucella abortus or tetanus toxoid

are generally reported as being normal in R.A. (Waller, Ellman

and Toone, 1966; Rhodes et al, 1969), but in Bristol Bucknall et al (1978)

found decreased response, both 1° and 2°, to $\emptyset$ x, an antigenic

bacteriophage not previously met by normal subjects. On the other

hand, the response to endogenous antigens (i.e., autoantibodies to

self antigens such as thyroglobulin) appears to exceed that of age - and - sex - matched controls (Muller, 1971).

The role of helper or suppressor cells in R.A. has not been evaluated, the lack of human assay systems being the main reason, particularly for suppressor cells. Also the role of such cells in vivo could only be guessed at.

Recently the cytotoxic potential of rheumatoid lymphocytes has been investigated. PHA induced cytotoxicity is reduced in R.A. (Wisloff, Froland and Natvig, 1975), this may be related to the reduced thymidine incorporation of R.A. lymphocytes cultured with PHA. Increased ADCMC has been reported for R.A. lymphocytes and related to FcR bearing cells (Sany et al, 1977). However, Diaz-Jouanen et al (1976) and Panayi and Corrigal (1977) reported unchanged ADCMC in R.A.. Isturizi et al (1976) found decreased ADCMC in R.A. The possibility of cytotoxicity towards an antigen in the rheumatoid synovium has been investigated and been found to be increased (Person, Sharp and Lidsky, 1976) and normal (Griffiths et al, 1976) in R.A. Panayi, (1976) has found increased cytoxicity towards Chang human liver cells in R.A. and suggested a possible antigenic similarity between rheumatoid synovial membrane and Chang cells.

The identification of an antigen (s) responsible for the intra-articular immune response is obviously one of the most important steps in solving the mystery of R.A. Many animal models have been used in those studies to assay the effects of a range of antigens (Cooke and Jasin, 1972). These studies have a limited usefulness, but they have shown that antigen persistence is not essential for the maintenance of chronicity

in R.A., the initial antigen possibly inducing the formation

of a second antigen that persists in the absence of the first.

R.A. lymphocytes produce MIF if cultured with aggregated or

complexed IgG (Eibl and Sitko, 1975). Rheumatoid factor has

mitogenic properties in inducing lymphocyte transformation (King,

Messner and Williams, 1968). Antigen-antibody complexes also stimulate

lymphocytes, particularly in the presence of complement, (Moller,

1969; Bloch-Schtacher et al, 1968) and may be involved in

maintenance of the inflammation by providing an altered IgG structure

which acts as an autoimmunogen.

To counteract the involvement of lymphocytes in R.A. a number

of drug treatments have been tried. Glucocorticoids may work by

being immunosuppressive and cytotoxics, such as azathioprine and

cyclophosphamide have been used with some success (Bacon et al,

1978). Antilymphocyte serum has been used in systemic lupus

erythematosus (SLE), (Nossa et al, 1969). Drainage of the thoracic

duct lymph has been used as immunosuppressive treatment in glomerulo-

nephritis (Bonomini et al, 1970), myasthenia gravis (Bergstrom

et al, 1975), multiple sclerosis (Brendel, Ring and Seifert,

1975) and in preparation for allotransplantation. Thoracic duct

drainage (TDD) of R.A. patients has been used by Paulus et al

(1977) and produced significant clinical improvement, as shown by a

number of parameters, supporting the hypothesis that some lymphocytes

in thoracic duct lymph are essential for the continued activity of the

inflammation associated with rheumatoid arthritis. 51-Chromium-

tagged reinforced autologous lymphocytes were demonstrated in the

inflamed synovium and synovial fluid and had a slower rate of

disappearance from these sites than from the liver or spleen. If

'homing' was occurring only a few cells were participating. Edgren

et al (1976) found that extracorporeal irradiation of thoracic

duct lymph in R.A. patients produced a marked clinical improvement

and found that peripheral blood T lymphocytes were reduced after

reinfusion of the irradiated lymph. Indeed, both groups of workers

suggest that removal of thoracic duct lymphocytes may cause depletion

of suppressor T cells.

IV LYMPHOCYTE ACTIVATION IN JOINTS

The lymphocyte functions described above depend upon certain

lymphocytes being activated at one time or another. Some of them

will be activated in joints and others, possibly, in lymph nodes,

spleen or peripheral blood. Very few studies have been undertaken,

into the occurrence and nature of activated lymphocytes in the

rheumatoid patients. Loewi, Dorling and Howard (1974) found

increased numbers of large lymphocytes in R.A. synovial fluid;

plasma cells were also found and it was suggested that these account

for the increased numbers of null cells found in R.A. synovial fluid.

Traycoff, Pascual and Schumacher (1978) found lymphoblasts in R.A.

synovial fluid but rarely   in synovial fluid from patients

with crystal-induced synovitis or bacterial infections. Patients with

ankylosing spondylitis and psoriatic arthritis had small numbers of

lymphoblasts which were non-T cells. Both groups suggested that the

identification of lymphoblasts in synovial fluid may be a useful

marker for rheumatoid arthritis. Electron microscopic examination

of perivascular infiltrates of the rheumatoid synovium showed large

numbers of plasma cells grouped together in certain areas and

transitional areas containing both plasma cells, immunoblasts

and small lymphocytes (Ishikawa and Ziff, 1976). Most of the
immunoblasts contained rough endoplasmic reticulum, well-
developed Golgi areas and mixtures of mono - and polyribosomes
in the cytoplasm, indicating that they were plasmablasts (i.e.,
destined to become plasma cells). About a third of the blasts
had T blast characteristics. Macrophages were seen to be
intimately associated with both T and B blasts suggesting release
of mediators of cell mediated immunity and cell-cell inter action.
The authors suggested that plasma cells were common in R.A.
synovium because of their relative immobility compared with re-
circulating T cells. So. although most of the small lymphocytes
in R.A. synovium are T cells, most of the blasts are of B cell
origin. Isolation of lymphoid cells from rheumatoid synovium
by de Vries et al (1977) showed that a minority of blasts
(30-40%) formed E- rosettes, but that no blasts formed EAC-
rosettes and interpreted this as a lack of B-blasts in
rheumatoid synovium. Their assumption that B blasts retain
C_3 receptors has been shown to be incorrect (Jondal, 1974 a,b)
and thus many B blasts may be present due to the low number of
T blasts shown.

V.PERIPHERAL BLOOD IMMUNOBLASTS

The demonstration of immunoblasts in rheumatoid synovium does
not mean that the lymphocytes are activated and/or transformedthere.
Horwitz, Stastny and Ziff (1970) found increased numbers of
proliferating lymphoid cells in the peripheral blood of patients
with inflammatory diseases, including rheumatoid arthritis.
Increased thymidine incorporation was found in acute polyarthritis,

the highest levels being in acute juvenile R.A. The authors

were not sure if these cells were haematopoietic stem cells or

lymphocytes stimulated to divide by a specific antigen.

Bacon, Sewell and Crowther (1975) showed that these proliferating

mononuclear cells were immunoblasts and were raised in R.A.

by comparison with healthy subjects, particularly in early R.A.

and in acute exacerbation of disease activity. These cells were

identical to those found in idiopathic thrombocytopenic purpura,

other auto-immune disorders, infections and following immunization.

The authors suggested that the circulating immunoblasts in R.A.

may account for the changes in lymphocyte function and sub-populations

and may be related to events within the synovial membrane. Delbarre,

Le Go and Kahan (1975) also found increased numbers of immunoblasts

in R.A. and again found a positive correlation between numbers and

disease activity. Immunoblast numbers were not related to

inflammation, patients with psoriatic arthropathy having normal immuno-

blast levels. Increased numbers of immunoblasts have also been found

in polymyalgia rheumatica (Eghtedari, Esselinckx and Bacon, 1976) and

ankylosing spondylitis (Eghtedari, Davis and Bacon, 1976).

The immunoblast may have a central role in the pathogenesis

of rheumatoid arthritis. It may be responsible for the spread of the

disease or merely a non-specific response to immune complexes or a

host of other functions between these two. Once the occurrence

and nature of immunoblasts has been determined more work can be

carried out to find out the origin and fate of these cells.

The work described was carried out with the aim of

enumerating immunoblasts in R.A. and to characterize them by

means of surface marker techniques with a view to investigating

their role in the disease pathogenesis.

CHAPTER TWO

MATERIALS AND METHODS

SECTION 1

MATERIALS

All chemicals and reagents were of analytical grade whenever available and, unless otherwise stated, came from normal commercial sources such as British Drug Houses Ltd., Poole, Dorset.

All tissue culture media and fluorescein conjugated anti-Ig were obtained from Wellcome Reagents Ltd, Beckenham, Kent or Flow Laboratories, Irvine, Scotland.

All Sephadex and Sepharose gels, Ficoll and Ficoll/ Paque were from Pharmacia, Uppsala, Sweden.

Radiolabelled (^{3}H-) thymidine was from the Radiochemical Centre, Amersham, Berks.

Preservative-free Heparin was from Evans Medical Ltd., Liverpool.

Physiological solutions and tissue culture media

1. **Phosphate buffered saline (P.B.S.) - Dulbecco**

   Dulbecco A

   Formula: mg/litre

Sodium chloride	8000
Potassium chloride	200
Disodium Hydrogen phosphate	1150
Potassium Di-hydrogen phosphate	200

   in distilled water (D.W.)

   Dulbecco B

   Formula: mg/litre

Calcium chloride	100
Magnesium chloride	100

   in distilled water (D.W.)

   Five ml of Dulbecco B was slowly added, with stirring to 1 litre of Dulbecco A to make the complete Dulbecco phosphate buffered saline (pH 7.4) which was filter sterilised into sterile glass bottles and stored at +4°C.

2. **TC 199**

   Formula:

TC199 (10X concentrate)	100 ml
Sterile water	830 ml
penicillin/streptomycin	20 ml
4.4% Sodium bicarbonate	C.50 ml

Sodium bicarbonate varied to adjust to pH 7.4 at 20°C .

Filter sterilised into sterile glass bottles.   Stored at +4°C.

3.   TC199 + 10% new born calf serum

   Formula:

TC199 (10 X concentrate)	100 ml
Heat inactivated new born calf serum	100 ml
Penicillin/streptomycin (5000 iu/ml and	
5000 mcg/ml)	20 ml
Glutamine (200 mM)	10 ml
Distilled water	720 ml
4.4% Sodium bicarbonate	C. 50 ml

   pH adjusted to 7.4 with sodium bicarbonate.   Filter
sterilised into sterile glass bottles and stored at +4°C.

SECTION 2

METHODS

a. <u>Venous Blood</u> - Defibrination and anti-coagulation

Blood was drawn from a **cu**bital vein into a sterile

disposable syringe and immediately expelled into 10 ml sterile

bottles, each containing 300 iu heparin (preservative-free)

and gently shaken for one minute. Alternatively, the blood

was expelled into 20 ml bottles, each containing two large

steel **paper** clips and gently, but constantly, shaken until

the blood was completely defibrinated. The fibrin coated paper

clips were then removed from the bottle.

b. <u>Lymphocyte preparations</u>

  i) <u>Methyl Cellulose and Carbonyl Iron</u>

  <u>Materials</u> 1% Methyl Cellulose (BDH) in 0.9% saline.

        Carbonyl Iron (BDH)

  <u>Method</u>

20 ml blood was mixed with 2.5ml methyl cellulose solution

and 50 mg carbonyl iron in a 30 ml McCartney bottle. This was

shaken gently at $37^{o}C$ for 30 minutes.

The bottle was then allowed to stand on a strong magnet

at $37^{o}C$ for 30 minutes. During this period the erythrocytes

were clumped by the methyl cellulose and sedimented and the

polymorphonuclear leucocytes and monocytes, which had phagocytosed

the iron, were pulled to the bottom of the bottle by the magnetic

field.

The lymphocyte rich supernatant was aspirated with a pasteur pipette and collected in glass centrifuge tubes.

ii) Ficoll/Triosil and Ficoll-Paque

Materials Ficoll/Triosil - 10 ml 9% Ficoll in distilled water, 4ml 50% Triosil in distilled water mixed, filter-sterilised and stored at -20°C.

Ficoll-Paque   - (Pharmacia)

Method

20 ml of blood was diluted 1:1 with PBS, mixed and carefully layered on to Ficoll/Triosil in glass centrifuge tubes at the ratio of 6ml of diluted blood to 4ml of Ficoll/Triosil. The tubes were then centrifuged at 400g maximum at 18°C for 30 minutes. During this period the Ficoll caused the erythrocytes to agglutinate and sediment, the Triosil was phagocytosed by the dense polymorphonuclear cells, which increased in density and sedimented. The interface between the upper plasma layer and the lower Ficoll/ Triosil compartment became a site for accumulation of lymphocytes and some monocytes.

Taking care to avoid contamination with Ficoll/Triosil the lymphocyte rich interface was carefully removed and ejected into a glass centrifuge tube.

On some occasions the lymphocyte rich supernatant from the methyl cellulose-carbonyl iron method was further purified on Ficoll/Triosil.

Ficoll-Paque was sometimes used instead of Ficoll/Triosil and the cell yield and contamination levels were similar enough to allow interchange of the two media as availability required.

c. Measurement of total cells and viability

   Materials White cell counting fluid (WCCF) comprising:

   0.1% Methylene Blue
   3.0% acetic acid } in distilled water

   Trypan Blue - 0.16% in PBS

For total cell count an aliquot of the cell suspension was diluted with WCCF which lysed the red cells and stained the leucocytes a pale blue, and the concentration microscopically determined in a counting chamber.

To assess viability an aliquot of the cell suspension was diluted in Trypan blue stain and viability determined in a counting chamber. According to Boyse, Old and Chouroulinkov (1964) viable cells exclude the stain but non-viable cells cannot and stain dark blue. Hence, percentage viability may be recorded. Other dyes have been used, such as Eosin Y (Terasaki, Vredevoe and Mickey, 1967) and nigrosin (Kaltenbach, Kaltenbach and Lyons, 1958). Trypan blue is not a sensitive test of damage of lymphocytes (Tennant, 1964); cells that appear under phase contrast to be in an advanced state of degeneration may nevertheless exclude the dye. Although such cells may be alive at the time of testing there can be no confidence in their prolonged survival.

d. Cell smears

Permanent preparations of cell suspensions were made by smearing one drop of concentrated cell suspension on one glass slide with another of lesser width, to reduce surface tension effects.

In some instances a Shandon cytocentrifuge was used to prepare permanent slides.

All slides were left to air dry ($\geqslant$ 10 mins), fixed by flooding

with methyl alcohol and then, when dry, stained. Most

preparations were stained with Wrights stain (0.2% in methyl

alcohol) in the following manner: the slide was flooded with

Wrights stain for two minutes. After this period the stain

was diluted 1:1 with a phosphate buffer (pH 5.8), and left

to differentiate for eight minutes. The stain was washed off

the slide with the phosphate buffer and left to air dry prior to

microscopic examination.

e. E- rosettes

Materials   Sheep Red blood cells (SRBC) (in Alsevers

solution). (Oxoid-fresh weekly)

SRBC absorbed heat inactivated new-born calf serum.

Preparation: 1ml packed washed (X3) SRBC mixed

with 10ml heat inactivated new-born calf serum.

Left overnight at 4°C. Centrifuged at 200g for

10 minutes. Supernatant aspirated and stored at

-20°C in 1 ml aliquots.

Method

One ml sheep blood was mixed with a large excess of PBS with a

pasteur pipette and centrifuged at 200g for 10 minutes to pellet

the cells. The cells were washed twice by resuspension in PBS and

centrifugation. The supernatant was carefully aspirated with a

pasteur pipette and 50 µl packed cells removed, mixed with 1 ml

SRBC-absorbed heat inactivated new-born calf serum and made up to

10 ml with PBS.

Lymphocytes from Ficoll/Triosil preparations were washed

three times with PBS by repeated sedimentation and resuspension

in fresh media, and adjusted to 2 X 10^6/ml.

Equal volumes (0.2ml) of lymphocyte suspension and SRBC suspension

were mixed in small, stoppered, plastic, round bottomed tubes (LP3

Luckham). Immediate incubation at 37°C for 10 minutes was followed

by centrifugation at 200g for four minutes. Refrigeration at 4°C

was maintained for two hours. During this period a 0.2% crystal

violet solution in PBS was prepared and filtered through cotton wool.

Three drops of crystal violet dye were added to each tube and the

contents resuspended in a controlled manner on a rotary turntable

- - - - (20 revolutions/min - Baird and Tatlock) for one minute. A drop of the

resuspended cells was placed in a counting chamber (Neubauer) and the

% rosetting lymphocytes determined. The lymphocytes stain dark blue and a

rosette was counted as a lymphocyte binding three or more SRBC.

E- rosette depletion

To deplete populations of E- rosetting lymphocytes the rosettes

were formed as above, but mixing 10 ml volumes of both lymphocytes and

SRBC instead of the 0.2 ml aliquots. The incubation and centrifugation

steps were as described. The twenty ml. of lymphocytes plus SRBC

was layered on to 4 X 4ml volumes of Ficoll/Triosil and centrifuged

at 400g for 30 minutes, at 18°C. During this period the SRBC sedimented

to the bottom of the tube, and any lymphocytes bound to the SRBC

sedimented with them. Hence, the interface between the upper plasma

layer and the lower Ficoll/Triosil compartment contained lymphocytes

depleted of E- rosetting cells. This interface was aspirated with a

pasteur pipette, ejected into a glass centrifuge tube and washed three

times with PBS.

f. EA- rosettes

Materials   Ox blood – fresh fortnightly   courtesy of

Rabbit anti ox RBC antiserum   Dr. J. Smith,

Regional Immunology

Service, Southampton.

The IgG fraction of the rabbit anti-ox RBC antiserum was
purified by affinity chromatography on Protein A-Sepharose CL-4B
(Pharmacia). The F_c portion of IgG specifically interacts with
Protein A (Forsgren and Sjöquist, 1966).

The gel was poured into a small chromatography column
(K9 Pharmacia) and washed with PBS. Rabbit anti-ox RBC antiserum
(2.5ml) was loaded on to the column and eluted with PBS to remove
all the serum proteins except IgG. The bound IgG was eluted with
1M acetic acid and the eluate concentrated to 2.0ml over an Amicon
Diaflo PM10 membrane (nominal exclusion weight 10,000 daltons) at
$4^{o}C$.

The protein content of the IgG fraction was assessed using the
Folin- Ciocalteu method (Lowry et al, 1951) and the concentration
adjusted to the normal concentration found in serum (8-16mg/ml).

Method

Ox blood (0.5 ml) was washed three times with PBS, pelleted,
the supernatant carefully removed by aspiration and discarded. 100µl
packed cells were made up to 5ml with PBS and mixed. Equal volumes
(1 ml) of ox RBC and rabbit IgG anti-ox RBC antiserum, at varying dilu-
tions in PBS, were mixed and incubated at $37^{o}C$ for 60 minutes. The
cells were sedimented at 200g, washed three times with PBS and made

up to 2 ml with PBS , resulting in a 1% suspension of IgG –

coated ox RBC.

During this period lymphocytes were prepared by isopycnic

centrifugation on Ficoll/Triosil, washed three times with CMFSS

and adjusted to 2 X 10^6/ml.

Equal volumes (200 µl) of 1% IgG coated ox RBC and

lymphocytes were mixed in LP3 tubes, centrifuged at 200g for

four minutes and incubated at 4°C for one hour.

After incubation the rosettes were stained with crystal

violet, resuspended and % rosetting lymphocytes determined

as for E- rosettes. (Section 2.e)

g. EAC- rosette depletion

This is the depletion of lymphocytes with receptors for the

C3 component of complement.

Materials Sheep blood in Alsevers Solution

Rabbit anti-SRBC (Flow Ltd.) diluted in PBS

Human serum

Method

1ml packed washed SRBC was mixed with 5ml 1/10,000 rabbit

anti-SRBC and incubated for one hour at 37°C. The SRBC were

pelleted and washed three times with PBS. After the third

sedimentation the supernatant was discarded and 0.5ml human

serum added as a source of complement, incubation at 37°C for

30 minutes was followed by three washes with PBS. 50 µl of packed

cells were made up to 10 ml in PBS.

Lymphocytes from a Ficoll/Paque separation were washed three times

and adjusted to 2 X 10^6/ml.

Equal volumes (10 ml) of SRBC and lymphocytes were mixed, incubated at 37°C for 10 minutes, sedimented at 400g for 10 minutes and left to incubate at 4°C for 30 minutes. The pellet was resuspended and an aliquot taken for determination of % EAC- rosetting lymphocytes. The rosettes were determined as for E- rosettes.(Section 2.e)

The remainder of the resuspended cells were layered on to 4 X 4ml volumes of Ficoll/Triosil and centrifuged at 400g for 30 minutes at 18°C. The interfaces, containing lymphocytes depleted of cells with C3 receptors, were harvested and washed.

h. Surface Ig

This is a technique in which a fluorescein isothio-cyanate (FITC) labelled anti-human Ig was used to enumerate cells expressing surface Ig.

Materials Sheep anti-human Ig (fluorescein conjugated) diluted in PBS (1:5)

Method

Lymphocytes prepared by Ficoll/Paque separation were washed three times, counted and 10^6 cells pelleted. The supernatant was discarded and 0.2 ml of FITC anti-human Ig added to the pellet, mixed and incubated at 4°C for 30 minutes. The cells were washed three times with PBS and the final pellet resuspended in three drops of 40% glycerol in PBS. The cells staining for surface Ig were assessed by their fluorescence under a UV microscope. Positively staining cells had a bright yellow cell membrane.

i. <u>Mitogen stimulation of lymphocytes</u>

    <u>Materials</u>   Phytohaemagglutinin (purified grade) (Wellcome Ltd.)

Micro-titre plates (sterilin) from Arnold R. Horwell, Ltd., Kilburn, London.

Oxford sample micropipetting system and polypropylene tips. Oxford Laboratories, International Corporation, Ireland.

Scintillant - PPO (2.5 diphenyl oxazole) : **12.5g**

POPOP (14 Di(2-(4-methyl 5 phenyloxazolyl Benzene): 0.75g

Toluene   : 2.5 l

Glass fibre filter papers (Whatman Ltd.)   : 2.5cm. GF/C

<u>Method</u>

Lymphocytes, obtained by aseptic Ficoll-Paque separation, were washed with sterile TC199 three times, resuspended in TC199 containing 10% heat-inactivated new-born calf serum at 10^6/ml. The suspension was aliquoted in 200 μl volumes into micro-titre plates and PHA added to give final concentrations within the range 0.2 - 100 μl/ml. All this preparation was carried out in a Microflow sterile cabinet. The microtitre plates were covered and incubated in 5% CO_2/95% air at 37°C at high humidity for three days.

The contents of each well were resuspended by repeated withdrawals and expulsions from an Oxford pipette system and

29Ci/mmol ^{3}H-thymidine added at a strength of 1μCi/ml cell suspension to each well. The cell cultures were returned to the 37°C, 5% CO_2 incubator for a further 60 minutes.

The contents of each well were again resuspended and transferred to a glass tube. The well was washed out with 200μl distilled water and the washings added to the cell suspensions. Approximately 1ml distilled water was added to each tube which was mixed with the cell suspension by a whirlimixer. This lysed the cells to allow release of the cell contents. 2ml of ice-cold 10% trichloracetic acid (TCA) was added to each tube which was whirlimixed again. Each tube was transferred to 4°C for one hour to allow the macromolecules to precipitate.

The tube's contents were resuspended on a whirlimixer, and the precipitate filtered on to a glass fibre filter and washed with 20 ml of a mixture of three parts ether to one part ethanol and then with 20 ml of ether alone. The filters were dried at 37°C for two hours and placed in 2" X $\frac{1}{2}$" glass vials covered with 3ml of the scintillant. Radio-activity incorporated into DNA was counted in a Phillips liquid scintillation analyser using an internal standard.

Whenever possible, all samples were tested in triplicate. The results were calculated as disintegrations per minute (d.p.m.) and used as a measure of DNA synthesis. Control values were for samples without PHA. Incorporation of test cultures was expressed as a percentage of the control.

CHAPTER THREE

FICOLL DENSITY GRADIENT SEPARATION

OF LYMPHOCYTES

A practical means of separating lymphocytes by size

was needed so that functionally and morphologically

different populations could be enriched, evaluated and

characterised.

3.2 <u>INTRODUCTION</u>

The purification and separation of lymphocytes and

sub-populations of lymphocytes from various tissues has been

accomplished by filtration of cells through columns of glass

wool, nylon fibres or glass beads (Cooper, 1968; Lewis and

Robbins, 1972; Cudkowicz, Bennett and Shearer, 1964), by

cannulation of lymphatic vessels (Koster and McGregor, 1970)

and by electrophoresis (Thomas and Cater, 1972). The ability

to fractionate a complete population of cells into sub-

populations differing in one or more **physical** characteristic

and still capable of normal function could be a very powerful

tool for the study of many problems in cellular biology,

particularly in the field of differentiation. Such a technique

could be used preparatively, to obtain a purified sub-population

of cells to be used in further experiments or analytically to

study the properties of a particular sub-population of cells

under varying conditions. The analogy with biochemistry is

obvious, a field in which physically based techniques such as

centrifugation, electrophoresis and chromatography are widely

used, both preparatively and analytically.

Microscopic examination of most cell populations show that

different cell types vary greatly in size, and that cell size is

often correlated with cell function. Thus a technique producing

fractionation of different sized   cells might also produce

fractionation of functionally different cells. There are a

number of techniques available for the separation of

lymphocytes on the basis of size.

Glass-bead column filtration, originally described by

Shortman (1966) is based on the simple (and, as it turns out,

oversimplified) physical proposition that if beads of sufficiently

small size are packed into a column, so that the pores between them

become comparable in size to the diameters of cells, the flow of

large cells will be hindered more than that of small cells. The

method is simple, rapid and can yield very pure small lymphocytes.

Dead and damaged cells are trapped, so that a high proportion of

the passed cells are viable. Using this technique, the immunological

potential of small lymphocytes has been investigated by Shortman and

Szenberg (1969); Shortman et al (1971); Lewis, Mitchell and Nossal

(1969); and Hunt, Ellis and Gowans (1972). However, the yields

are very low if large lymphocytes are to be eliminated effectively,

often a second passage through the column being necessary. Physical

adherence to the glass beads also tends to remove plaque-forming

(Plotz and Talal, 1967) or erythrocyte-binding (Wigzell and Makela,

1970) cells from spleen cell suspensions, indicating that thymus-

independent lymphocytes are more adherent than thymus-dependent

lymphocytes. The electrical charge of the lymphocyte is another factor

which the columns may distinguish (Sela et al, 1970). This method

was not used because of the total loss of large cells from the

lymphocyte preparations.

Large lymphocytes are preferentially destroyed during incubation

in unfavourable conditions, thus producing a relative enrichment of

small lymphcoytes. This is performed at a high cell concentration

($>$ 10^7/ml) shaking at 37°C overnight in serum-depleted medium,

(Gowans, 1962). It is simple and effective (contaminating large lymphocytes being reduced to less than 1 per cent) and leaves small lymphocytes still capable of immunological function (Lewis, Mitchell and Nossal, 1969; Ellis, Gowans and Howard, 1969; Gowans and Uhr, 1966). Its main drawbacks are the variable overall loss of small lymphocytes; there may thus be an undetected differential survival of sub-populations of small lymphocytes. Again, the large lymphocytes are not recoverable.

The main problems of the above techniques are the loss of cells in toto and the selective destruction of large lymphocytes. More desirable techniques allow the separation of cells by volume and density with recovery of all cell fractions at high yields.

Fulwyler (1965; 1970) has described an electronic cell sorter which separates cells on the basis of volume. Although significant separations can be achieved with this apparatus (Van Dilla et al, 1967) its complexity and the low rate at which it separates cells make it unsuitable for routine fractionations of heterogeneous populations of cells.

In theory it might be possible to use differences in sedimentation rate of separate cells on the basis of size. The sedimentation velocity of a spherical cell falling through a fluid under the influence of gravity is given by Stokes Law.

$$S = \frac{2}{9} \left(\frac{\rho - \rho'}{b} \right) g r^2$$

Where b is the coefficient of viscosity, ρ and ρ' the density of the cell and fluid medium respectively, g the acceleration due to gravity and r the radius of the cell. If it can be arranged that

($\rho - \rho'$) and η remain practically constant, it is evident that

the final sedimentation velocity becomes a sensitive function of

the size of the cell being proportioned to the square of its radius.

Variations in the density of the cells and of the medium become

relatively unimportant if ($\rho - \rho'$) is substantially greater than

variations in either ρ or ρ'. Thus, in the conditions of the

normal experimental procedure, although density may exert a small

influence, the major factor determining sedimentation velocity

is size.

Theoretically, the layering of a band of cells over a medium

of constant density should allow a separation of large from small

lymphocytes, the larger cells sinking more rapidly. In practice,

however, two complications interfere - the first concerns the

stability of the suspending medium during the introduction and

recovery of the cells; any turbulence due to mixing or convection

would ruin the separation. To avoid this, à very shallow density

gradient can be imposed on the medium, solely to stabilize it.

The gradient should be as shallow as possible in order to retain

the approximate constancy of ($\rho - \rho'$) and η . The function

of this gradient is totally different from those employed in

isopycnic centrifugation. The second complication is that Stokes

Law is not obeyed by cells at high concentrations where, instead,

characteristic 'streamers' of cells break away from the main band

and sediment at an excessively fast rate. Streaming, which can be

detected by careful visual inspection of the cell band, and by

analysis of the final profile of the sedimented cells, can be

overcome by lowering the cell concentrations to less than the

streaming limit. To separate large numbers of cells the size of

the sedimenting chamber has to be increased. However, pools

of enriched large lymphocytes have been obtained (Hunt, Ellis and

Gowans, 1972) and these pools were shown to be enriched

particularly in lymphocytes in their DNA synthetic ('S') phase,

since it has been shown that, in common with many other cell

types (MacDonald and Miller, 1970), 'S'-phase large lymphocytes

sediment more rapidly than those not in 'S'-phase (Hunt, Ellis and

Gowans, 1972; Yoshida and Osmond, 1971).

This technique has been used in analyses of mouse spleen

(Phillips and Miller, 1970; Osoba, 1970; Miller and Phillips, 1970)

and bone marrow (Edwards, Miller and Phillips, 1970) of human blood

Brubaker and Evans, 1968) rat bone marrow (McCool et al, 1970)

and in a partial separation of thymus-derived and thymus-independent

peripheral lymphocytes of the rat (Howard, Hunt and Gowans, 1972).

Functional studies using velocity sedimentation have included

the separation of cytotoxic T lymphoblasts from mouse spleen (Greenberg

1973), transformed lymphocytes from mouse malignant neoplasm

(Zettergren, Luberoff and Pretlow, 1973), cytotoxic effects on

lymphocytes generated by human mixed leucocyte reaction (MLR)

(Fradelizi et al, 1977), mouse helper and suppressor T lymphocytes

(Tse and Dutton, 1976) and killer cells infiltrating allografts

in mice (Roberts and Hayry, 1977).

There are a number of problems associated with velocity

sedimentation; firstly, the time taken for sedimentation is usually

in excess of four hours and the preparation of the gradient takes at

least another hour, reducing the number of samples separable in one

day. Second, it is virtually impossible to compare the size range

recorded from one sample with another sample if fine differences are

to be noted. Third, if the harvested fractions are to be cultured all

of the sedimenting chamber and gradient maker have to be kept

aseptic, not a simple operation if the equipment has to be left

standing for five or more hours on a laboratory bench. Fourth,

the sedimenting chamber has to be large in diameter, and hence

the gradient media is more susceptible to convection currents

and physical vibration.

The third, more commonly used, technique for lymphocyte

separation is density flotation (or isopycnic centrifugation).

This has been developed from the original work by Leif and

Vinograd (1964) who used centrifugation to equlibrium in an

albumin density gradient to effect separation between young and

old erythrocytes and to characterise an individual erythrocyte

population with a high degree of precision and reproducibility.

Other earlier workers (Hilal, et al, 1964) employed dextran

density gradients to separate cells of the myeloid series from

bone marrow.

The separation of cells by centrifugation to equilibrium

in density gradients should reflect differences only in cell

density, not cell size, since the cells come to density equilibrium

in a gradient which covers the range of densities in the cell

population. So, compared with velocity sedimentation the density

difference between the density flotation media and the cell

population is small and a much higher g force is used for density

separation, often due to the high viscosity inherent in solutions

with a density above 1.05 centipois (cps).

Although density gradient centrifugation does not separate cells

by size there is reason to predict an indirect relationship between

size and density. The nucleus of a lymphocyte should be more dense

than the cytoplasm. Thus, a small lymphocyte with proportionally

more nucleus, should be more dense than a large lymphocyte with

proportionally more cytoplasm. This was confirmed by Shortman

(1971), using thymocytes, who compared the volumes of cells from

gradient fractions of different densities. Using both Coulter

Counter size distribution data and direct morphological examination

of stained preparations it was possible to determine the density

distribution of cells of a given size range (Fig. 3.0). This

established that even a given size or morphological class of

lymphocyte was heterogeneous. Shortman also noticed that not all

large cells were light and not all small cells were dense, although

this general rule was adequate to obtain enriched populations of

lymphocytes of various sizes, if not pure ones. Such a distribution may

reflect the presence of several independent lymphocyte populations,

each with its own series of large, medium and small cells.

Alternatively it may reflect a series of definite metabolic stages

through which any one lymphocyte may pass in its development.

Recently evidence has shown that density flotation may be more

sensitive than velocity sedimentation in separating newly activated

lymphocytes. (Steinman et al, 1978). Velocity sedimentation

separates large cells, mainly those in the S and G + M phases of the

cell cycle, rather than blast transformed cells per se. This is

also a problem showed by ^{3}H-thymidine labelling of lymphocytes; the

recently transformed cells, in G_1 of the cell cycle are not identified

or characterised (Splinter and Reiss, 1974). On the other hand,

density flotation has been successfully used to identify and

characterise in vitro stimulated lymphoblasts in the G_1 phase

(Steinman et al, 1978). Low density blasts, identified morphologically

by basophilic cytoplasm, readily discernable nucleolus and abundant

Fig.3.0   "The average volume of normal rat thymus lymphocytes

banding in various regions of a density gradient.

The results are from a single animal, but are typical

of many experiments."

From: Shortman, K.(1971.) J. Cell. Physiol. 77: 319
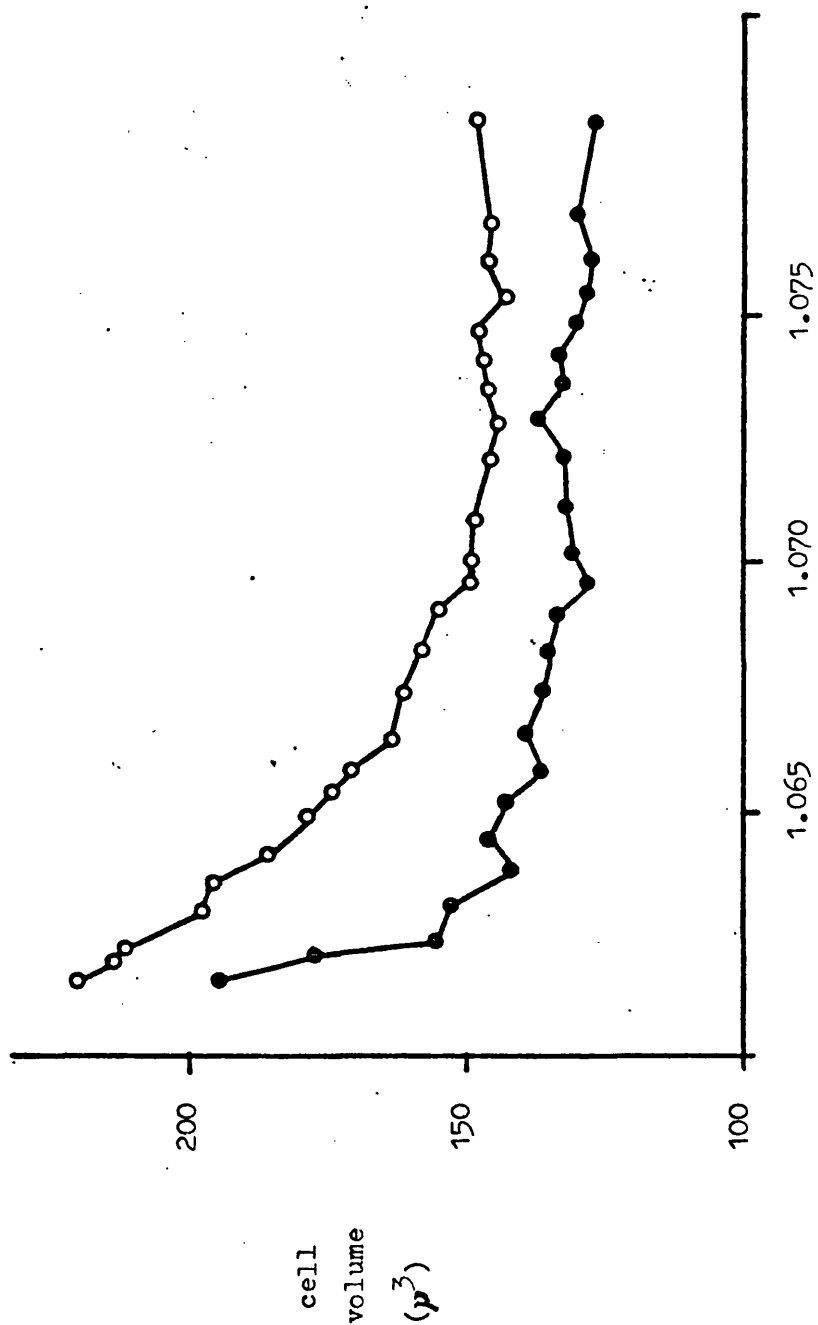
polyribosomes, were found within 20-24 hours of stimulation of·

mouse spleen cells by mitogens. These cells were not markedly

enlarged, but went on to enlarge and synthesize DNA after a

further 18-20 hours in culture.

It is not known why or when, during its initial cell cycle,

the transformed lymphocyte acquires a low density. Possibly

changes in the condensation of chromatin or in the water content

of the cell (? nucleus) are involved. It appears that the density

shifts occur before DNA synthesis begins. Gunthe et al (1974) obtain-

ed information that stimulated cells in G_1 are irreversibly

committed to entering S phase many hours before they actually

synthesize DNA. They noted that removing the stimulus (Con A)

after 20 hours of stimulation did not prevent the cells from

entering S phase after a day of additional culture.

Shortman et al (1967) showed that Vinblastine, a mitotic

inhibitor, when administered to antigen stimulated mouse

lymphocytes in vitro, caused an increase in the number of less dense

cells in a density gradient and a marked depletion of others.

As it is a drug that causes a build-up of cells in the process

of division and a relative depletion of product cells,this lends

further support for the usage of density gradient techniques to

separate and characterize activated and non-activated cells. The

ability to separate a number of samples concurrently is another

advantage over velocity sedimentation, as well as the separation

taking less time and media. Linked with the separation of newly

activated cells, not possible with velocity sedimentation, the·

merits of density flotation are clear.

Density gradient centrifugation has been used to identify

antigen sensitive cells (Haskill, 1967; 1969) and cells forming

19S haemolytic antibody in the rat (Haskill, Legge and Shortman,

1969) to enrich chicken blood lymphocytes initiating a graft-

versus-host reaction (Shortman and Szenberg, 1969), to demonstrate

several classes of killer cells found in allograft rejection

(Roberts and Hayry, 1977), to characterize cells involved in the

immune response to sheep erythrocytes (Haskill, Byrt and Marbrook,

1970) and to recover mouse spleen lymphoblasts generated in vitro

(Steinman et al, 1978).

There are two ways of using gradient centrifugation techniques;

continuous gradients and discontinuous gradients. The continuous

gradient is prepared in such a manner so that there is an

uninterrupted range of concentrations within the centrifuge tube

as illustrated in Fig. 3.1$_a$ This requires a gradient generator

to mix a light solution with a heavy solution and pump it into the

centrifuge tube. The discontinuous gradient is prepared by

carefully layering sequentially lighter solutions upon each other in

a centrifuge tube (Fig. 3.2a) Upon centrifugation of a continuous

gradient the cells find their density equilibrium at the relevant

point within the gradient. To harvest the separated cells a needle,

with a tube attached, is inserted into the bottom of the centrifuge

tube and the contents drained off in as many fractions as required.

In a discontinuous gradient the separated cells collect at the

interfaces between the layers of media. This gives a limited

number of fractions, but the harvesting is easily accomplished by

aspirating off the successive layers.

Loos and Roos (1974) compared the two methods and found that

the discontinuous system required less preparation, was easier to

Fig. 3.1.   The generation of density gradients

light   heavy

a) Continuous gradient

light   heavy
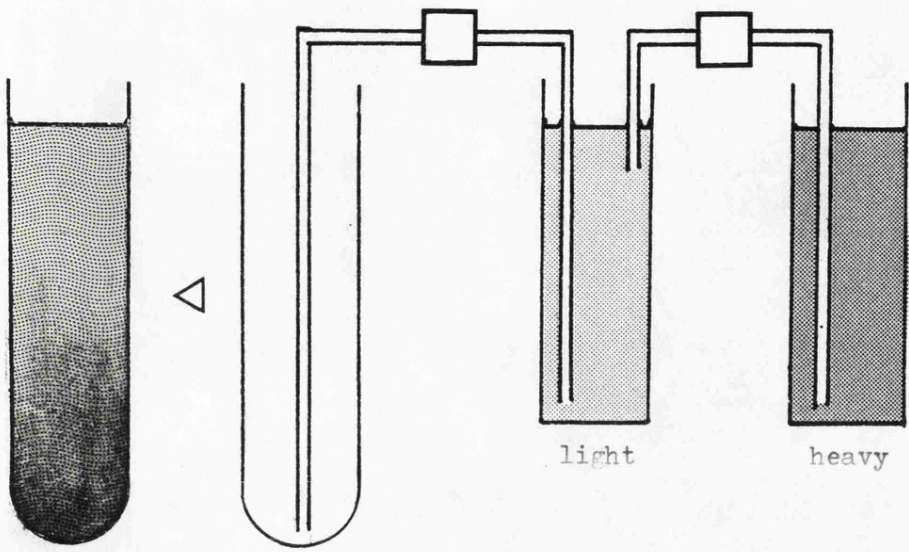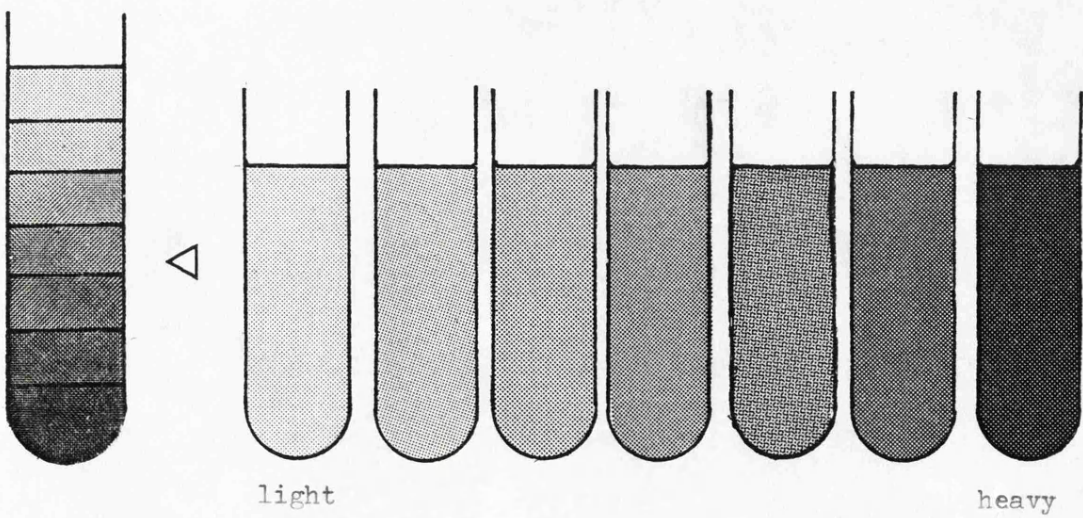
b) Discontinuous gradient

harvest, and gave a better yield of viable lymphocytes (approximately

50% more). They recommended its use for preparative techniques and

I employed it routinely for lymphocyte separation.

The separation of lymphocyte sub-populations on the basis

of density differences places particular demands on the materials to

be used for the preparation of the gradients. Obviously, the material

must be dense enough to produce gradients covering the normal

lymphocyte density range (1.055 - 1.095 g/ml). Besides, the material

must allow generation of gradients which are iso-osmotic and

with a constant pH through the whole gradient. Moreover, the material

should not influence the cells with respect to aggregation,

density or viability, nor should it be antigenic. Additionally, its

viscosity should be low in order to allow short centrifugation times

and easy measurement of its physical properties. Finally, the gradients

should give high cell yields and reproducible patterns.

Several materials have been used for the generation of density

gradients, continuous as well as discontinuous, among them sucrose,

gum acacia (Kinoshita et al, 1970), Renograffin (Grdina et al,

1973), albumin (Shortman, 1968; Williams, Kraft and Shortman, 1972;

Haskill, 1969), Ficoll-Hypaque (Day, 1972), Ficoll-isopaque (Loos

and Roos, 1974) and Ficoll (Bach and Brashler, 1970; Gorczynski,

Miller and Phillips, 1970). However, none of these materials is

completely without disadvantages. Sucrose permeates easily through

lymphocyte membranes and exerts a relatively higher osmotic pressure

at high concentrations. Gum acacia shows a non-linear relationship

between concentration and osmolarity and its viscosity is too high

for easy handling (Loos and Roos, 1974). Renograffin is not isotonic

throughout the gradient (Grdina et al, 1973). Bovine Serum Albumin

(BSA) and Ficoll are the two materials most widely used for lymphocyte

separations., Ficoll having superseded BSA to some extent in recent

years.

BSA has been strongly advocated for lymphocyte separation by

Shortman (Shortman, 1966; 1968; 1971; Shortman and Szenberg, 1969;

Shortman et al, 1971) and Haskill (Haskill, 1967; 1969; Haskill,

Byrt and Marbrook, 1970) whereas Ficoll has been favoured by

Bach and Brashler (1970), Gorczynski, Miller and Phillips (1970),

Glinski et al, (1976) and Yu et al (1973).

Shortman (1968) noted a marked heterogeneity in the density

distribution of lymphocytes from a number of tissues in continuous

BSA gradients. Many discrete bands or peaks were noted, suggesting

a far greater heterogeneity than expected from biological considera-

tions such as cytological criteria. Haskill, Legge and Shortman

(1969) also noted a heterogeneity of antibody producing cells

from mouse spleen. These authors thought that such distribution

patterns may reflect the presence of several independent lymphocyte

populations, each with its own series of large, medium and small

cells. Alternatively, they believed it may reflect a series of

definite metabolic stages through which any one lymphocyte may

pass in its development.

Gorczynski, Miller and Phillips (1970) pointed out that velocity

sedimentation of antibody-forming cells indicated that by size,

those cells are a homogeneous population (Phillips and Miller, 1970).

Although there was some heterogeneity in sedimentation velocity this

could almost entirely be explained in terms of cell volume changes

associated with cell growth through the division cycle. If there

was heterogeneity of the magnitude reported by Haskill et al

(1969), the distribution of sedimentation velocity for antibody-

producing cells should have been much broader than was observed

and should have shown some fine structure.

Worton, McCulloch and Till (1969) were disturbed about the lack
of reproducibility of the reports on BSA density gradient analyses
of mammalian cells. This could be caused by the nature of the
gradient material. BSA is a natural product and its isolation may
vary from one batch to another, variations in different preparations
can generate different density profiles for the same population
of cells (Shortman, 1968). In addition, because BSA is a charged
molecule, it will bind ions and decrease the osmolality of the
solution (Scatchard, Batchelder, and Brown, 1946). Changes in
osmolality are known to radically alter the density distribution of
some classes of cells (Shortman 1968; Gorczynski et al, 1970).
Also, to use BSA successfully the pH has to be quite low, e.g.,
Shortman (1968) used pH 5.1. However, the heterogeneity he
reported for lymphocyte density distribution is almost certainly
due to the osmotic gradient formed within the density gradient.
The inability of a peak of harvested cells to reband at the same
point in a second BSA gradient indicates that the BSA affects
the density of the cells in the gradient.

In contrast, Ficoll, a large polymer of sucrose, is not a natural
product and can be synthesized in large amounts to a required
standard. It contains no ionized groups and hence does not need
the rigorous desalting that must be applied to BSA before its use
in a density gradient. Ficoll has a very low permeability towards
cell membranes and hence cannot change cell density in the same way
that, for instance, unpolymerized sucrose may do.

Cells will associate or aggregate in Ficoll unless the divalent
cations calcium and magnesium are omitted from the media in which the

Ficoll is dissolved (Walder and Lunseth, 1963; Yu et al, 1973).
Alternatively, a dispersing agent, 2-naphthol-6,8-disulphonic acid
(dipotassium salt), has been used successfully (Shortman, 1968).
The pH may be maintained at a physiological level in a Ficoll
gradient whereas with BSA aggregation can only be reduced by
lowering the pH to 5.1, which increases lymphocyte density (Legge
and Shortman, 1968).

Gorczynski et al (1970) found that continuous Ficoll gradients
are isotonic throughout the density range used for lymphocyte
separation and that Ficoll is neither toxic nor stimulatory for
small lymphocytes, a report confirmed by Loos and Roos (1974) and
Yu et al (1973). The basic test to determine the validity of
a density distribution profile of rebanding a single peak of
lymphocytes in a second gradient has been used to evaluate Ficoll
gradients, both continuous and discontinuous (Loos and Roos,
1974; Bach and Brashler, 1970; Yu et al, 1973; Glinski et al,
1976; Williams, Kraft and Shortman, 1972). This was successful
in all cases, but depends upon the media being isotonic to the
plasma osmolarities, varying between 260 and 320 milli-osmoles
(Williams, Kraft and Shortman, 1972).

Ficoll appears to be fully established and acceptable as a
density gradient material and in some ways preferable to BSA,
not least being its relative cheapness. Ficoll was used throughout
for the analysis and preparation of lymphocyte populations in
discontinuous density gradients.

3.3 DISCONTINUOUS FICOLL DENSITY GRADIENT METHODOLOGY

The method described was that routinely employed for lymphocyte

density separation. During the design of this method a number of

variables were examined and these will be described in the next

section.

a. Lymphocyte preparation

Lymphocytes were prepared from peripheral blood by isopycnic

centrifugation on Ficoll/Triosil as described in chapter 2. 2b.

Calcium-and-magnesium-free media (CMFSS) was used throughout for

blood dilutions and lymphocyte washings.

<p style="text-align:center">Calcium-,and-magnesium-free salt solution (CMFSS)</p>

Sodium chloride	8000 mgl^{-1}
Glucose	1600 mgl^{-1}
Potassium chloride	400 mgl^{-1}
Potassium di-hydrogen phosphate	60 mgl^{-1}
Di-sodium hydrogen phosphate	48 mgl^{-1}

<p style="text-align:center">pH 7.3 at 18°C</p>

Occasionally phenol red ($17mgl^{-1}$) was added to enable a

constant visual check on the pH during storage.

The media was usually made up as a 10 X concentrate, filter

sterilized and stored in 125ml bottles at 4°C. Each bottle was

diluted with distilled water and the pH adjusted with concentrated

KH_2PO_4 or NA_2HPO_4.

b. Discontinuous Ficoll gradient generation

Ficoll 400 was dissolved with stirring overnight at 4°C in

CMFSS. A stock solution of 30% Ficoll was prepared in CMFSS and

filter sterilized into sterile 30ml McCartney bottles for storage

at -20°C.

Prior to use the Ficoll was thoroughly thawed, mixed and

left to stand to allow air bubbles to escape. Dilutions were

made in test tubes as follows:-

%Ficoll	30% Ficoll (ml)	CMFSS (ml)
16	2.13	1.86
17	2.26	1.73
18	2.40	1.60
19	2.53	1.46
20	2.66	1.33
21	2.80	1.20
22	2.93	1.06
23	3.06	0.93
30	4.00	0

The tubes were thoroughly mixed on a whirlimixer and left to

stand to allow air bubbles to escape.

Eighteen ml. glass centrifuge tubes (110 X 15mm) were used

for the density gradient centrifugation. Consequently lighter

2ml volumes of Ficoll solution were carefully layered above each

other as shown in Fig 3.2, excepting the 16% Ficoll. Two density

gradients were always prepared for strict control in sample

Fig. 3.2.   Discontinuous Ficoll gradient   81

Fig. 3.2.   Discontinuous Ficoll gradient
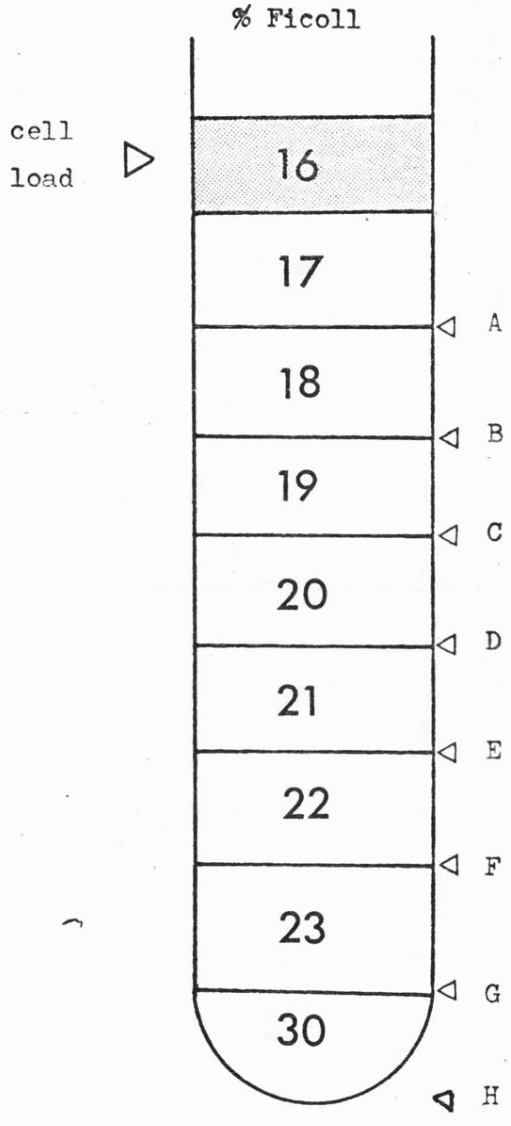
comparisons of density distribution.

c. Monocyte depletion

The cell pellet, consisting of washed lymphocytes and monocytes, was resuspended in 2ml of 16% Ficoll in a glass tube and incubated at 37°C for 30-60 minutes. This lymphocyte rich supernatant was decanted and carefully layered at the top of the density gradient.

d. Density gradient centrifugation

The separation was in the swing-out head of a refrigerated centrifuge (MSE Mistral 2L) at 10°C. A fixed temperature was used to prevent convection currents and the changes in viscosity and density which occur with temperature variations (Shortman, 1968). The centrifuge speed was increased slowly to prevent mixing of the gradient and a force of 3,000g, at the tip of the tube, maintained for one hour. The brake was not used for running down the centrifuge motor, again to prevent mixing of the gradient.

The lymphocytes localised at the interfaces between the gradient layers. To harvest the cells the layers were sequentially withdrawn using a pasteur pipette, rotating the pipette around the inside wall of the centrifuge tube while aspirating. In this manner the contamination by adjacent interfaces was minimal.

Each interface suspension was ejected into a graduated tapered glass centifuge tube, made up to approximately 10ml with CMFSS, thoroughly mixed by repeated withdrawals and expulsions in a pasteur pipette and the cells sedimented by centrifugation at 200g for 10 minutes. The cell-free supernatant was decanted and discarded and the cell pellet made up to 0.5ml with CMFSS and thoroughly mixed. The

lymphocyte count was determined by diluting an aliquot in white

cell counting fluid (WCCF) and counting the cells in a

haemocytometer using the low power (X10) of a binocular microscope.

3.4   EXPERIMENTAL DESIGN - INVESTIGATION OF PARAMETERS

A range of techniques for density gradient centrifugation using
Ficoll have been described (Zettergren, Luberoff and Pretlow, 1973;
Loos and Roos 1974; Yu et al, 1976; Bach and Brashler, 1970;
Glinski et al, 1976; Glinski, Gershwin and Steinberg, 1976;
Minderhoud and Smith, 1972; Williams, Kraft and Shortman, 1972;
Gorczynski, Miller and Phillips, 1970).

a.   Density gradient material

Ficoll is a synthetic high polymer (MW 400,000) made by the
co-polymerization of sucrose and epichlorodydrin. The molecules
have a branched structure and have a high content of hydroxyl groups
leading to a very good solubility in aqueous media. No ionized
groups have been found in Ficoll (Pharmacia Information Services).

Ficoll has been used in a mixture with Isopaque (sodium-,
calcium-, and magnesium-N-methyl-3,5-diacetamido-2,4,6-tri-
iodobenzoate) to form density gradients for lymphocyte separation
(Loos and Roos, 1974). This was used successfully with both contin-
uous and discontinuous gradients, showing similar density distribu-
tions and with good cell yields, particularly in the discontinuous
gradients.

My attempts to repeat these experiments were fruitless. The
Ficoll/Triosil mixture detailed in Chapter 2.2b for lymphocyte
isolation was used diluted with phosphate buffered saline to produce
a density range of 1.040 - 1.080 g/ml in six steps as a discontinuous
gradient. Upon applying the lymphocyte sample to the surface of
this gradient and centrifuging at 3,000g the cells aggregated to a
large extent and formed a monolayer at one of the gradient
interfaces. Possibly Triosil is unsuitable by virtue of it having
a slightly different formulation to Isopaque, even though both

are mainly sodium-, calcium- and magnesium salts of metrizoic

acid.

Ficoll/Triosil was rejected as a density gradient medium and

Ficoll alone in solution was the next medium investigated. Initial

attempts used a full PBS media but the divalent cations, calcium

and magnesium, caused aggregation of lymphocytes in the gradient

and the cells formed a monolayer at the first gradient interface,

i.e. did not distribute according to density. The calcium-, and

magnesium-free salt solution w s based on the Flow Catalogue

Formulation for Hanksbalanced salt solution and prevented

lymphocyte aggregation to a great extent. Similar results were

found by Bach and Brashler (1970) and Yu et al (1973) who remark

on the importance of eliminating cell aggregation. Aggregation

of lymphocytes of varying densities may seriously distort the

density distribution pattern. An association between a large

light cell and a small dense cell would result in a doublet with

a buoyant density less than the arithmetic mean of the two densities.

The mean cell volume of cells in the less dense region of the gradient

is usually greater than cells in the more dense region. Thus,

an increasing proportion of doublets and larger aggregates with

higher cell concentrations would be expected to cause a small decrease

in the apparent average buoyant density. More importantly, preparative

techniques would be rendered impractical due to the contamination

of the density gradient fractions and further analysis of gradient

fractions would be of only limited value.

The density range of various Ficoll concentrations is shown in

Fig. 3.3 The range of Ficoll concentrations used here produces a

similar range of specific gravities to those used by other authors

to separate lymphocytes into different density classes (Shortman, 1968;

Fig. 3.3.   Density of Ficoll as a function of

concentration.
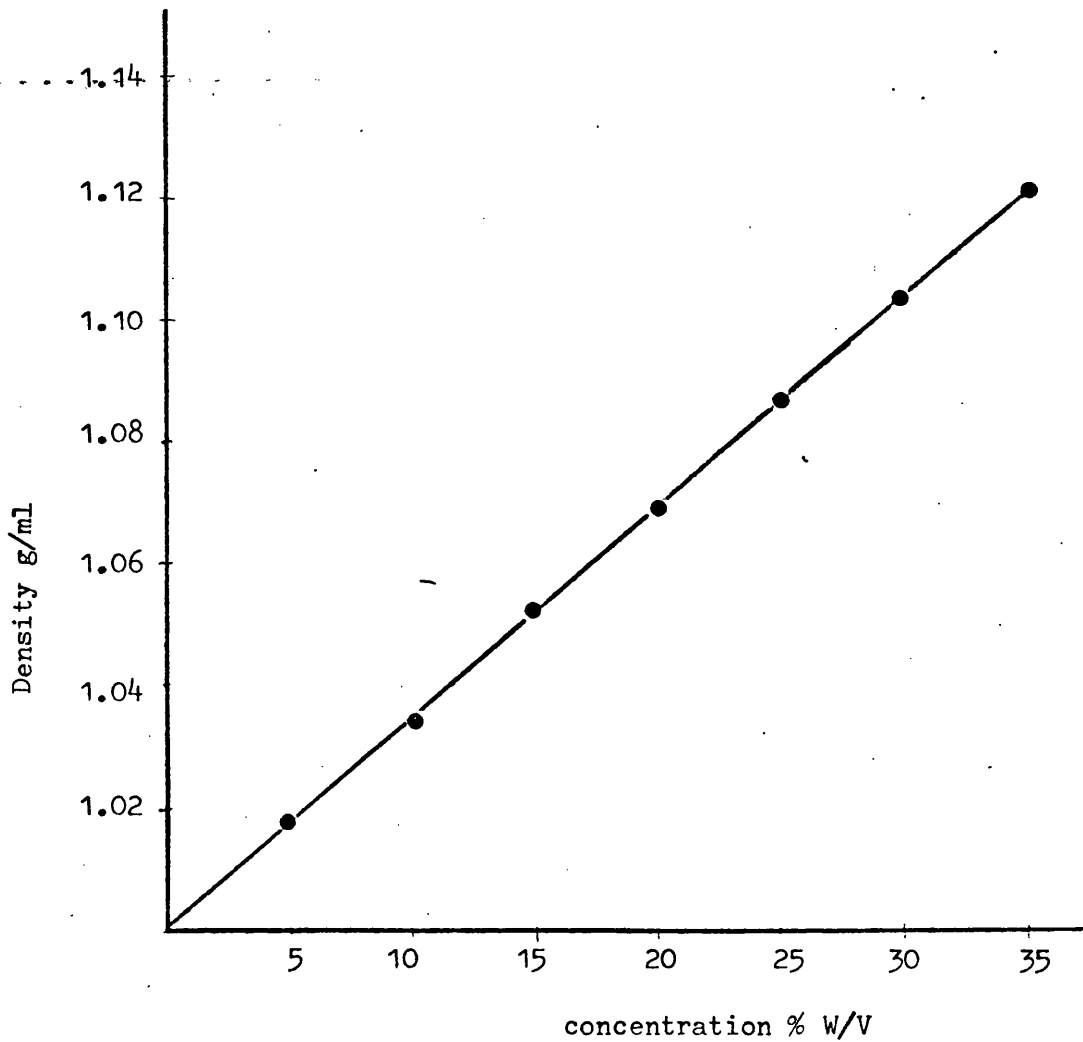
Haskill, Legge and Shortman, 1969; Haskill, Byrt and Marbrook, 1970; Loos and Roos, 1974) although upon close examination the mean density of lymphocytes varies by as much as 0.020g/ml from one group to another.

My initial experiments with the density gradient had a wider range of Ficoll concentrations than finally employed. After several lymphocyte separations it became clear that the lymphocytes were only separating in the 16-23% Ficoll layers. This was determined by visual examination of the intact gradients to detect the presence or absence of cells. The final gradient range means that many lymphocytes were found in fraction G and so produced a wider, better density distribution analysis of the less dense cells than of the dense cells.

Fortunately, Ficoll solution is viscous enough to allow solutions varying only by 0.004g/ml to be layered above each other by hand with a pasteur pipette and bulb. The process is slow however, and requires a certain amount of practice to ensure a clear interface Schlieren between layers.

The Schlieren line between the layers disappears after a few minutes but the interfaces and layers stay intact much longer. This was demonstrated by colouring, with a small amount of 1% Trypan blue, the 17%, 19%, 21% and 23% Ficoll layers before forming a gradient. After the gradient had been made it was left to stand at 4°C for 36 hours during which time the coloured layers had not mixed at all with the adjacent clear layers, confirming the stability of the gradient.

Centrifugation up to 3,000g maximum with gentle acceleration and deceleration, did not disturb the layers.

In many published studies on density gradients the cells were

suspended in the most dense solution of the gradient because

suspending cells in the light layer may cause distortion of the

density profile by the "wall effect" (Anderson, 1955). This

was examined by Leif and Vinograd (1964) who found that a certain

proportion of cells were lost by sedimentation against the

cylindrical walls of the tube. More recently, Yu et al (1973)

examined this phenomenon in discontinuous gradients of Ficoll

and found the density distribution profiles very similar whether

the cells were resuspended in 5% or 30% Ficoll. Because of the

differences in viscosity, it is easier to disperse cells in a

low density Ficoll solution than a high density Ficoll solution.

This prevents aggregation which has been shown to seriously

distort the density profile. Similar results to those of Yu

et al (1973) with Ficoll gradients have been shown with dis-

continuous BSA density gradients (Adler, Peavy and Smith,

1970). For these reasons I always suspended the cells in the

lowest density Ficoll in my experiments.

b. Duration and force of centrifugation

The duration of centrifugation required for equilibrium was

tested. The duration finally employed (60 minutes) was more

than sufficient for equilibrium and is similar to that used by

Raidt, Mischell and Dutton (1968) in their discontinuous albumin

gradients.

For confirmation of this lymphocytes were prepared from 50ml

venous blood from a healthy person, (S.C.),divided into two equal

amounts and separated in two Ficoll gradients. One was centrifuged

at 3,000g for 40 minutes and the other at 3,000g for 60 minutes.

Cells were seen only at the interfaces of both gradients, suggesting

equilibrium had been attained. The density distribution profiles were virtually identical (Fig. 3.4), suggesting that 60 minutes at 3,000g was more than adequate to permit equilibriation.

There are several factors to consider when determining the optimum centrifugation speed. The force applied needs to be high enough to bring the system to equilibrium in a reasonable time, and in addition, high enough to overcome the forces causing light and dense cells to associate together and thus to band in a region of intermediate density. However, to reduce the possibility of high forces damaging cells, the speed should be as low as practicable. The force used (3,000g) is at the low end of the range of force used by a number of workers which extends up to 55,000g yet, as it appears to allow equilibrium, there is no need to go any higher.

c. <u>Cell load</u>

Cell to cell association affects density distribution, and possibly the cell load affects cell interaction. Shortman (1968) and Yu et al (1973) have investigated this parameter to see if cell load changes caused variations in lymphocyte density distribution profile. They have suggested upper limits of at least 5×10^8 cells and 3×10^8 cells per tube respectively before the profile changes. The maximum number of cells loaded in any experiments described here is 1×10^8/tube, below either reported maximum.

d. <u>Rebanding of a gradient fraction</u>

A basic test to determine the validity of a density distribution profile is to reband a single peak or fraction of cells in a second gradient. This fraction should then reform only one fraction, at the same density position. In tests with rat thymus cells

Fig. 3.4

The effect of centrifugation time on the

density separation of healthy lymphocytes.

60 ml of peripheral blood was taken from a healthy subject

and the lymphocytes prepared (Chapter 2.2.b ii). The monocytes

were depleted by adherence (Chapter 3.3.c) and the lymphoid cells

separated by density in a Ficoll gradient (Chapter 3.3.d.) at 3,000g

for either 40 minutes or 60 minutes. The interfaces were harvested

and counted and the results plotted as the percentage of the

total recovered lymphocytes found in each fraction.

Fig. 3.4.
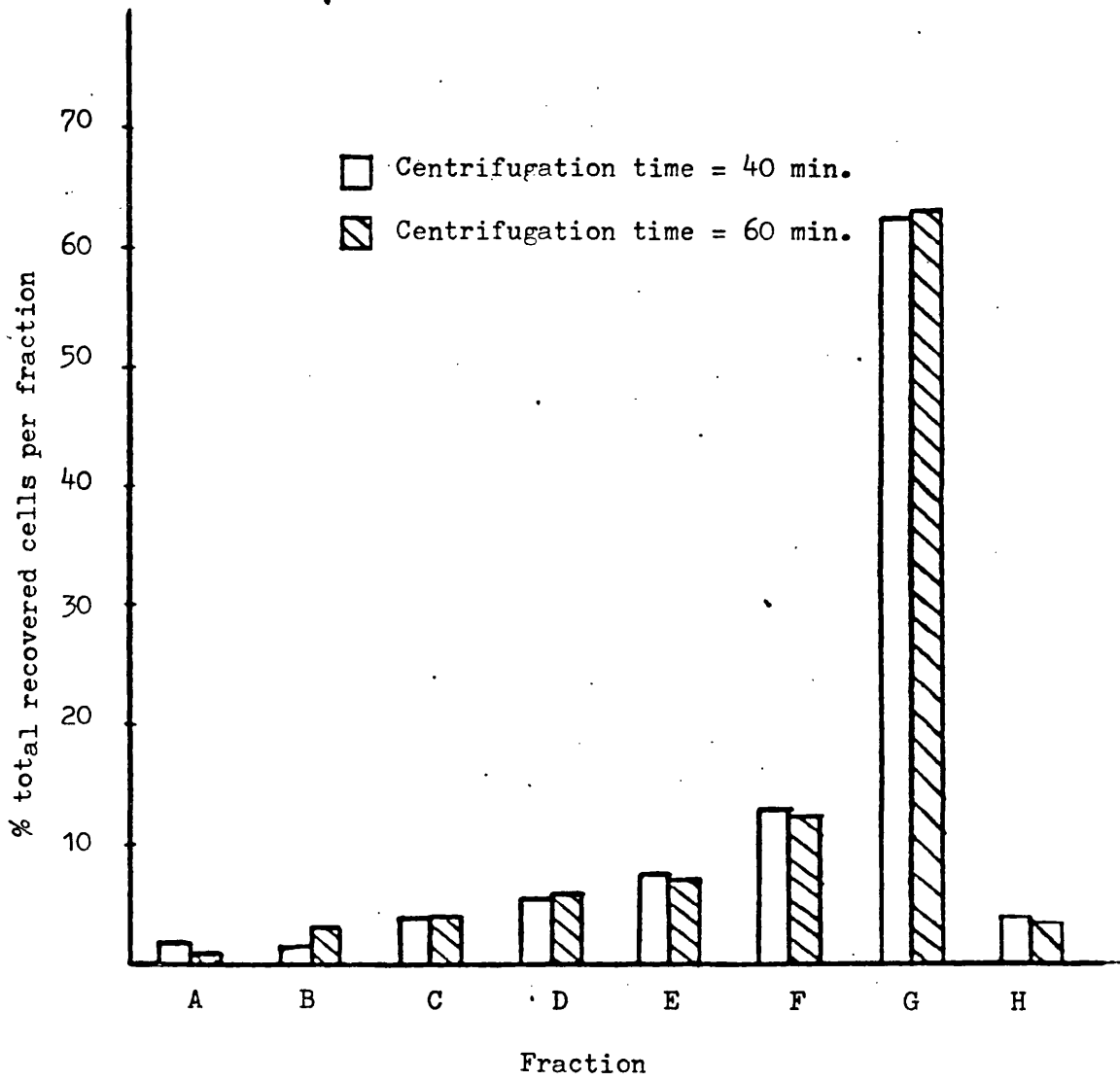
(Shortman, 1968) an isolated fraction formed only one peak on a second gradient, but with a shift to the more dense regions. This was shown to be due to stress caused by the high force used in the second centrifugation. The relatively low centrifugal force used in these experiments should not cause any change in optimal rebanding of a fraction of cells.

Rebanding experiments have been used to validate density gradients of BSA (Shortman, 1968; Adler, Peavy and Smith, 1970; Williams, Kraft and Shortman, 1972), Ficoll-Isopaque (Loos and Roos, 1974) and Ficoll(Bach and Brashler, 1970; Gorczynski, Miller and Phillips, 1970; Yu et al, 1973; Glinski et al, 1976). Two purposes are fulfilled by successful rebanding. First, the reproducibility of the gradients and the degree of resolution may be shown. Second, cell to cell association in the first gradient would show itself in the second gradient by the appearance of cells in more fractions than that being rebanded. If no major spreading of the rebanded fraction occurred it would show that large and medium lymphocytes were banding at their own buoyant density and were not just non-specifically trapped by their association with small lymphocytes.

Lymphocytes from a healthy donor were separated on a discontinuous Ficoll gradient, the fractions harvested and washed with CMFSS. The cells from fraction G were pelleted, resuspended in 16% Ficoll and separated on a second Ficoll gradient. The fractions were harvested, washed and cell counts performed. The results are shown in Fig. 3.5. The majority of the cells (84%) from fraction G from the first gradient reisolated

Fig. 3.5

Re-centrifugation of lymphocytes from a Ficoll density
gradient fraction in a second gradient.

Lymphocytes were harvested from 30 ml venous blood
(Chapter 2.2.b.ii) and the monocytes depleted by
adherence (Chapter 3.3.c.). The lymphocytes were
separated by density in a Ficoll gradient at 3,000g
for one hour. The fractions were harvested and counted
and fraction G re-centrifuged in a second Ficoll gradient.
The fractions were again harvested for lymphocyte counts.
The results are plotted as percentage of total recovered
cells in each fraction.

Fig. 3.5.
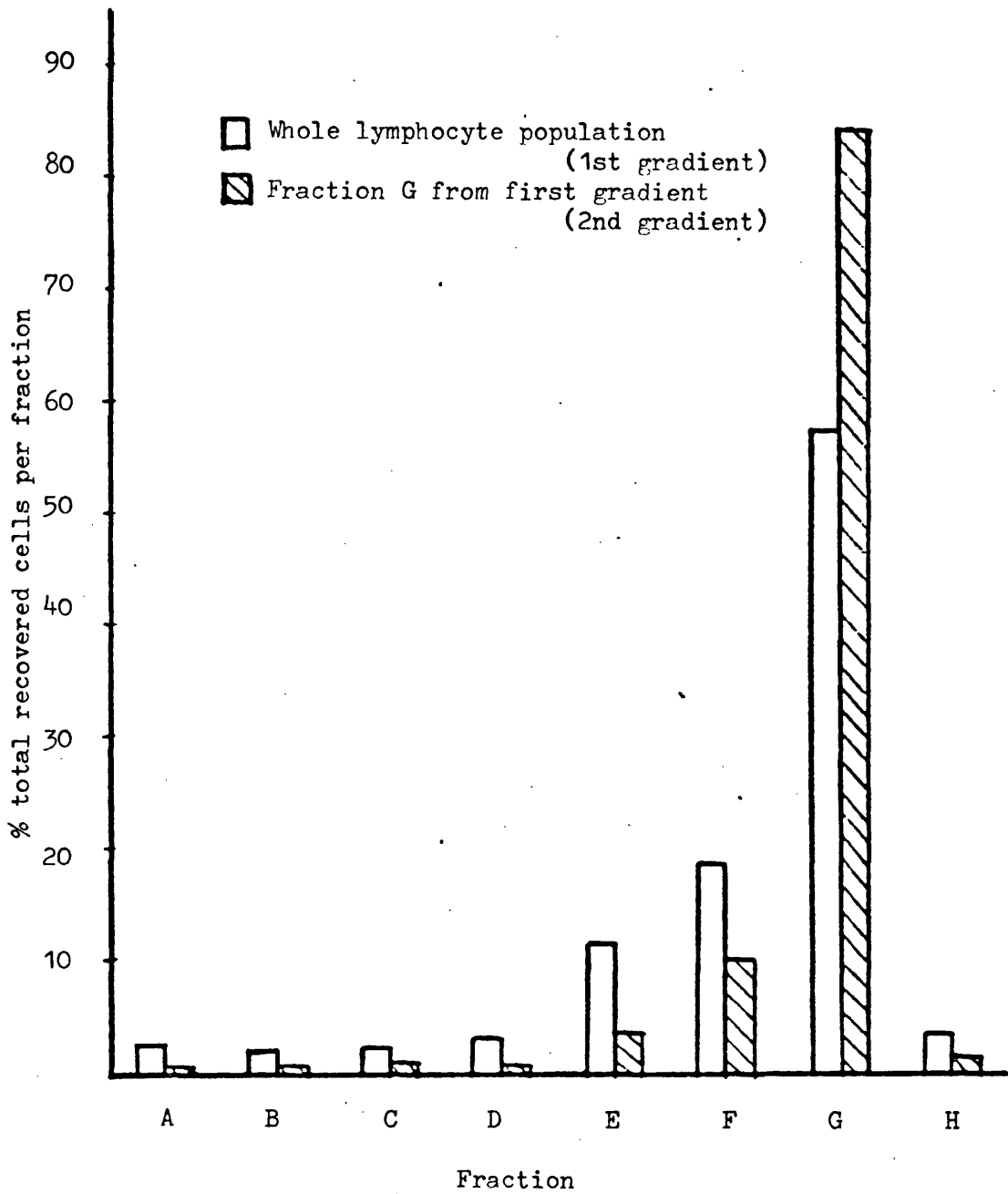
at G in the second gradient. This degree of enrichment in the reisolation is enough to suggest that the cells are separating by their buoyant density. This is a better re- isolation than some authors have obtained, particularly so with the narrow density range employed here. The second gradient separation was performed as soon after the first as possible, because other authors have reported that prolonged exposure to gradient material may affect lymphocyte density (Shortman, 1968; Williams, Kraft and Shortman, 1972; Bach and Brashler, 1970; Loos and Roos, 1974), particularly by increasing the density of the reisolated lymphocytes. This was not found to be a problem in this experiment.

e. Reproducibility

To test the reproducibility of replicas a sample of healthy peripheral blood lymphocytes was divided into two equal aliquots of 2×10^{7} cells and put through two separate density gradient tubes. Fig. 3.6 shows that the differences between the two tubes are small and that comparisons of density profiles of cells in identical gradients, prepared and centrifuged together, are valid.

If serial studies of density distribution profiles from the same subjects are to be attempted, the day to day, week to week and month to month variation should be considered. The various cells involved in the immune response are constantly being turned over, lymphocytes with varying functions have different life spans (Gowans, 1966). It is important to know if the density distribution profile of various healthy subjects lymphocytes is representative of a status quo in the lymphocyte dynamics or a phase in an ever fluctuating pattern.

During the course of experimentation the lymphocytes from some

Fig. 3.6

Reproducibility of Ficoll density

gradients

60ml of blood was taken from a healthy subject

and the lymphocytes separated (Chapter 2.2.b ii).

The monocytes were depleted by adherence (Chapter 3.3.c.)

and the sample separated in two Ficoll gradients

(Chapter 3.3.d.). The interfaces were harvested

the lymphocytes counted and the results compared.

Fig. 3.6.

Fig. 3.6.
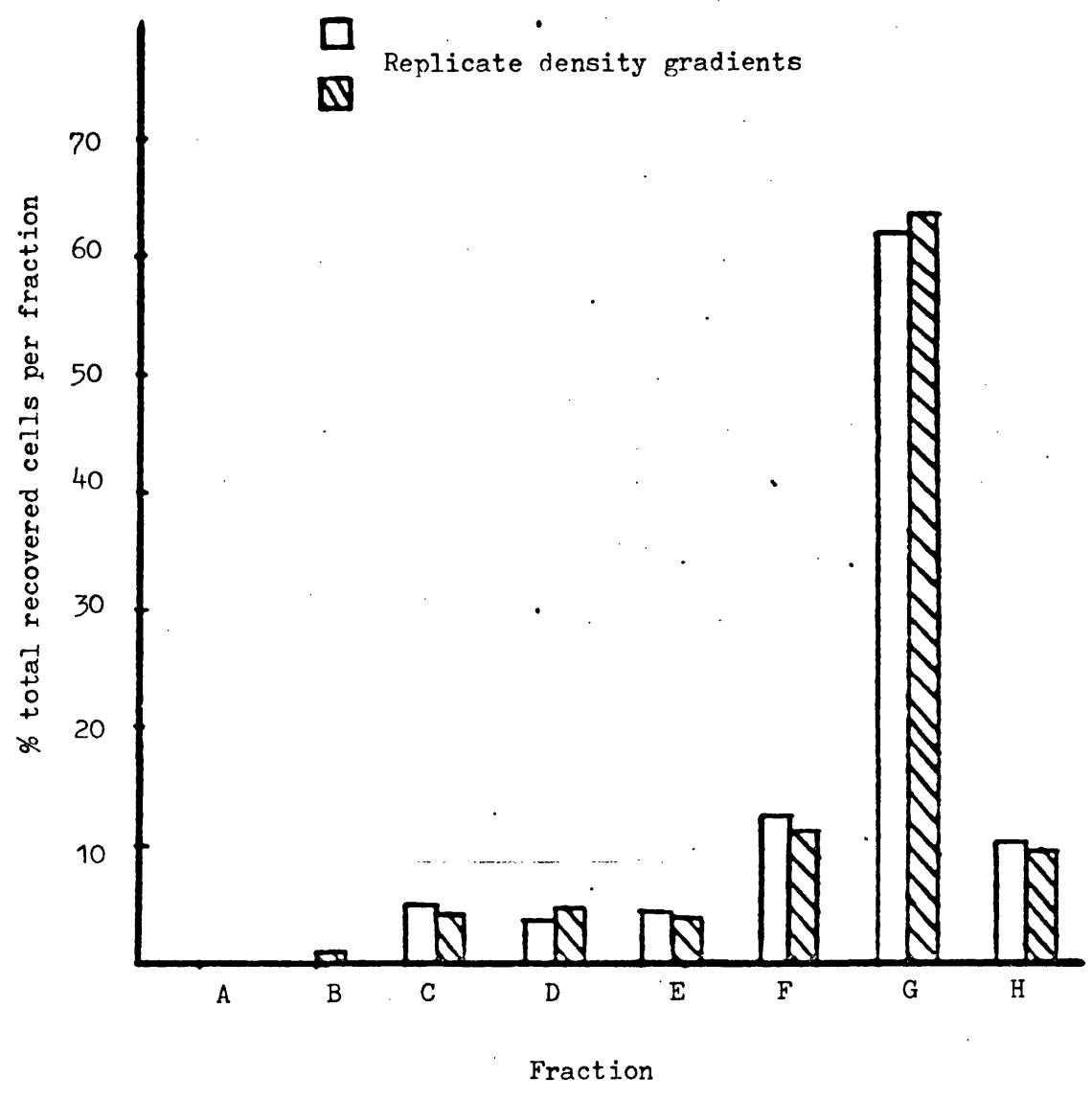
healthy individuals, notably laboratory personnel, have been used

several times for comparison with patients suffering from rheumatic

diseases. One example is shown in Table 3.1. The density

distribution profiles are for the lymphocytes from one person

determined at approximately three monthly intervals and show little

variation within each fraction. All blood samples were taken at the

same time of the day to eliminate any diurnal variation. The stable

pattern shows that in a healthy person there is an equilibrium

in the range of lymphocytes as detected in this system, in the

peripheral blood. This pattern is expected to change during

immunization or infection as shown by cytological examination

(Crowther, Hamilton-Fairley and Sewell, 1969).

f. Yield and viability

The yield of lymphocytes from the gradient was found to vary

between 30% and 60%. This is similar to the results of other

authors. Higher yields have been reported for BSA (Shortman,

1968; Haskill, Legge and Shortman, 1969) and the addition of

human albumin (1%) to Ficoll solutions has also been reported

to increase the yield from a Ficoll gradient (Loos and Roos, 1974).

The cell viability in each fraction was assessed by the Trypan

Blue dye exclusion test. Incubating the lymphocytes in the

16% Ficoll layer to remove monocytes by adherence had a beneficial

side effect. The Ficoll conferred a degree of protection to the

lymphocytes during the gradient centrifugation, particularly

to the less dense cells which are more susceptible to damage.

(Shortman, 1968). This phenomenon of cell protection by

concentrated solutions of Ficoll has been reported previously

TABLE 3.1   <u>DENSITY DISTRIBUTION PROFILES OF A HEALTHY</u> .

<u>SUBJECT'S (S.C.) PERIPHERAL BLOOD LYMPHOCYTES</u>

<u>(AT APPROXIMATELY THREE MONTHLY INTERVALS)</u>

CONSECUTIVE SAMPLES

FRACTION	1	2	3	4	5	X ± 1 S.D.
A	0	0	0	0.5	0.2	0.1 ± 0.19
B	0	0	2.9	1.5	0.3	0.9 ± 1.12
C	5.2	2.2	2.1	0.8	1.2	2.3 ± 1.54
D	3.6	3.2	2.7	4.5	3.5	3.5 ± 0.59
E	4.4	3.2	6.2	4.1	6.0	4.8 ± 1.15
F	12.3	9.2	10.9	9.0	7.8	9.8 ± 1.58
G	74.5	79.0	72.2	77.0	75.0	75.5 ± 2.31
H	0	0.9	3.0	2.7	6.0	2.5 ± 2.07

(All figures % of total recovered cells

in each fraction)

(Stuart and Simpson, 1970) and was found, in this work, to radically improve both yield and viability of density gradient fractions. The viabilities of lymphocytes prior to and after including pre-incubation in Ficoll as a medium for monocyte depletion is shown in Table 3.2. The Ficoll pre-incubation increases the cell viablity, particularly in the less dense fractions. The poor viability in fraction H is due to the fact that most of the cells in this fraction are there because they are dead. When a lymphocyte lyses one of the first events is the appearance of holes in the cytoplasmic membrane. Then the cytoplasm is lost through these enlarging gashes leaving only the nucleus, which is denser than cytoplasm, and hence the cell becomes dense and sinks within the density gradient to equilibriate in a lower position than most of the viable cell population. The poor viabilities in the less dense cell fractions have been previously reported by Shortman, (1968) and he noted that most of the damaged cells were large lymphocytes. The fragility of large lymphocytes has been reported by other authors (Hall, 1966; Sewell, R. L., personal communication) although no reasons for this have been put forward.

g. Cytological examination of density gradient fractions

The cell suspensions of each fraction were washed in CMFSS, centrifuged to a pellet and smears made on duplicate glass slides. These were air dried and stained with Wrights stain (chapter 2.2d.). Under a microsoope the less dense fractions contained more cells with the cytological appearance of activated lymphocytes and plasma cells.

Table 3.2

VIABILITY OF LYMPHOCYTES IN FICOLL

DENSITY GRADIENT FRACTIONS

Fraction	Pre-incubation in CMFSS	Pre-incubation in 16% Ficoll
A	12	64
B	10	61
C	34	60
D	35	75
E	81	91
F	88	92
G	89	90
H	15	30

All figures % viable cells as assessed by

dye exclusion

The dense fractions were relatively depleted of activated
lymphocytes and plasma cells compared with the original
population. Quantitative analysis was difficult because many
of the cells in the low density fractions were damaged,
proportionally more than shown in the viability data,
indicating that at least some cells were damaged during the
making of a smear, providing more evidence that large lymphocytes
are relatively more fragile than small lymphocytes. In addition,
all the damaged cells in the low density fractions had typical
nuclear features of activated lymphocytes. The degree of
enrichment of these large cells was thus difficult to determine
accurately by normal microscopical means although inspection suggested
that large lymphocytes were less dense than small lymphocytes.

To reduce cell damage some slides were made from cell suspensions
using a cytocentrifuge. The cell morphology was distorted but more
of the large lymphocytes were undamaged. Accurate measurement
of cell diameter was impractical, but a visual assessment of
lymphocyte activation on Wrights stained preparations was made on
the basis of cell size, nuclear characteristics, nuclear: cytoplasm
ratio, number of nucleoli and basophilia of cytoplasm. The results
(Table 3.3) are from a patient with rheumatoid arthritis and a
chronic chest infection who had large numbers of less dense cells.
Increased numbers of large cells are shown in the less dense
fractions and decreased numbers in the denser fractions.
Contaminating granulocytes were usually found in fraction H,
but a few ($<5\%$) were occasionally found in fraction G. Granulocytes
density (>1.085 g/ml) is more than that of lymphocytes ($1.050 -$
1.080 g/ml) and so the result is expected. This is the means of

Table 3.3

DIFFERENTIAL COUNT OF LYMPHOCYTES IN FICOLL

DENSITY GRADIENT

Lymphocyte Size

Fraction	Small	Medium	Large
A	51	32	17
B	42	35	23
C	39	36	25
D	40	35	25
E	46	39	15
F	78	22	0
G	96	4	0
H	91	9	0

All figures % of total in each fraction

separating lymphocytes and granulocytes on Ficoll/Triosil or Ficoll-Paque (isopycnic centrifugation).

Erythrocytes were only a minor problem as contaminants and only then if present in such large numbers that auto rosettes formed and lymphocyte density was affected by cell to cell association. This was detected before cell separation on density gradients and the sample was then rejected.

h. ^{3}H-thymidine incorporation

Proliferating lymphocytes are actively involved in DNA production and as such will incorporate labelled thymidine. The degree of proliferation may be determined from the rate of incorporation of the labelled thymidine. The incorporation of ^{3}H-thymidine by density gradients fractions was investigated to compare proliferation within the separated sub-populations.

Lymphocytes (30×10^6), from a healthy donor, were re-suspended in 10ml TC 199 containing 10% heat-activated new-born calf serum and 2mm glutamine. ^{3}H-thymidine (29 Ci/mmol) was added at 2μ Ci/ml. Incubation at 37°C for one hour was followed by two washes with CMFSS. The cells were resuspended in 2ml of 1% Ficoll, incubated at 37°C for 30 minutes and then separated on the Ficoll density gradient. The harvested fractions were washed, counted, adjusted to 10^6/ml with CMFSS and 200 µl aliquots taken. The ^{3}H-thymidine incorporation of each aliquot was determined as in Chapter 2.2i. The results (Table 3.4) show that fractions C ,D and, to a lesser extent, E incorporate more ^{3}H-thymidine than the denser fractions, indicating that these low density fractions contain the bulk of the proliferating cells in

Table 3.4

^{3}H-THYMIDINE INCORPORATION OF A HEALTHY SUBJECT'S

FRACTIONS FROM A FICOLL DENSITY GRADIENT.

(MEAN OF TWO EXPERIMENTS)

Fraction	^{3}H-thymidine incorporation (d.p.m)
A	290
B	409
C	930
D	905
E	581
F	352
G	208
H	71

(All figures for incorporation of

2 X 10^5 cells for one hour)

peripheral blood. Similar results have been published by August

et al (1970) on their studies of human tonsil lymphocytes

fractionated on BSA density gradients. This assay has been called

the To test by Dicke, Tridente and van Bekkum (1969) in their studies

of haematopoietic stem cells from mouse spleens and August et al

(1970) reported that the distribution of human tonsillar lymphocytes

with high To tests was almost identical to that obtained with

mouse spleen. Low density lymphocytes, incorporating ^{3}H-thymidine

at a high rate, have also been found in human thoracic duct lymph

by discontinuous Ficoll density gradient analysis (Yu et al, 1973).

In vitro generated blasts, using mouse spleen cells stimulated by

mitogens, have been shown to have a low density and incorporate

^{3}H-thymidine. (Steinman, et al, 1978).

i. Density distribution of mitogen-stimulated lymphocytes

    Mitogens, such as phytohaemagglutinin (PHA), concanavalin A

(con A) and Pokeweed mitogen (PWM) have been used to activate

lymphocytes non-specifically in vitro. Once activated those

cells then transform into large lymphocytes which morphologically

resemble those found as a result of in vivo antigen stimulation.

Many of the morphological (Douglas, 1971; Hirschhorn et al, 1963;

Janossy and Greaves, 1971) and biochemical changes (Naspitz

and Richter, 1968; Ling, 1968) have been investigated and shown

to have parallels with antigen stimulated cells.

    From a practical point of view, mitogens are of value because

large numbers of lymphocytes may be stimulated compared to specific

antigens. Low dose PHA has been shown to preferentially stimulate

a sub-population of T cells (Hirschhorn et al, 1971) and recent

work by Morgan (1978) has shown that in excess of 30% large

lymphocytes may be found after three days of culture with this

mitogen. Mouse spleen lymphoblasts stimulated to divide in vitro

by lipopolysaccharide (LPS) Foetal Calf Serum (FCS) and Con A,

acquire a low density and may be separated by isopycnic techniques

(Steinman et al, 1978). Human lymphoblasts stimulated in vitro,

have a high cytoplasmic:nucleus ratio (Ling, 1968) and should have

a low density. Thus, this system was used to prepare large numbers

of activated lymphocytes to assay the ability of the Ficoll density

gradient to separate activated from non-activated cells.

Lymphocytes (30×10^6) from a healthy subject, isolated by

isopycnic centrifugation on Ficoll Paque were incubated with purified

PHA at $1\mu g/ml$ (this contains relatively more mitogenic activity

and less agglutinating activity than the crude preparation) as

described in chapter 2. After three days in culture the cells were

sedimented, washed twice in CMFSS, resuspended in 16% Ficoll and

separated on a Ficoll gradient. Simultaneously, more lymphocytes

from the same donor were prepared fresh and separated on an identical

gradient. The fractions were harvested, washed, counted and cell

smears made on a cytocentrifuge.

The density distribution profiles are shown in Fig. 3.7. The

peak of cells in fraction G is much reduced in a PHA activated

population and a new peak appears at C. The intermediate fractions

D. E. and F. show that PHA activation is not entirely synchronous,

as new cells are continuously being recruited (Steinman et al, 1978).

The large number of less dense cells shows that this technique

of density gradient separation is sensitive to activation of lympho-

cytes.

Fig. 3.7.

The effect of culturing with PHA

on the density of lymphocytes in a

Ficoll gradient

30 ml of blood was taken and .the lymphocytes prepared

aseptically (Chapter 2.2.b.ii). The lymphocytes were

cultured in 5% CO_2 at $37^{\circ}C$ at 1×10^6ml with PHA ($1\mu gml^{-1}$)

for. three. days. The cells were washed X2 with CMFSS

and separated on Ficoll gradient. Freshly prepared autologous

lymphocytes were also separated in a parallel gradient.

The gradient fractions were harvested and the lymphocytes counted.

The results are expressed as percentage of total recovered

cells in each fraction.

Fig. 3.7.
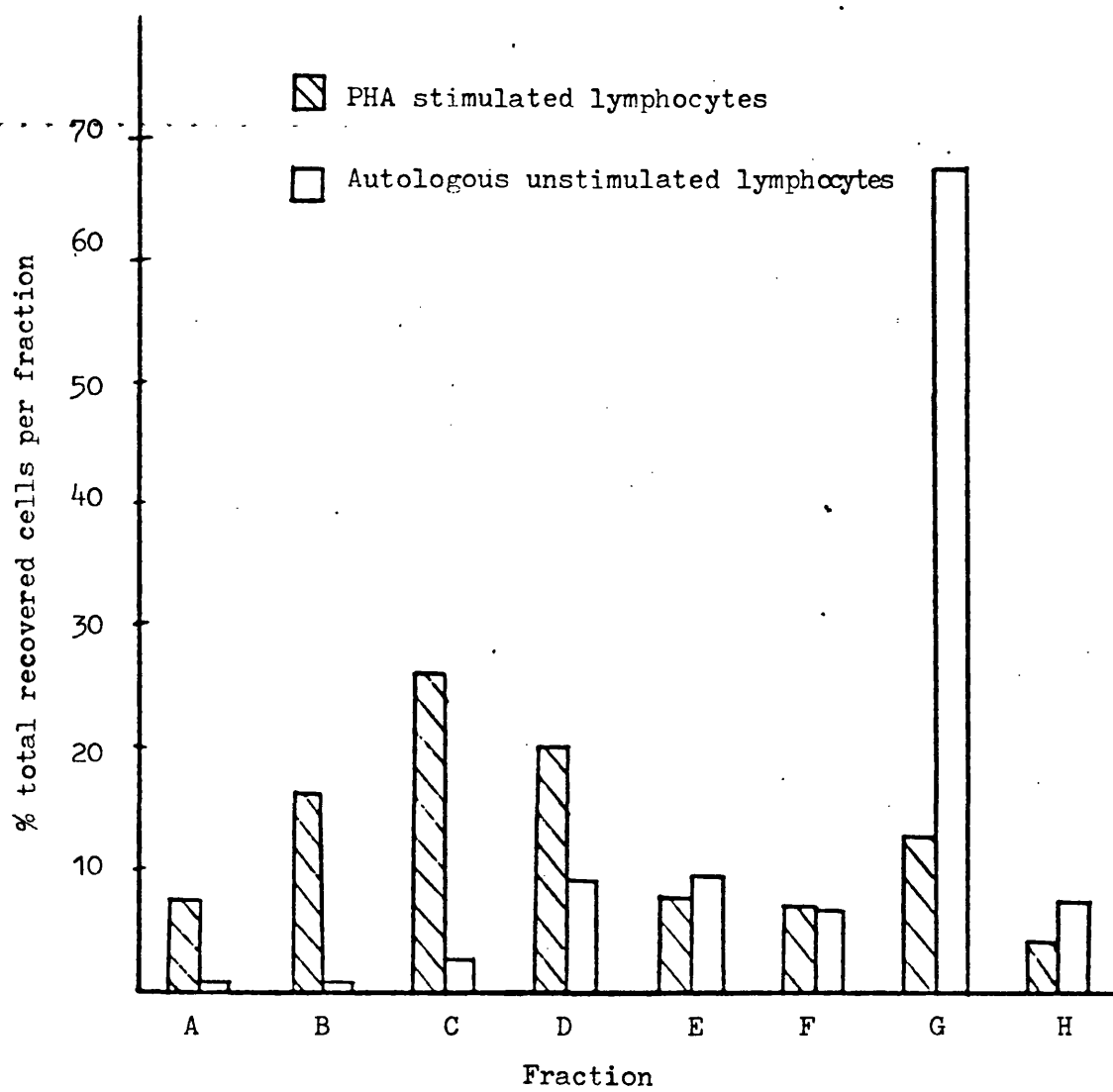
The cytocentrifuged smears were examined after staining with
Wrights stain. Again, a cytocentrifuge was used to prepare the
slides as the shearing forces resulting from a slide over slide
smear cause cell damage particularly to large lymphocytes.
The results of differential counts are shown in Table 3.5. The
larger cells are primarily of low density and the smaller cells
are of high density. The density distribution is related to the
size of the cell, but quantitative measurements were not possible
because although cytocentrifuged slides help to keep cells intact
they are distorted in the process and micrometric measurement
cannot be accurate. The cells were, instead, graded on gross hist-
ology. Plasma cells were not seen in any preparations, neither
were polymorphonuclear leucocytes or monocytes/macrophages.

In this experiment the Ficoll density gradient method described
has been shown to be able to enrich and separate large lymphocytes
from small lymphocytes.

j. Monocytes

Ficoll-Paque and Ficoll/Triosil are good media for separating
lymphocytes from whole blood, almost all red cells and polymorpho-
nuclear leucocytes are removed. However, monocytes are still
present in the lymphocyte preparation and need to be removed
before density gradient analysis. This is because the density
of human monocytes (S.G. ≃1.055) is within the range of lymphocyte
densities, (S.G = 1.050 - 1.080) (Dacie and Lewes, 1963).
Investigations of allograft rejection in mice by Roberts and
Hayry (1977) have shown monocytes to distribute within the
lymphocyte density range but not in the low density fractions.

Table 3.5

DIFFERENTIAL COUNTS OF DENSITY GRADIENT FRACTIONS OF

PHA-STIMULATED LYMPHOCYTES

	Lymphocyte Size		
Fraction	Small (%)	Intermediate (%)	Large (%)
A	2	21	77
B	5	21	74
C	5	28	67
D	12	27	61
E	29	37	34
F	60	29	11
G	86	14	0
H	100	0	0

Elliot, Haskill and Axelrad (1975) have shown that mouse macrophages sediment at a rate (10-15mm/h) within the lymphocyte range as shown by velocity sedimentation. Monocytes are actively phagocytic, a property not shared by lymphocytes. Monocytes also adhere readily to glass, which lymphocytes do not, although B cells adhere to a certain extent. These properties have been used to separate monocytes from lymphocytes in order to purify the lymphocytes prior to density gradient analysis to check on any interference of monocytes in the density profile.

Lymphocytes were obtained from peripheral blood by two means. In the first method carbonyl iron was incubated with whole blood (chapter 2.2b) for 30 minutes at $37^{o}C$. The suspension was then left to sediment on a magnet. The supernatant was applied to Ficoll/Triosil (chapter 2.2b). The lymphocytes were harvested from the interface after centrifugation, washed twice with CMFSS and separated on a Ficoll density gradient.

In the second method lymphocytes were prepared from whole blood solely on Ficoll/Triosil and washed twice with CMFSS. 60% of the cells were pelleted resuspended in 10ml of Eagles medium containing 10% heat inactivated mouse serum and incubated in Falcon flat plastic flasks (50ml capacity) for three hours at $37^{o}C$. The flask was gently swirled and the cell suspension decanted. This contained non-adherent cells. Adherent cells were removed by adding 5ml CMFSS to the flask and gently rubbing the flask's interior surface with a polypropylene-covered spatula prior to decanting. This washing and rubbing was repeated to harvest as many adherent cells as possible. These two populations were spun down, washed twice with CMFSS, resuspended in 16% Ficoll and immediately isolated on Ficoll density gradients. The remaining lymphocytes from the Ficoll/Triosil

separation were resuspended in 16% Ficoll in a glass tube and incubated for 30 minutes at 37°C prior to separation on a Ficoll density gradient.

The four density gradients were centrifuged simultaneously, the fractions harvested, washed and counted. The results are shown in Table 3.6. The adherent cells equilibriated predominantly in fractions A. B. C. and D. The relatively high numbers in fraction G are probably contaminating small lymphocytes which had not been removed in the initial decanting of non-adherent cells. These are probably B cells which are believed to adhere by 'physical' means as opposed to the active adherent process of monocytes. It has been shown that by reducing the temperature to 4°C to inhibit cell metabolism, B cells still adhere, whereas monocytes will not (Shortman et al, 1971). This principle has been used to enrich plaque-forming (Plotz and Talal, 1967) and erythrocyte-binding (Wigzell and Makela, 1970) cells.

The three methods of monocyte depletion produce very similar density gradient profiles on the resulting population. Stained smears from those three populations showed no identifiable monocytes (1,000 cells were examined in each sample).

To confirm this observation a monocyte-specific stain was used, usually referred to as non-specific esterase. The staining is due to the specific classes of enzymes in the cytoplasm of monocytes. The method used was based on the original work of Yam, Li and Crosby (1971).

METHOD

The stain was applied to cytocentrifuged-prepared smears,

table 3.6

MONOCYTE AND LYMPHOCYTE DENSITY DISTRIBUTION

(leucocyte source - healthy subject)

Means of monocyte depletion

Fraction	3 hours incubation in Eagles		30 minutes inc. in 16% Ficoll	carbonyl iron
	Non-adherent	Adherent		
A	0.1	18.2	0.2	0.2
B	0.1	31.8	0.3	0.3
C	1.0	4.5	1.2	0.3
D	2.4	9.1	3.5	1.0
E	8.6	0	6.0	4.0
F	11.6	0	7.8	8.3
G	75.0	27.3	75.0	79.9
H	1.2	9.1	6.0	6.0

Adherent cells $\mp$ 9.6% of total leucocytes

air dried and fixed in buffered formal acetone.

Stain Preparation

A) 50mg. α - napthyl acetate was dissolved in

3ml methoxy ethanol and added to 40ml phosphate

buffer pH 7.6.

B) 1ml 4% Sodium nitrite was added to 1ml

pararosanilin and shaken for one minute.

Solutions A and B were mixed, adjusted to pH 6.2 using

2N HCl, filtered into a coplin jar, the slides immersed for

one hour and then counter-stained with methyl green (1%)

for 45 seconds.

The α- napthyl acetate acts as a substrate for the specific

enzymes and monocytes have a diffused brick red cytoplasm upon staining.

The only other cells which may stain are some T cells which sometimes

have a small dense staining body, but not the characteristic

diffuse staining of the monocyte.

Samples of cell suspensions were tested before and after

adherence and density gradient fractions were also analysed.

(table 3.7). Adherence adequately removed the monocytes, but the

adherent population had only a few contaminating non-myeloid

cells, a point to be remembered as the loss of some cells at

this stage would not have allowed representative density distribu-

tion analyses of lymphocytes. Non-specific esterase staining

of the Ficoll gradient fractions showed that very few monocytes

contaminated the gradients (less than 1% of the cells). As

expected, these few cells which have a high cytoplasm/nucleus

ratio, were found in the low density fractions B and C. The maximum

contamination (3% of fraction B) was regarded as being minimal and

not high enough to distort either the lymphocyte density distribution

Table 3.7

MONOCYTE DEPLETION FROM MONONUCLEAR

CELL SUSPENSION BY ADHERENCE TO GLASS

AND PLASTIC

a) 30 minutes incubated in flask

	Pre-incubation	Non-adherent	Adherent
%NSE +ve*	4	< 1	> 95

b) 30 minutes incubation prior to

Ficoll gradient separation

Fraction	Pre-inc.	A	B	C	D	E	F	G
%NSE +ve* per fraction	5	0	3	<1	0	0	0	0

* NSE +ve = Non-specific esterase staining **positive**

data or the surface marker studies.

Carbonyl iron depletion produced a relative depletion of cells in the low density fractions, C. D. and E. This is a more physically demanding method of separation than adherence and the more fragile cells may well be damaged during the 30 minutes agitation with the carbonyl iron. Large lymphocytes are fragile so this may explain the relative loss of cells from the low density fraction. Incubation with carbonyl iron does not produce such a high lymphocyte yield from blood as Ficoll/Paque or Ficoll/Triosil (chapter 2.).

Adherence, thus, seems to be an adequate means of monocyte removal and 30 minutes at 37°C in 16% Ficoll in a glass tube seems adequately effective.

CHAPTER FOUR

ENUMERATION OF CIRCULATING

ACTIVATED LYMPHOCYTES IN

RHEUMATOID ARTHRITIS

ENUMERATION OF CIRCULATING ACTIVATED LYMPHOCYTES IN

RHEUMATOID ARTHRITIS

4.1 INTRODUCTION

Increased numbers of immunoblasts have been shown in peripheral

blood during infection (Wood and Frenkel, 1967) and immunization

(Crowther, Fairley and Sewell, 1969). Previous workers have shown

increased numbers of immunoblasts in the peripheral blood in R.A.

(Bacon, Crowther and Sewell, 1975), ankylosing spondylitis

(Eghtedari, Davis and Bacon, 1976) and polymyalgia rheumatica

(Eghtedari, Esselinckx and Bacon, 1976). In R.A. the high

incidence of immunoblasts correlated with increased disease

activity and early onset of the disease. No studies on the

effect of drugs on immunoblast numbers in R.A. have been

reported. In almost all cases of lymphoid cell examination the

blood (or a lymphoid cell-enriched preparation) was smeared,

air dried and then differentially stained. Identification of

immunoblasts is relatively easy; they are large (12-25μm diameter)

have a high cytoplasm: nucleus ratio, prominent nucleoli and

intensely basophilic cytoplasm. Occasionally, methyl green/pyronin

has been used to highlight the pyroninophilic cytoplasm, mainly

to eliminate cells of the monocyte/macrophage series.

Enrichment of the mononuclear cell population helps to

prevent confusion of PMN precursors as immunoblasts. Some workers

have removed monocytes/macrophages from mononuclear cell preparations

by using adherence (Panayi, 1976) or by ingestion of iron particles

(Eghtedari, Davis and Bacon, 1976).

DNA synthesis has been used to identify proliferative cells,

usually uptake of tritiated thymidine. However, only about 30%

of immunoblasts are in the DNA synthetic phase of cell growth
(Crowther, Fairley and Sewell, 1969). Autoradiography of smeared
labelled cells is particularly useful for identification of the
proliferating population, while scintillation counting of labelled
cell suspensions is purely quantitative.

Lymphocyte size distribution has been used to compare
healthy controls and patients with immunodeficiency status
(Heyn, Tubergen and Althouse, 1973) and juvenile rheumatoid
arthritis (Brungard, Moser and Althreya, 1975). In both
reports the patients had more large cells than the controls.

Density gradients have been used for lymphocyte fractionation,
the density of small lymphocytes being invariably higher than the
density of larger cells (Gorczynski, Miller and Phillips, 1970).
Systemic lupus erythematosus (SLE) patients peripheral blood lymphocytes
have been compared with healthy controls on Ficoll density gradients
(Glinski et al, 1976). To date, no report has appeared concerning
the density distribution of R.A. peripheral blood lymphocytes.

A study was therefore undertaken to determine immunoblast
numbers, lymphocyte size distribution and density distribution
analysis in rheumatoid patients and to relate these parameters
to disease activity and chrysotherapy.

4.2 MATERIALS AND METHODS

4.2.a. PATIENTS AND CONTROLS

All patients were receiving only analgesic or non-steroidal anti-inflammatory drug (NSAID) treatment unless otherwise stated. Patients receiving gold, penicillamine, cortico-steroid or cytotoxic therapy were excluded, as were all patients with infections. Controls were healthy hospital staff, age and sex matched for R.A. patients wherever possible, particularly for the density gradient analysis.

4.2.b. LYMPHOCYTE PREPARATION AND MICROSCOPIC EXAMINATION

Venous blood was collected and defibrinated or heparinized (Chapter 2.2.a.). Lymphocytes were prepared by separation with methyl cellulose/Carbonyl iron and/or Ficoll-Triosil (Chapter 2.2.b) cell smears were made and differentially stained with Wrights stain (Chapter 2.2.d.) and examined under a binocular microscope.

Immunoblasts,identified as outlined in 4.1, were expressed as a percentage of lymphoid cells, at least 200 cells being counted.

Lymphocyte diameters were measured using an eyepiece graticule which had been calibrated with a stage graticule, 100 cells being measured in each instance.

In any method of purifying lymphocytes from whole blood there is cell loss. More important, there may be a selective loss of certain sub-populations. Johnson and Madsen (1978) found no difference in human lymphocyte sub-populations in heparinized and defibrinated blood. I had a greater total loss of lymphocytes in defibrinated blood and eventually used heparinized blood for all experiments.

Thomson, Bull and Robinson (1966) found that activated lymphocytes were particularly susceptible to damage and death during lymphocyte preparation. Loos and Roos (1974) found a loss of activated lymphocytes from mouse spleen cell preparations when purifying the lymphocytes. This problem was investigated in this study by assaying the loss of mitogen-stimulated cells in a Ficoll-Paque separation so that I could enumerate any loss of activated cells.

Peripheral blood lymphocytes prepared by Ficoll/Paque isopycnic centrifugation (Chapter 2.2.b.) from four healthy donors were incubated with PHA at $1\mu g/ml$ for three days (Chapter 2.2.i.). An aliquot of these cells (1×10^6) was taken for ^3H-thymidine incorporation. The remainder (approx. 10×10^6) were subjected to isopycnic centrifugation on Ficoll-Paque at $2 \times 10^6/ml$. The interface cells were washed and an aliquot (1×10^6) taken for ^3H-thymidine incorporation. The relative incorporation was determined by equal cell numbers before and after Ficoll-Paque purification. The results (Table 4.1) show that although there was a large cell loss associated with Ficoll-Paque separations, this did not cause a relative depletion of proliferating lymphocytes as estimated by ^3H-thymidine incorporation. In fact there was an increase in the incorporation per cell. This is probably due to the decreased cell density of the mitogen stimulated population. The unstimulated cells, which are denser, pass through the Ficoll-Paque and are not recovered. Thus, there is no selective loss of proliferating lymphocytes with Ficoll-Paque. This conclusion has its limitations if extended to the separation of in vivo stimulated blasts from blood. The slight leucoagglutinating activity of the purified PHA may prevent the stimulated cells from passing into the Ficoll-Paque during centrifugation; also the agglutination may be protective

TABLE 4.1

RECOVERY OF PROLIFERATING CELLS AT

FICOLL-PAQUE INTERFACES

Subject	Lymphocyte numbers (10^6)			Radioactivity (^3H) (dpm)		
	Pre F-P	Post F-P	% recovery	Pre F-P	Post F-P	% recovery
PM	7.9	2.1	27	31031	39375	126.9
NM	7.4	3.6	48	31263	39729	127.1
BS	9.7	4.0	41	44813	50850	113.5
JC	11.2	4.2	38	35284	39492	111.9
					Mean	119.9

to the proliferating cells. However, these are imponderables

and it would seem that it is reasonable to use Ficoll-Paque

to prepare samples of lymphocytes which contain activated

cells.

4.2.c. LYMPHOCYTE VOLUME ANALYSIS

Lymphocytes were prepared by isopycnic centrifugation on

Ficoll-Triosil (Chapter 2.2.b2) and monocytes depleted by adherence

at $37^{\circ}C$ for 30 minutes (Chapter 3.3.c.)

Contaminating erythrocytes were removed by repeated washing in

0.83% ammonium chloride. The cells were washed three times with

PBS which had been rendered particle-free by two passages through

0.45μm Millipore filters. During these washings the cells were

pelleted at 200g to reduce contamination by platelets. Cell

viability assessed by dye exclusion was $>95\%$. Cell volume was

determined with a celloscope model B. A probe with a pore diameter of

60μm was immersed in the washed lymphocyte suspension (C. 5×10^4

lymphocytes/ml). The suspension was sucked into the pore and

as each cell passed through the pore it caused a fluctuation

in the movement of the mercury manometer used to draw in the

suspension by negative pressure. The small movement of the

mercury column was recorded as a change of electrical potential

between two electrodes. This was detected by a mercury manometer

and the results were accumulated by a multi- channel data analyser

(Nuclear Data, Reading) and were displayed as a volume distribution

curve on a cathode ray tube. Once a sufficient number of cells

had been measured to produce a large distribution curve this data

was printed on to graph paper via a flat bed pen recorder. Approx-

imately 5,000-8,000 lymphocytes were measured for each sample.

4.2.d DENSITY DISTRIBUTION ANALYSIS

The methodology for this is described in Chapter

3 Section 3. Gradient tubes were run in parallel in

every case, taking as much care as possible in age matching

the control (who was always sex matched) for the patient.

The patients disease activity was clinically assessed by

Dr. P. A. Bacon (Consultant Rheumatologist) as being

broadly "active" or "inactive". A large number of the active

patients were new admissions to the R.N.H.R.D. who had received

no previous "second-line" drugs, i.e. had never had any other

drugs apart from analgesics or NSAIDs. Inactive patients

were those whose disease was well controlled by NSAID or were in

remission.

A number of patients (8) receiving gold therapy were also

used. One patient (P.H) who had not responded to any drug

therapy was subjected to whole body irradiation (500 rad) and

the effect of this treatment on his peripheral blood lymphocytes

density distribution was monitored. A patient with sarcoidosis

was also tested: he had been diagnosed as an early rheumatoid at

the time of testing.
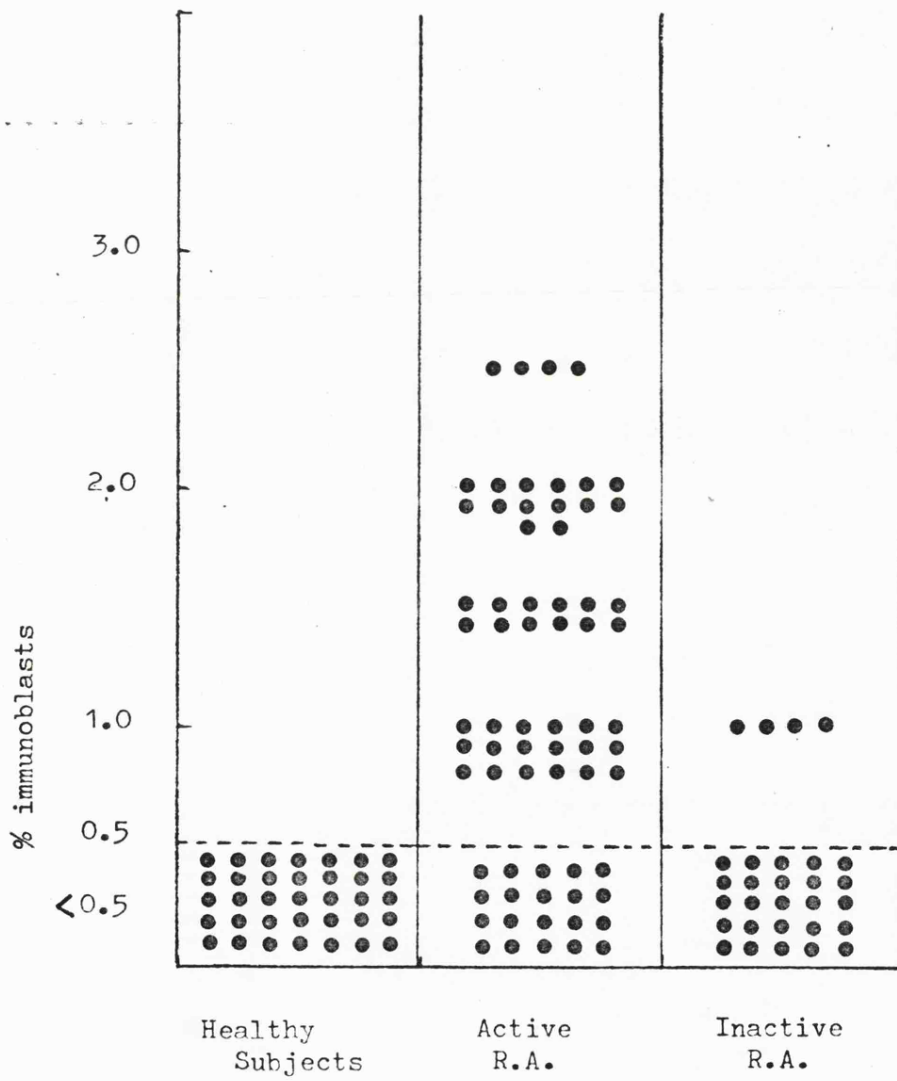
4.3 RESULTS

a) IMMUNOBLAST NUMBERS

Lymphoid cells with the morphology of immunoblasts

in my preparations were rarely of the great size reported by

other authors (Horwitz et al, 1970; Delbarre et al, 1975).

Many lymphoid cells which had the cytological characteristics of

immunoblasts were only 10μm diameter, cells above 20μm were only

seen in a few exceptional cases, even in patients with chest

infections and infectious mononucleosis. Nearly all authors

characterize immunoblasts as being above 15μm diameter, in fact

Delbarre et al (1975) judged immunoblasts to be 20-30μm diameter.

In this study immunoblasts were defined as being above 10μm and poss-

essing a kidney-shaped, eccentrically placed nucleus with prominent

nucleoli, a high cytoplasm/nucleus ratio and hyperbasophilic

cytoplasm. However, to include an analysis of increased cell

size, lymphocyte diameters were measured (see next Section).

During the preparation of cell smears surface tension can result

in large cells being carried towards the edges and tail of the

smear. Thus, when examining the stained smears, each was system-

atically scanned to ensure that the data was truly representative.

The percent incidence of immunoblasts in controls and active and

inactive R.A. is shown in Fig. 4.1. Increased immunoblasts are shown

predominantly in active disease with more than half of these patients

having immunoblasts raised above the normal level (0.5%). Only

four of 29 patients with inactive R.A. had raised levels of

immunoblasts and then only to 1% of the lymphoid cell population.

Fig. 4.1

The incidence of immunoblasts

in the peripheral blood lymphoid population

of healthy and rheumatoid subjects

Peripheral blood lymphocytes were prepared from

10 ml venous blood (chapter 2.2.b.ii) and stained

smears examined microscopically. The immunoblast

was recorded as a mononuclear cell about 10μ diameter

with a large eccentrically placed, kidney-shaped nucleus which

possessed two or more nucleoli. A high cytoplasm: nucleus

ratio and hyperbasophilic cytoplasm were also used as

positive criteria. The results are expressed with the

immunoblasts as a percentage of the lymphocyte population.

Each point is for one subject.

Fig. 4.1.

b) LYMPHOCYTE DIAMETERS

The eyepiece graticule allowed accuracy of 0.7μm in the measurement of lymphocyte diameters. Again, the slides were systematically scanned and only intact cells were measured. The results are shown in Fig. 4.2.a. In both rheumatoid (22 patients) and healthy subjects (17patients) most lymphocytes (90%) are in the normal range. The main difference between the two subject groups is at the high end of the range (4.2.b). R.A. patients have more lymphocytes above 10μm diameter, 3.1% compared with 0.7% of 11μm diameter for healthy subjects. Healthy subjects did not have lymphocytes above this size whereas R.A. patients had 0.75% circulating lymphocytes of 11.8μm diameter. Lymphocytes above 12.5μm were exceptional (<0.1%).

The results do not show a great increase in the numbers of large lymphocytes in R.A. However, this method of measurement is long and subjective and measures cell volume as found on a stained, dried smear. As a result distortion occurs and the larger cells in R.A. did not always have the morphological characteristics of blasts.

c) ELECTRONIC SIZING

The equipment employed (a celloscope) measures the volume of cells in suspension. For this experiment it was assumed that lymphocytes in a physiological medium are spherical. Apart from measuring more cells than can practically be measured by microscopy , this system measured the cells alive and undistorted by the various forces inherent in the making of cell smears. The number of lymphocytes measured is indeterminate and hence renders statistical analysis diff-icult. For this reason the y axis is marked as percentage peak height.

A total of eight R.A. patients and seven healthy subjects were used in this experiment. The results shown in Fig. 4.3. are representative of five of the six patients with active R.A. In the other three patients the distribution profiles for R.A. and control were similar. The abnormal pattern shown for the R.A. patient shows an increase in the number of cells of above average volume. In fact, up to 10% of the peak number are found in channels 265-293. This pattern of results is similar to the more qualitative figures shown in Fig. 4.2.

The pattern of lymphocyte size was not considered to demonstrate an adequate number of large lymphocytes to permit their separation by velocity sedimentation. With the knowledge that lymphocyte density is changed before a significant increase in cell size is recorded (Steinman et al, 1978) the separation of lymphocytes by density was performed.

d) LYMPHOCYTE DENSITY DISTRIBUTION

The results of a comparison between 23 healthy subjects and 23 R.A. patients are shown in Fig. 4.4. In healthy subjects most of the lymphocytes (67%) were found in the dense fraction (G), with decreasing incidence in the less dense fractions.

Compared with healthy subjects the R.A. patients selected from the whole spectrum of clinical activity, had a depletion of dense cells and increased numbers of low density cells. Fraction G contained only 50.5% of the lymphocytes in R.A. by comparison with 67% in healthy subjects. Fraction F contained 21% and 11%, fraction E 12.5% and 8%, fraction D 6.5% and 4% and fraction C 4% and 3% respectively.

Fig. 4.2

The mean diameter of peripheral blood lymphocytes

in healthy and rheumatoid subjects

Peripheral blood lymphocytes were prepared from venous

blood (Chapter 2.2.b. ii) and stained smears examined microscop-

ically. 100 lymphocyte diameters were determined with an eye-

piece graticule whilst systematically scanning the slide.

The results are expressed as percentage lymphocytes

of a given diameter.

a) shows the full range of results

b) highlights the difference between R.A. and healthy

subjects at the higher end of the range.

Fig. 4.2.a

Fig. 4.2.b

Fig. 4.2.b

A graph with y-axis labelled "% lymphocytes" (values 5, 10, 15) and x-axis labelled "mean diameter (μm)" (values 10, 11, 12). Legend: ● R.A., ○ Healthy subjects.
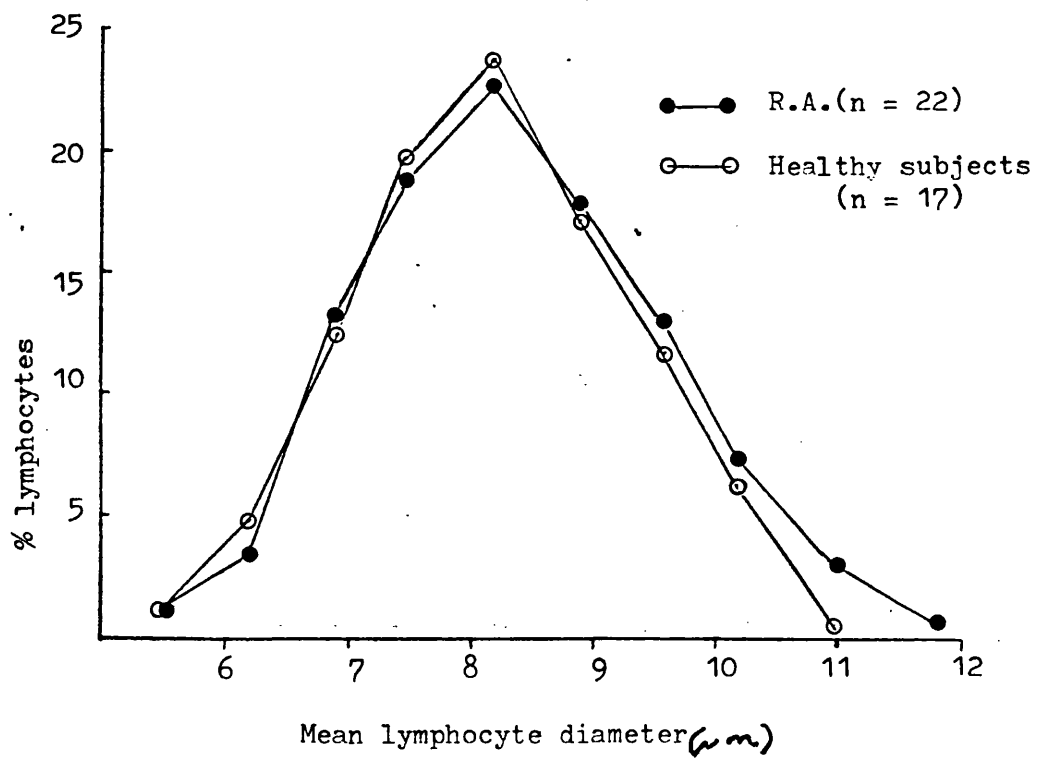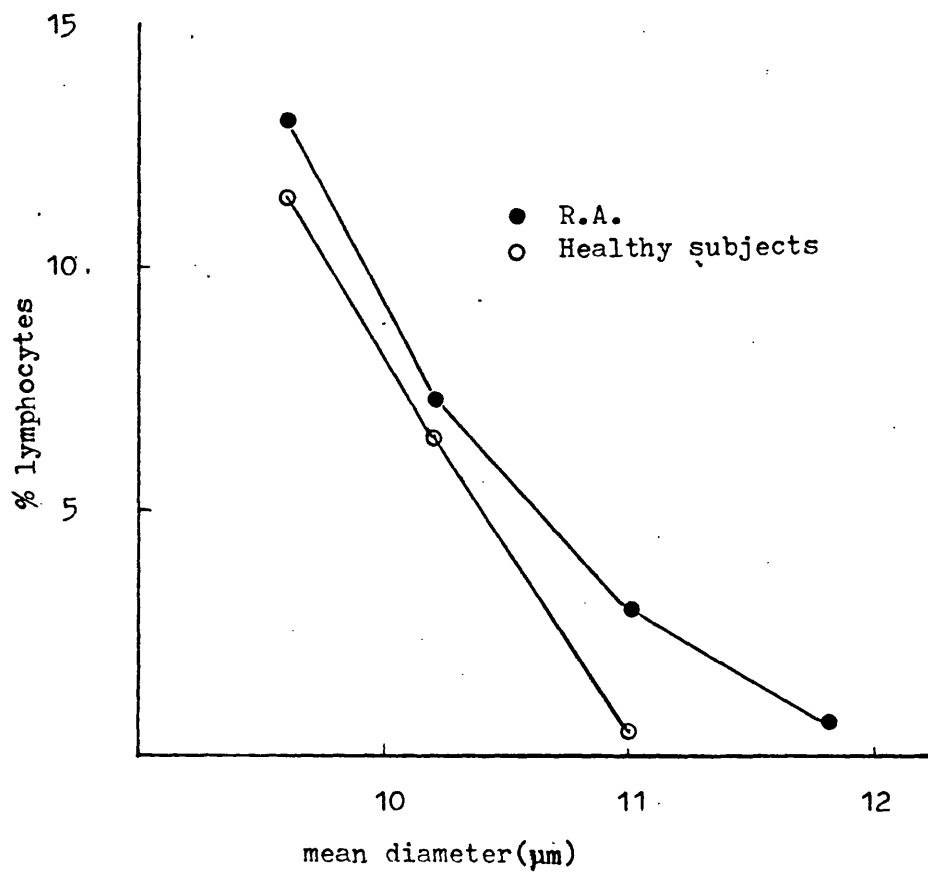
Fig. 4.3

Lymphocyte volumes of healthy and

R.A. subjects (measured with a Celloscope).

Lymphocytes were prepared from peripheral blood

(Chapter 2.2.b.ii) and washed twice with filtered

PBS. Cell volume was determined using a Celloscope (model

B) (4.2c). The results are expressed as percentage

of peak height in each channel.

Fig. 4.3.

Fig. 4.3.
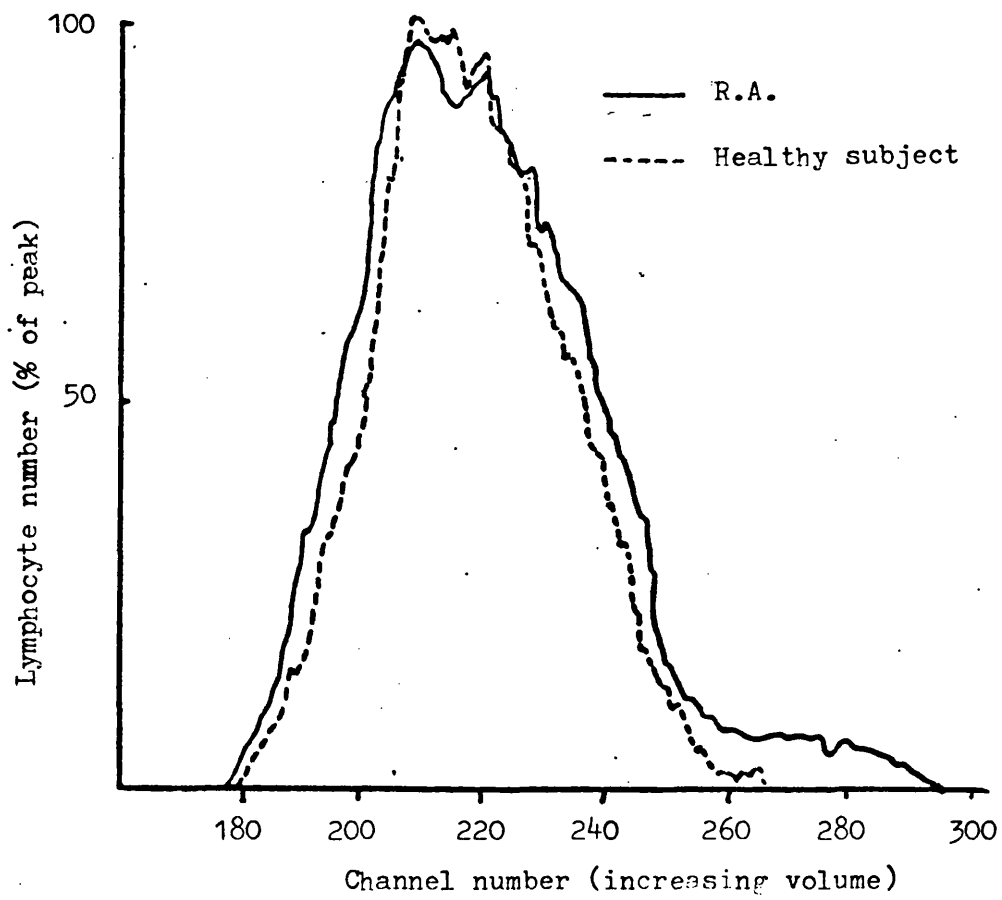
Fig. 4.4

Density distribution profiles of peripheral blood

lymphocytes from healthy and R.A. subjects

Peripheral blood lymphocytes were prepared from

30 ml venous blood (chapter 2.2.b.ii). Monocytes were depleted

(Chapter 2.3.c.) and the lymphocytes separated by density

in a Ficoll gradient. The fractions were harvested and counted

(Chapter 3.3.d.). R.A. patients were analysed in parallel

with age and sex matched healthy subjects. The results are

expressed as the mean percentage of total recovered lymphocytes

in each fraction $\pm$ 1 standard deviation (S.D.).

The differences between R.A. and healthy subjects were

statistically significant (Student t-test) in the following

fractions:

Fraction	p
A	< 0.02
B	NS
C	< 0.01
D	< 0.001
E	< 0.01
F	< 0.001
G	< 0.001
H	NS

Fig. 4.4.
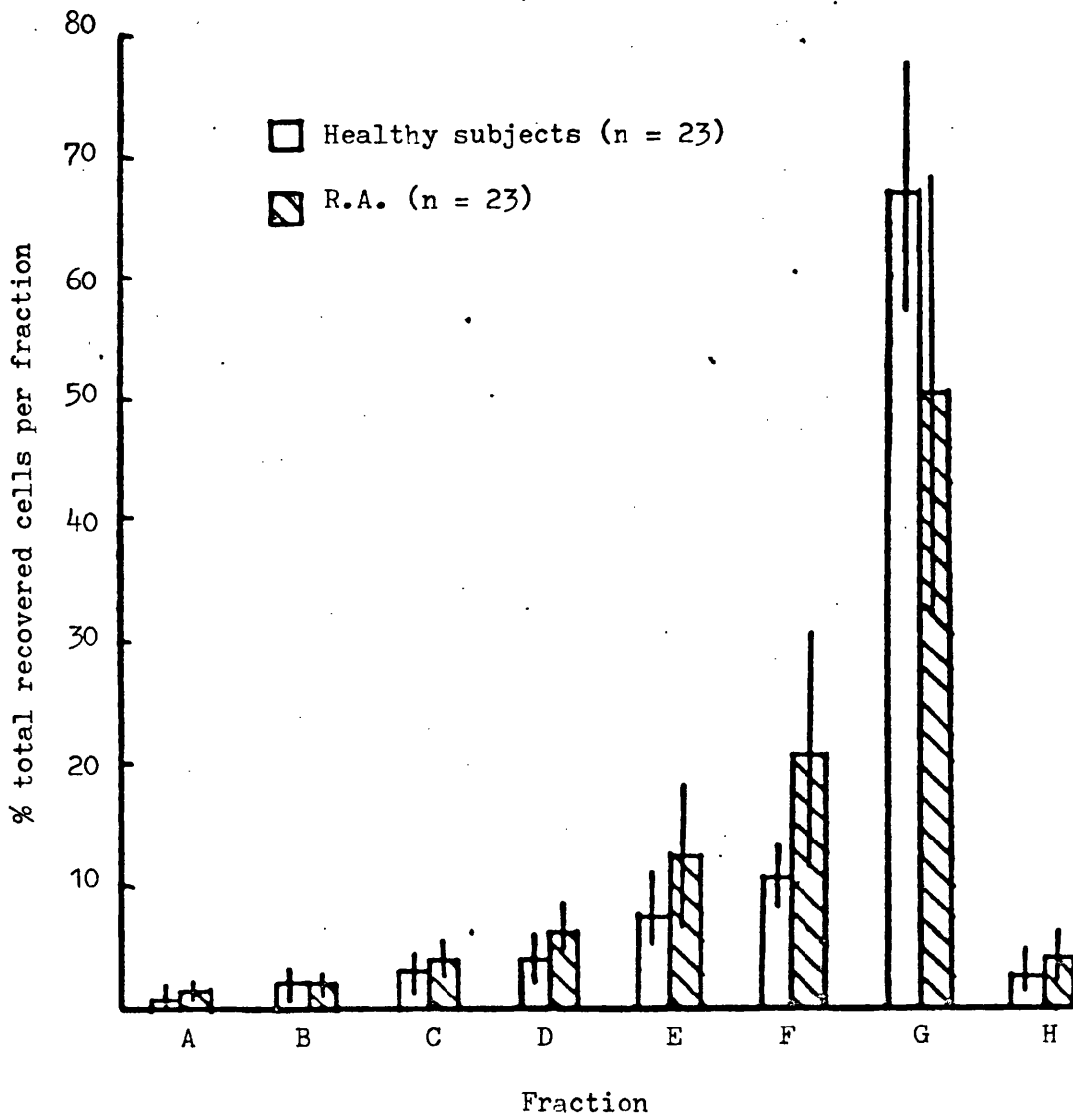
The proportions in fractions A and B were similar for R.A.
and healthy subjects. The differences between R.A. and
healthy lymphocyte percentages were highly significant in
fractions D, E, F and G (P $<$ 0.001).

Upon comparing a separate group of patients with clinically
active R.A. (n=11) with healthy subjects (n=23) the
results were more strikingly different (Fig. 4.5). The number
of lymphocytes in fraction G was further reduced to 37%
and fractions C, D, E and F increased to 5.5%, 8.0%, 16.0%
and 27.5% respectively. From this result it can be seen
that it is mainly the active patients who have the increased
number of low density cells. The comparisons between active R.A.
and healthy subjects are highly significant in fractions C,
D, E, F and G (P $<$ 0.001).

The density distribution profiles of a separate group of
12 clinically inactive patients peripheral blood lymphocytes
are shown in Fig. 4.b and shown to be dissimilar to patients
with active R.A. In fact fraction G contains 63% of the
lymphocytes, almost as many as in healthy subjects (67%).
The fractions E and F each contain less than 15% of the lymphocytes
and C and D are less than 5% each of the total. The
differences with clinically active patients are significant
in fractions D (P $<$ 0.01), C and E (P $<$ 0.02) and F, G and H
(P $<$ 0.001).

Conversely, eight patients receiving gold therapy had a mean
density distribution pattern similar to that for healthy subjects

Fig. 4.5

Density distribution profiles of peripheral

blood lymphocytes from healthy subjects

and clinically active R.A. patients.

Peripheral blood lymphocytes from healthy subjects

and clinically active R.N. patients were separated by density

as described in the legend to Fig. 4.4 and the results

presented accordingly.

The differences between clinically active R.A. and healthy

subjects were significant in the following fractions:

Fraction	p
A	NS
B	NS
C	< 0.001
D	< 0.001
E	< 0.001
F	< 0.001
G	< 0.001
H	NS

Fig. 4.5.

□ Healthy subjects (n = 23)

▧ Active R.A. (n = 11)

% total recovered cells per fraction

80

70

60

50

40

30

20

10

A   B   C   D   E   F   G   H

Fraction
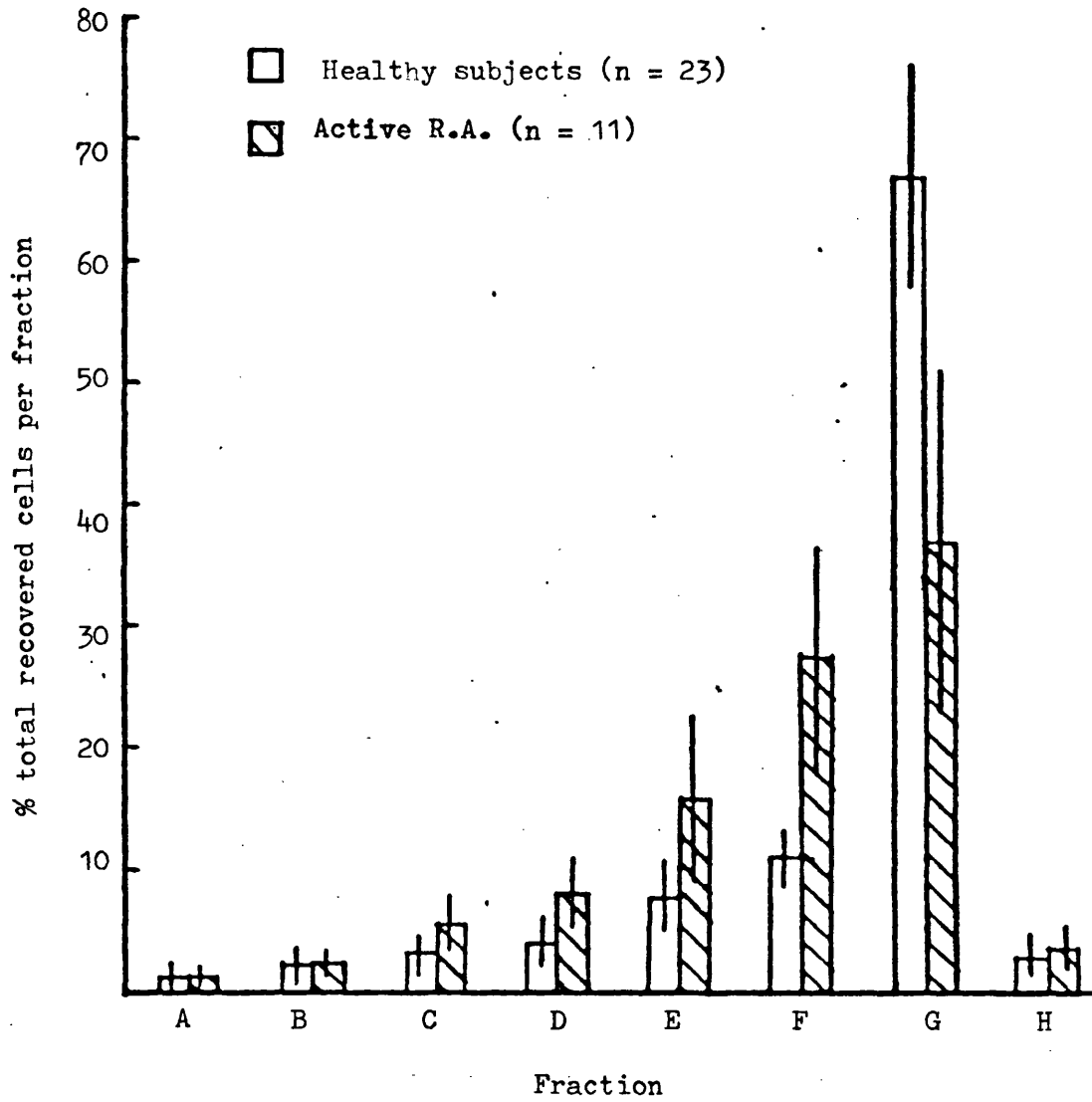
Fig. 4.6   Density distribution profiles of peripheral

blood lymphocytes from R.A. patients with

clinically active and inactive disease.

The lymphocytes were prepared and separated by density

as described in the legend to Fig. 4.4. The results are

presented in the same format, as Fig. 4.4, and statistically

significant differences between the patient groups were

recorded as follows:

Fraction	p
A	NS
B	NS
C	< 0.02
D	NS
E	< 0.02
F	< 0.001
G	< 0.001
H	< 0.001

Fig. 4.6.
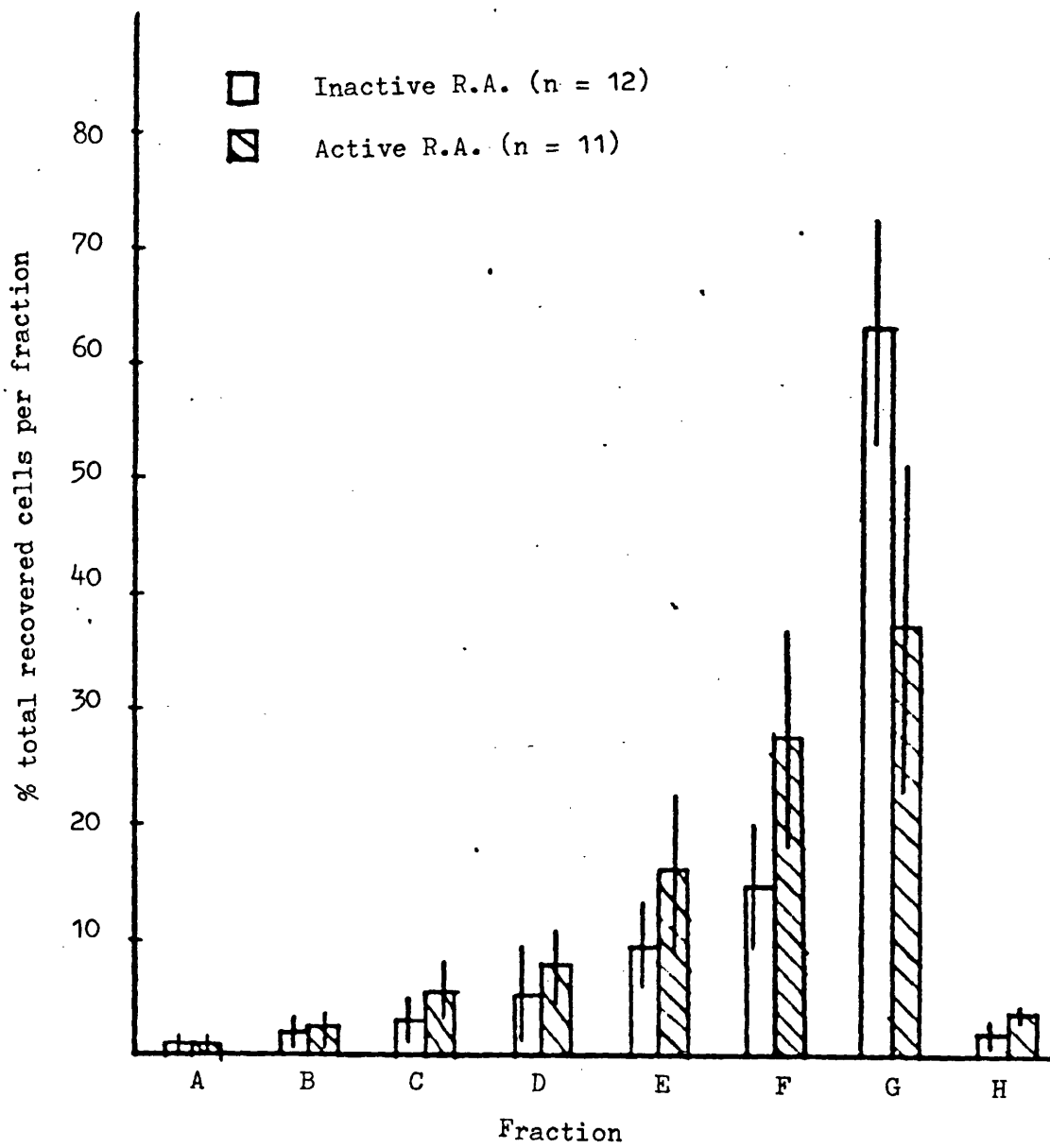
(Fig. 4.7). Differences between the fractions for the

two subject groups were not significant. Compared with

active R.A. patients (Fig. 4.8), the patients on gold

had fewer cells in fractions C and D (5%) E (7%) and F

(12.5%). Fraction G contained 68% of the lymphocytes - a very

similar figure to the healthy value (67%).

Total body irradiation of a clinically active R.A.

patient (P.H) in two half-body treatments caused a shift of the

lymphocyte density distribution profiles towards the low density

fractions (Table 4.2). The peak of lymphocytes on day 0

(48%) was in fraction G, but at three days (two days post-

irradiation) was in fraction F (78.3%) and after the

second irradiation, at 14 days, was in fraction E (48.7%). At

31 days and 93 days (17 and 79 days after the second irradiation)

the distribution profile was shown to be slowly resuming to that

recorded before treatment, at 93 days the peak (59.1%) being

in fraction F.

The patient with sarcoidosis had the most abnormal density

distribution profile of the study. The majority of the lymphocytes

(58%) were found in fraction E in contrast with only 9% for

healthy subjects. Fraction G was reduced to 5% of the total compared

with 67% for healthy subjects. Fractions C and D had increased cell

numbers relative to healthy subjects (Fig. 4.9.).

Fig. 4.7

Density distribution profiles of peripheral

blood lymphocytes of healthy subjects and

R.A. patients receiving gold therapy.

The lymphocytes were prepared and separated by density

as described in the legend to Fig. 4.4 and the results

presented in the same format. There was no statistically

significant difference between the two subject groups.

Fig. 4.7.

Legend:
- ☐ Healthy subjects (n = 23)
- ◩ R.A. on gold (n = 8)
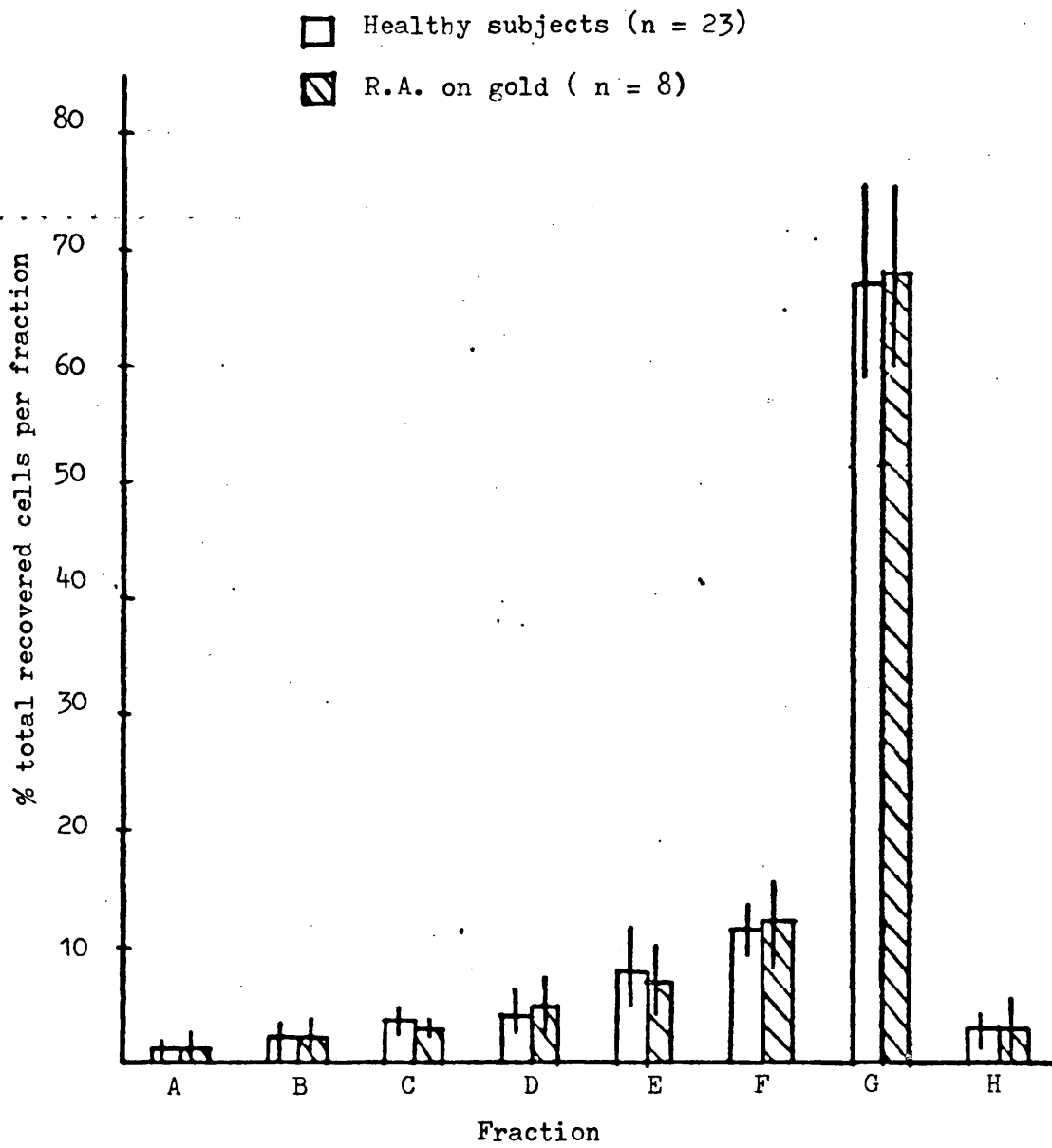
Fig. 4.8

Density distribution profiles of peripheral

blood lymphocytes from R.A. patients with

clinically active disease or receiving gold

therapy.

The lymphocytes were prepared and separated by density
as described in the legend to Fig. 4.4. The results are
expressed in a similar manner and statistically significant
results were shown in the following fractions:

Fraction	p
A	NS
B	NS
C	< 0.05
D	< 0.05
E	< 0.01
F	< 0.001
G	< 0.001
H	NS

Fig. 4.8.
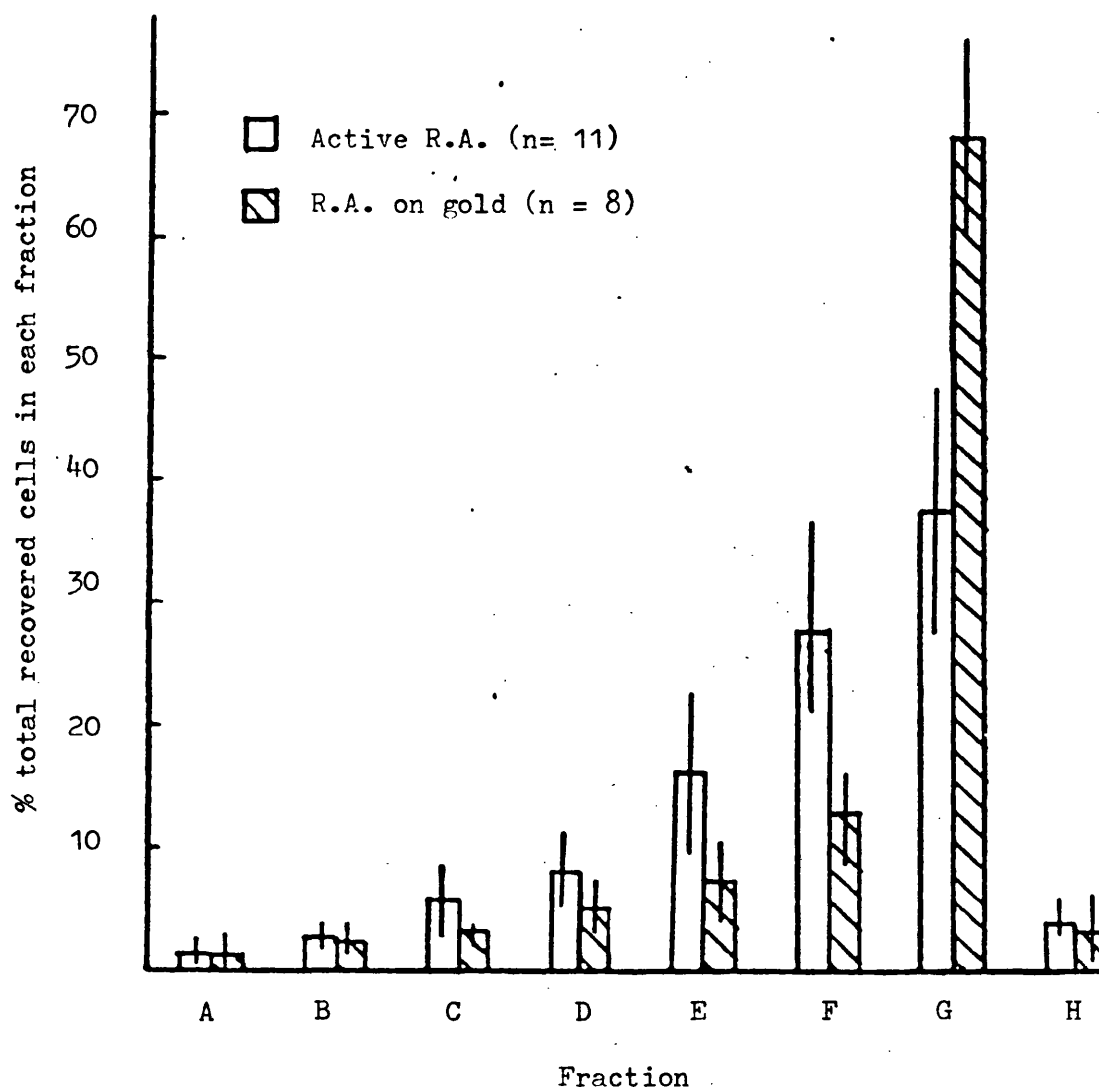
TABLE 4.2

DENSITY DISTRIBUTION PROFILES OF A CLINICALLY

ACTIVE RHEUMATOID ARTHRITIS PATIENT (P.H.). THE EFFECT OF

WHOLE BODY IRRADIATION.

		Days Post-irradiation			
Fraction	0	3	19	31	93
A	0.8	0.5	0.4	0	0.2
B	0.7	1.1	2.1	0.7	0.7
C	2.5	1.4	6.3	4.4	0.7
D	6.2	1.6	28.4	9.9	1.0
E	14.8	10.5	48.7	37.8	2.0
F	22.3	78.3	11.1	34.6	59.1
G	48.5	5.5	2.8	12.7	35.5
H	4.1	1.1	0.2	0	0.8

Two half body irradiations (500 rad) on day 1 and day 14.

Figures = % total recovered cells in

each fraction

Fig. 4.9

Density distribution profile of a patient

with sarcoidosis. Comparison with healthy

subjects.

The peripheral blood lymphocytes were prepared from

venous blood and separated by density as in the legend

to Fig. 4.4

Fig. 4.9.
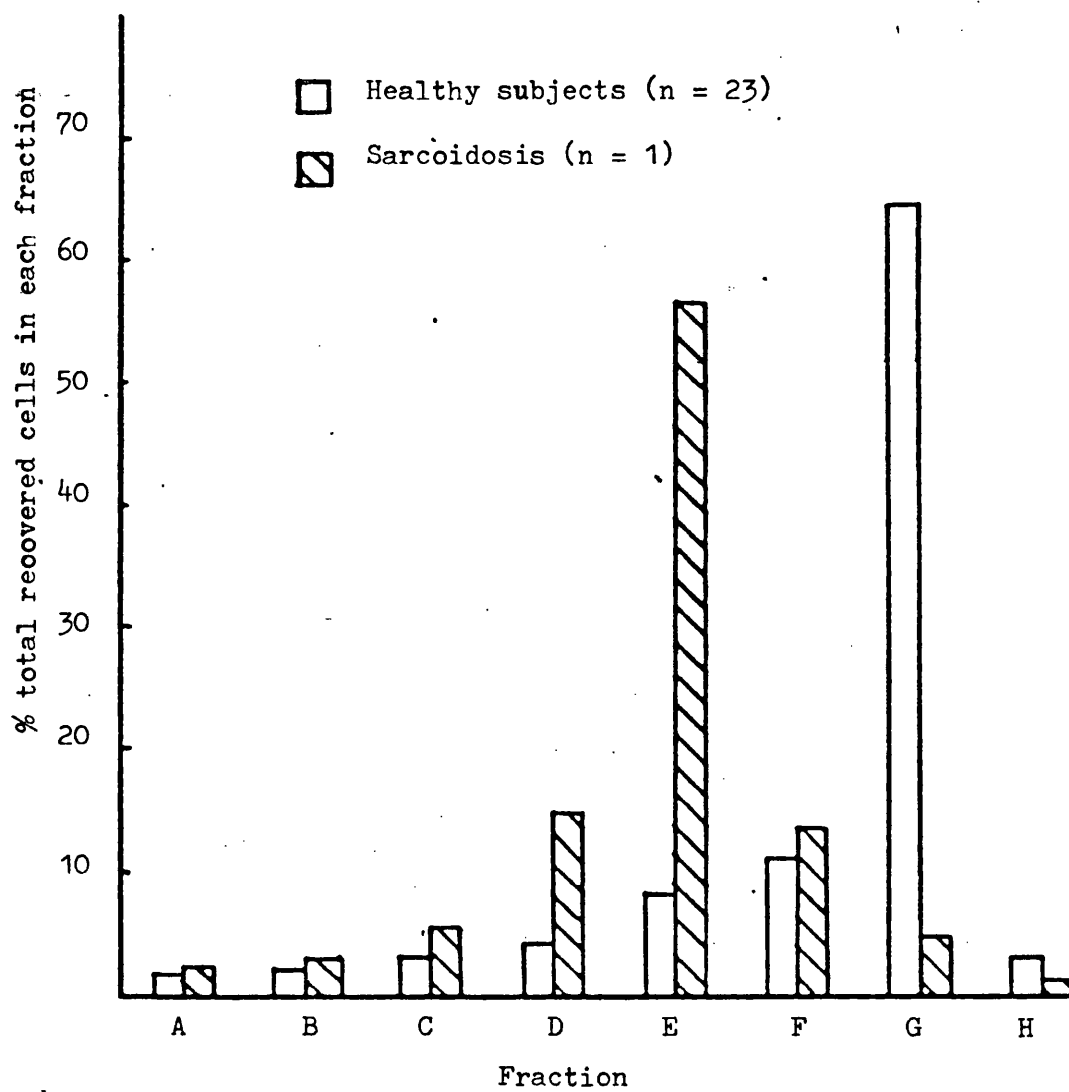
4.4 DISCUSSION

The activation of peripheral blood lymphocytes investigated

in this chapter will be discussed in relation to the surface

marker studies in Chapter 6. This discussion will deal only

with the results of the patient who was subjected to whole body

irradiation and the patient with sarcoidosis.

Whole body irradiation resulted in an increase in the numbers

of low density lymphocytes from two days up to 18 days post-

irradiation. Bond et al (1961) found a large decrease in the

percent DNA synthesizing cells 24 hours after irradiation of

man at a similar level (500 rads). However, the number of

those proliferating cells were increased above the normal level

from two days onwards and were still supra-normal after 50

days. Normal levels were attained at 141 days. These workers

also found a drop in DNA synthesizing cells in irradiated dogs

for 24 hours, followed by a sharp increase which peaked

after seven days and had returned to normal levels after 14 days.

The mitotic index of bone marrow also rises after an initial

fall (Fleidner et al, 1959). For irradiation to have such a lasting

effect, as shown by Bond et al (1961) and in this study, more than

circulating cells were being affected. The bone marrow is certainly

affected and the increased numbers of the proliferating population

and the low density population may be due to increased bone

marrow output. Edgren et al (1976) showed that T lymphocytes,

irradiated extra-carporealy, were destroyed or incorporated into

the intravascular lymphoid pool upon re-infusion. This was

also reflected by a decrease in the number of circulating T cells,

suggesting that T cells are more radiosensitive than B cells.

Whilst the density distribution profile was not, in this study, determined within 24 hours of irradiation, it is the proliferating population of lymphocytes which are believed to be the most radiosensitive (Waithe and Hirschorn, 1973). However, the shift in the densities of the circulating lymphocytes is such that this cannot be totally explained by an increase in bone marrow output. The change in density is more sensitive than any noted change in lymphoid morphology with respect to activation.

Sarcoidosis gave the most abnormal density distribution profile found in any disease state analysed in this study. This disease is characterized by a hypereactivity of the antibody producing system and a depression of delayed hypersensitivity. Both of these abnormalities could be due to antigen induced activation of both populations (Cole, 1977), an antigen having been found in sarcoid spleen (Kvein, 1941). Polyclonal activation could explain the density distribution profile.

CHAPTER FIVE

THE NATURE OF THE LYMPHOCYTE

SUB-POPULATIONS SEPARABLE BY

DENSITY IN HEALTH AND R.A.

5.1   INTRODUCTION

The previous chapter has shown a significant difference in the density distribution profiles of circulating lymphocytes from healthy and rheumatoid subjects. This difference was greater than the size differences shown by microscopy and electronic sizing, particularly for clinically active R.A. patients. Steinman et al (1978) believe that a lowering of density is one of the first indications of lymphocyte activation, which is reflected in an increased cytoplasmic refractive index (Metcalf et al, 1976). The preparative properties of discontinuous density gradient analysis allow further studies on the nature of the lymphocytes in the various fractions.

If the increased numbers of less dense lymphocytes shown in R.A. (Chapter 4) are activated cells it would be of great importance to find out their origin and function. Surface marker studies may help to do this, and this has been examined here.

Some lymphocyte sub populations retain their markers when activated, some may lose them. The literature shows that T cells do not lose their receptors for sheep erythrocytes upon activation and B cells do not lose integral surface Ig, but lose their C3 receptors and possibly their Fc receptors also (Jondal, 1974 b, c; Miyama et al, 1978). Conversely, activated T cells may acquire Fc receptors (Van Boxel and Rosenstreich, 1974), notably suppressor T cells (Moretta et al, 1977). The expression of surface markers by newly activated lymphocytes is unknown. Preparative continuous Ficoll gradient analysis allows examination of any such changes as a result of activation.

The data for T and B cell numbers in peripheral blood in R.A.
is equivocal (Hoyerall, Froland and Wisloff, 1975; Brenner, Schein-
berg and Cathcart, 1975; Froland, Natvig and Wisloff, 1975).
The variations in results may well be due to technical differences
in the assaying of both T and B lymphocytes.

Recent evidence has shown an increase in EA- rosette formation
by circulating lymphocytes in rheumatoid arthritis (Sharpin and
Wilson, 1977 a). This has been shown to be due to the binding
of the Fc region of IgG, which is not due to cell bound rheumatoid
factor. The enhanced EA- rosette formation by peripheral blood
lymphocytes from R.A. patients occurred through a range of concentra-
tions for sensitizing antibody, suggesting that the Fc receptors
on R.A. lymphocytes are more avid for EA than receptors on
lymphocytes from healthy subjects (Sharpin and Wilson, 1977 b). A
similar EA- rosette system, using sensitized rabbit (Fawaz-
Duhaut et al, 1975) or human erythrocytes (Bach, Delrieu and Delbarre,
1970; Sany et al, 1975) has detected increased EA- rosette formation
in R.A. A number of authors (Williams et al, 1973; Brenner,
Scheinberg and Cathcart, 1975) have commented on the increased
numbers of circulating non T, non B (null) cells in R.A.

This chapter describes the results of studies designed to
characterize the lymphocyte surface markers in Ficoll density gradient
fractions. In both health and R.A. clear trends were shown in the
incidence of surface markers within the lymphocyte density range.

5.2 MATERIALS AND METHODS

a) DENSITY DISTRIBUTION OF ENRICHED SUB-POPULATIONS

i) DENSITY DISTRIBUTION OF E-ROSETTE DEPLETED CELLS

T cells were depleted from the peripheral blood lymphocyte population of four rheumatoid patients as described in chapter 2.2e and the remaining cells subjected to Ficoll density gradient analysis (Chapter 3.2), in a comparison with autologous unseparated lymphocytes.

ii) DENSITY DISTRIBUTION OF EAC-ROSETTE DEPLETED CELLS

Cells bearing receptors for the C3 component of complement were depleted from the peripheral blood lymphocyte population of one R.A. patient by EAC- rosette depletion (Chapter 2.2g). The remaining cells were separated in a Ficoll density gradient (Chapter 3.2) in a comparison with autologous unseparated lymphocytes.

b) SURFACE MARKERS ON LYMPHOCYTES IN FICOLL DENSITY
GRADIENT FRACTIONS

Peripheral blood lymphocytes from R.A. patients and age and sex matched healthy subjects were fractionated, in parallel, in Ficoll density gradients (Chapter 3.2). The cells from each fraction were counted and washed three times with CMFSS.

i) E- ROSETTES

The lymphocytes from each fraction were pelleted and adjusted to 2×10^6/ml using PBS. Equal aliquots (0.2ml) of lymphocytes and washed sheep erythrocytes were mixed and the percentage E- rosettes determined (Chapter 2.2e).

ii) EA- ROSETTES

The lymphocytes from each fraction were adjusted to

2×10^6 with CMFSS. Ox red blood cells were washed, coated with IgG antibody and rewashed and mixed with the lymphocytes to form EA- rosettes as described in Chapter 2.2f.

The concentration of coating antibody to be used in these experiments was determined by a number of titration experiments. The range of concentrations used was 1/200, 1/400, 1/800, 1/1600 1/3200. The highest concentration. (1/200) was the strongest sub-agglutinating concentration. Whole lymphoid populations, both healthy and R.A., were tested in this way. From this range of titres two concentrations were chosen with the aim of determining high avidity EA- rosette formation and low + high avidity EA- rosette formation. These two concentrations were used for the determination of EA- rosette formation in Ficoll density gradients.

iii) SURFACE IMMUNOGLOBULIN

Surface immunoglobulin was assayed using fluorescein isothiocyanate (FITC) conjugated rabbit anti-human Ig as described in Chapter 2.2.h. To simplify the labelling and washing of large numbers of cell fractions the whole mononuclear cell population prepared on Ficoll/Paque was labelled with the FITC anti-Ig for 30 minutes at 4°C. However, only as much of the labelled conjugate was added to 30×10^6 lymphocytes as usually added to 1×10^6 cells. To assay the validity of this approach doubling dilutions of a lymphocyte suspension were prepared in PBS, pelleted at 200g and 0.2ml of the conjugate (1:5 in PBS) added to each pellet, mixed and incubated for 30 minutes at 4°C before reading under a UV microscope. The results (table 5.1) show that cell number does not appear to affect the efficiency of staining with the labelled conjugate.

TABLE 5.1

THE EFFECT OF CELL NUMBER ON THE LABELLING

OF LYMPHOCYTES WITH FITC- ANTI HUMAN Ig.

Cell number $(X10^6)$	% Surface Ig positive
1	19
2	20
4	21
8	20
16	23
32	22

The lymphocytes were thus labelled before density gradient fractionation (chapter 3.2). The number of cells per fraction was determined and then the cells were pelleted for estimation of percent FITC- labelled cells in each fraction.

5.3 RESULTS

The R.A. patients examined in this chapter included

the whole range of disease activity, patients with the more

clinically active disease being chosen where possible.

a) ROSETTE DEPLETION

i) E- ROSETTE DEPLETION

The effect of E- rosette depletion on the lymphocyte

density distribution profiles of four R.A. patients is shown in

Fig. 5.1. In comparison with the autologous whole lymphoid

population the E- rosette depleted population had a decrease

in the number of dense cells and a proportional increase in the

number of less dense cells. The E- rosette depleted population

had a reduction of lymphocyte percentage from 52.5% to 21.5%

in fraction G. Fraction F was reduced from 23% to 11%. The profile

of E- rosette depleted cells showed a shift to the left and the peak

was found in fraction E (28%), compared with the non E- rosette

depleted cells. Fractions A, B, C and D, were also proportionally

increased, fraction D being the most affected by the depletion,

with the percentage of cells in this fraction rising from 6%

to 20%. The differences in fractions B to G are highly significant.

(C, D, E: $P < 0.02$; B, F, G: $P < 0.01$).

The inference from these results is that the low density R.A.

lymphocytes in the Ficoll density gradient are predominantly non-T.

The depletion of lymphocytes from Fraction G was great, only 21.5% of

the remaining lymphocytes were found in this fraction. This may be

a result of a loss of denser lymphocytes on reapplication to a

second Ficoll-Paque separation, the step used to remove E- rosetted

Fig. 5.1.

The effect of depletion of E- rosetting

cells on the density distribution profiles

of peripheral blood lymphocytes in R.A.

60 ml of venous blood was taken and the lymphocytes separated

(Chapter 2.2.b.ii). The E- rosetting cells were depleted

from half of the lymphocyte sample (Chapter 2.2.e.) and the

non E- rosetting cells (from the Ficoll/Paque interface)

and the whole lymphoid population were separated in parallel

Ficoll gradients (Chapter 3.3.d.).

The gradient interfaces were harvested, counted and

the results expressed as a percentage of the total recovered

lymphocytes in each fraction.

The differences between the separated samples were

statistically significant (Students t-test) in the following

fractions:

Fraction	p
A	0.002
B	0.01
C	0.02
D	0.02
E	0.02
F	0.01
G	0.01
H	NS

Fig. 5.1

☐ R.A. (E- rosette depleted) (n = 4)

▨ R.A. (Whole lymphocyte pop^n.) (n = 4)

% total recovered cells per fraction

60

50

40

30

20

10

A   B   C   D   E   F   G   H

Fraction

lymphocytes. This possibility may be overcome by the direct

testing of density gradient fractions for E- rosette formation.

However, the rapidity of the former method allowed relatively

quick assessment of the surface markers in the density gradient

fractions; a relevant point considering the fragility of large

lymphocytes.

ii) EAC- ROSETTE DEPLETION

The effect of EAC- rosette depletion on the

lymphocyte density distribution of one R.A. patient is shown

in Fig. 5.2. By comparison with autologous unseparated lymphocytes

there was a depletion of lymphocytes from fractions F, G and H.

Quantitation of this can only be of limited value as only one

patient was tested. The implications are that relatively few of

the low density lymphocytes have receptors for the C3 component

of the complement. Conversely, many of the cells with C3 receptors

would be found in fractions F and G. Due to the need to use two

Ficoll-Paque separations, as described in the previous section,

this approach was discarded. The relative lack of C3 receptors

on activated B cells also reduced the value of this data to

some extent.

b) DIRECT SURFACE MARKERS

The lack of sufficient numbers of lymphocytes, particularly

in the low density fractions, necessitated restricting the analysis

to one marker study per subject sample.

i) E- ROSETTES

The results for each patient are shown in Fig. 5.3 and the

group mean values summarized in Fig. 5.4. In Fig. 5.3 the percentage

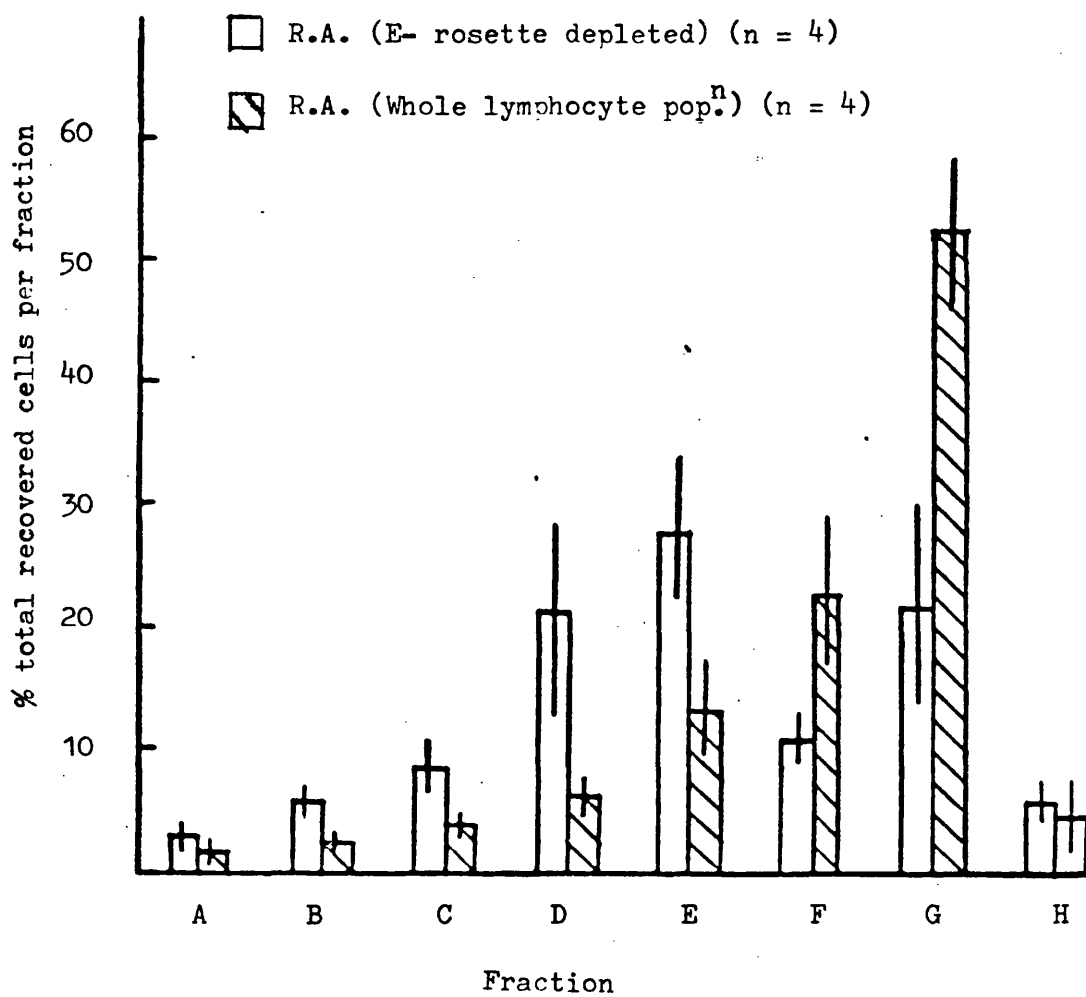of E- rosettes is seen to be increased in the denser fractions (fraction

Fig. 5.2   The effect of depleting EAC- rosetting cells

on the density distribution profiles of peripheral

blood lymphocytes in R.A.

60 ml of venous blood was taken and the lymphocytes

separated (Chapter 2.2.b.ii). The EAC- rosetting cells were

depleted (Chapter 2.2.g.) from half of the lymphocyte sample

and the non EAC- rosetting cells (from the Ficoll/Paque interface)

and the whole lymphoid population were separated in parallel

Ficoll gradients (chapter 3.3.d.).

The gradient interfaces were harvested, counted and

the results expressed as a percentage of the total recovered

lymphocytes in each fraction. There was no statistical

analysis.

Fig. 5.2
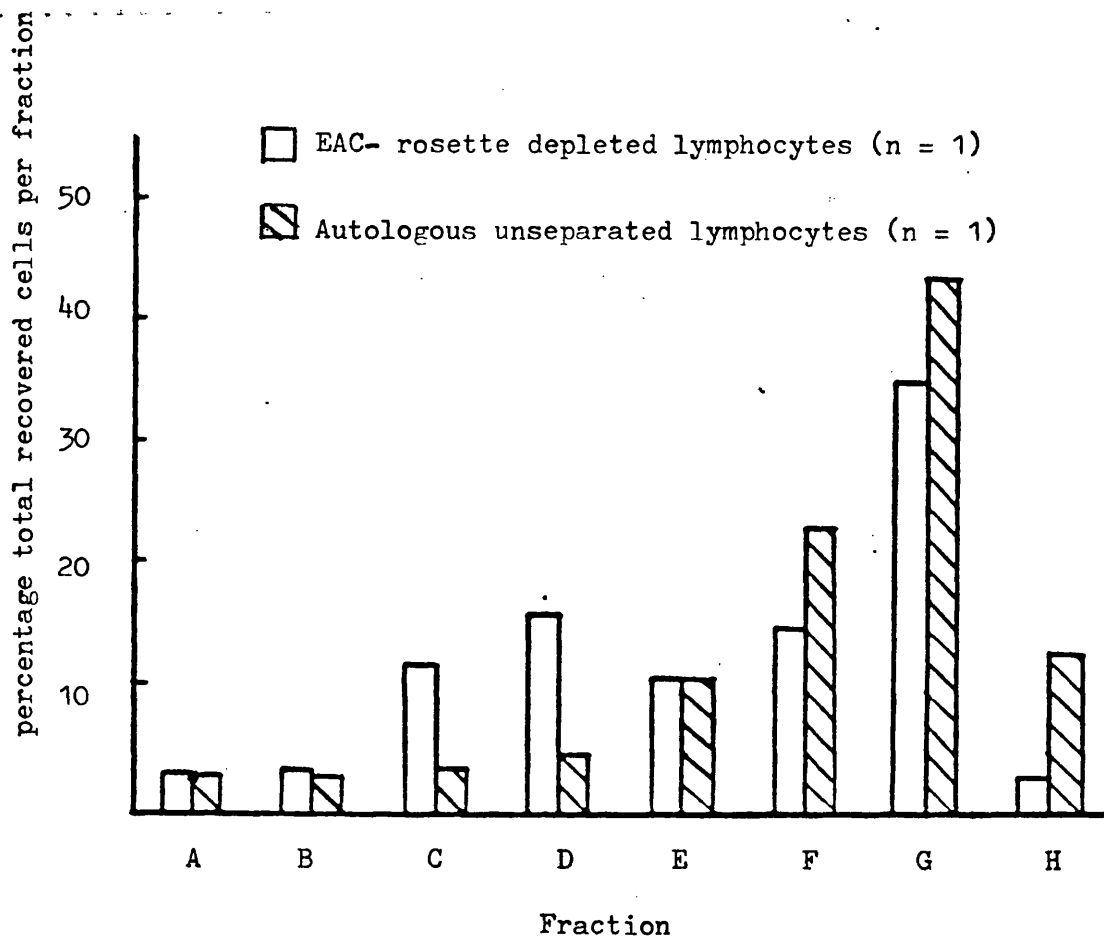
H excepted). The range of results is increased in the denser

fractions, subjects varying by up to 38% in fraction G (40-

78%). The mean of these results (Fig. 5.4) shows that both

healthy and rheumatoid subjects display the trend of increasing

E- rosette formation in the denser fractions. The difference

between R.A. and healthy subjects is most noticeable in fraction

G in which R.A. patients have a mean of 54% E- rosettes compared

with 61% for healthy subjects. This difference is not

statistically significant (Students t- test), neither are the

small subject group differences in fractions B, C, D, E and F.

The relative numbers of E- rosettes in the Ficoll gradient fractions

are shown in Fig. 5.5. It can be seen that most of the E- rosettes

are in fraction G, more so in healthy subjects. R.A. patients have

relatively more E- rosettes in the low density fractions,

suggesting some T cell activation. Accumulating the E- rosettes

in the gradient fractions shows that healthy subjects lymphocytes

formed 51% E- rosettes compared to 47% for R.A. patients.

Statistical analysis of this data is not possible as it is a

function of two results, each with their own population deviations.

ii) EA- ROSETTES

The mean percentages of EA- rosettes formed by whole

lymphoid populations from R.A. and healthy subjects at various

erythrocyte antibody concentrations are shown in Fig. 5.6. At

all concentrations the R.A. patients formed more EA- rosetting

cells, suggesting more avid EA- rosette formation (1/400 $P <$

0.02; 1/800 $P < 0.01$; 1/1600, 1/3200, 1/6400 $P < 0.001$).

To analyse EA- rosette formation in the Ficoll gradient

fractions two antibody coating concentrations were used.

Fig. 5.3

5.4   The density distribution of lymphocytes forming

5.5   E- rosettes in Ficoll density gradient fractions.

Comparison of healthy subjects and those with R.A.

Peripheral blood lymphocytes were prepared from 30 ml venous

blood (Chapter 2.2.b.ii), the monocytes depleted (Chapter 3.3.c.)

and the lymphocytes separated by density in a Ficoll gradient

(Chapter 3.3.d.). The interface fractions were harvested, counted

adjusted to 2 X $10^6 ml^{-1}$ and E- rosettes determined (Chapter 2.2.e.).

The results (Fig. 5.3) are expressed as percentage E- rosetting

lymphocytes in each fraction and each point represents the lymphocytes

from one patient in each fraction.

The mean result (± 1 standard deviation of the mean) for each

fraction is shown in Fig. 5.4. There were no statistically significant

differences between R.A. and healthy subjects.

The E- rosetting lymphocytes of each fraction are presented

with the mean total cell proportion in each fraction (Fig. 5.5.). This

figure was obtained by multiplying the % incidence of E- rosettes by the

proportion of lymphocytes in each fraction., e.g., in Fraction G

for healthy subjects the E- rosettes are 41 of 67% (approx 2/3) of

the total lymphocytes in the gradient.

Fig. 5.3

Fig. 5.3

o Healthy subjects

● R.A.

% E— rosettes per fraction

Fraction
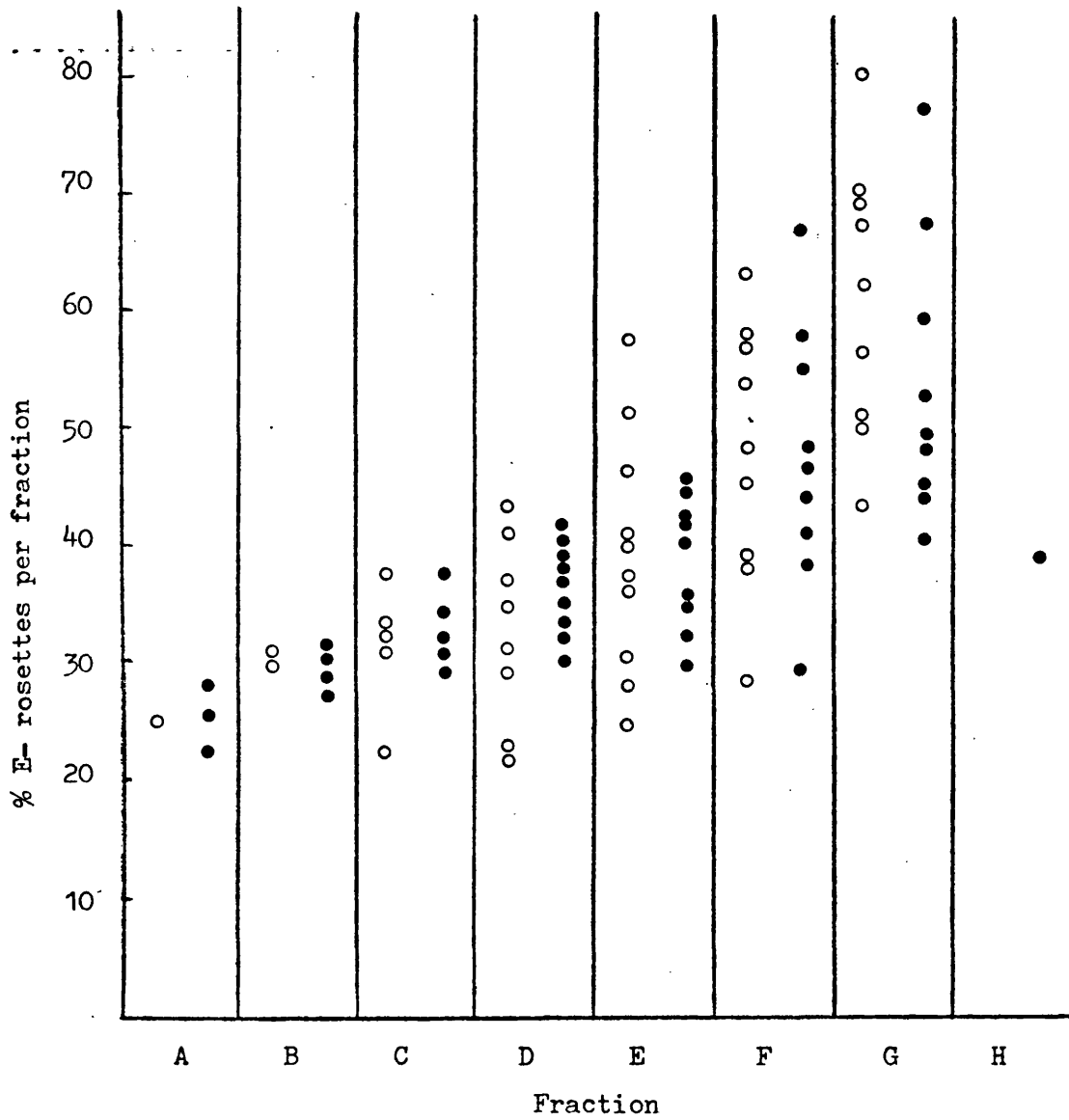
Fig. 5.4
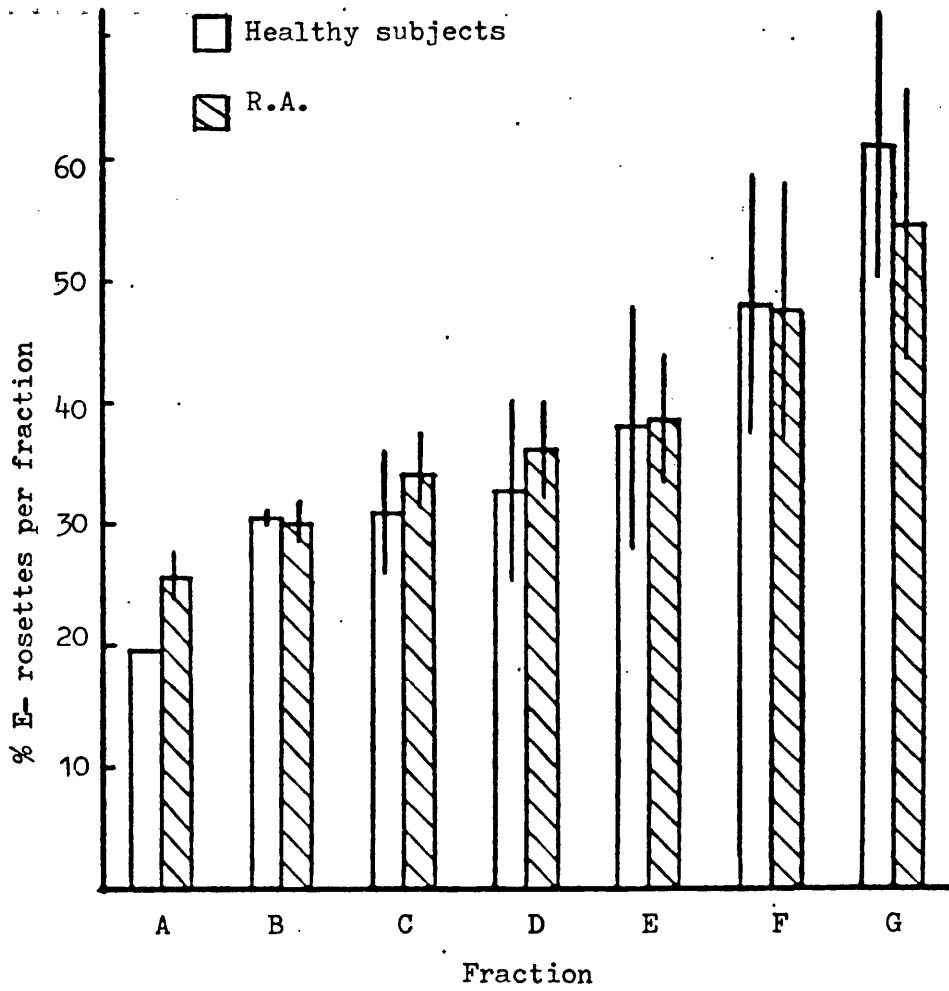
Fig. 5.5.
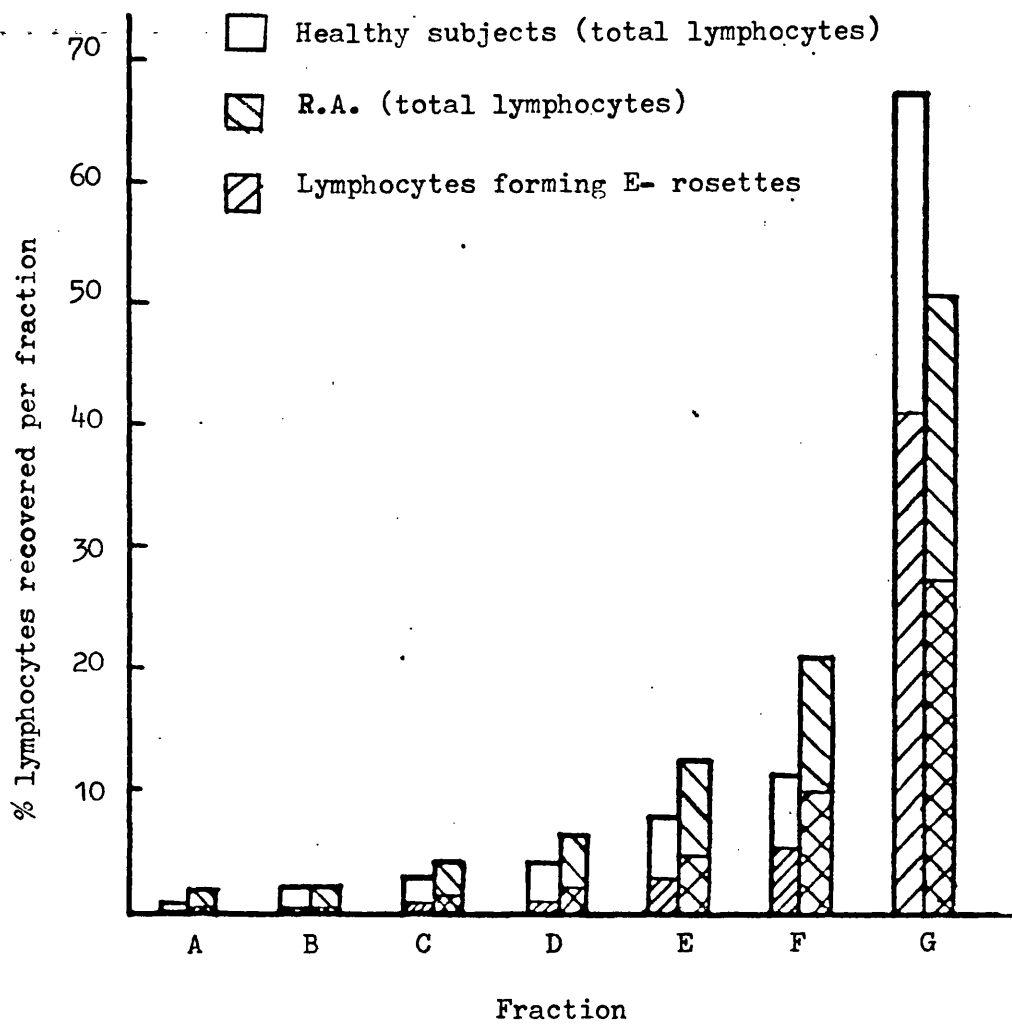
Fig. 5.6

EA- rosette formation of healthy and

rheumatoid whole lymphocyte populations

Peripheral blood lymphocytes were prepared from

venous blood (Chapter 2.2.b.ii). EA- rosette formation

was determined using various titres of rabbit IgG anti ox red cell

antisera (Chapter 5.2.b.ii). The results are plotted

as the mean $\pm$ 1 standard deviation. The differences between

R.A. and healthy subjects were significant at the following

concentrations:

Concentration	Numbers tested		p
	R.A.	Healthy	
1/200	8	8	NS
1/400	10	10	<0.02
1/800	10	10	<0.01
1/1600	34	19	<0.001
1/3200	26	18	<0.001
1/6400	7	7	<0.001

Fig. 5.6.
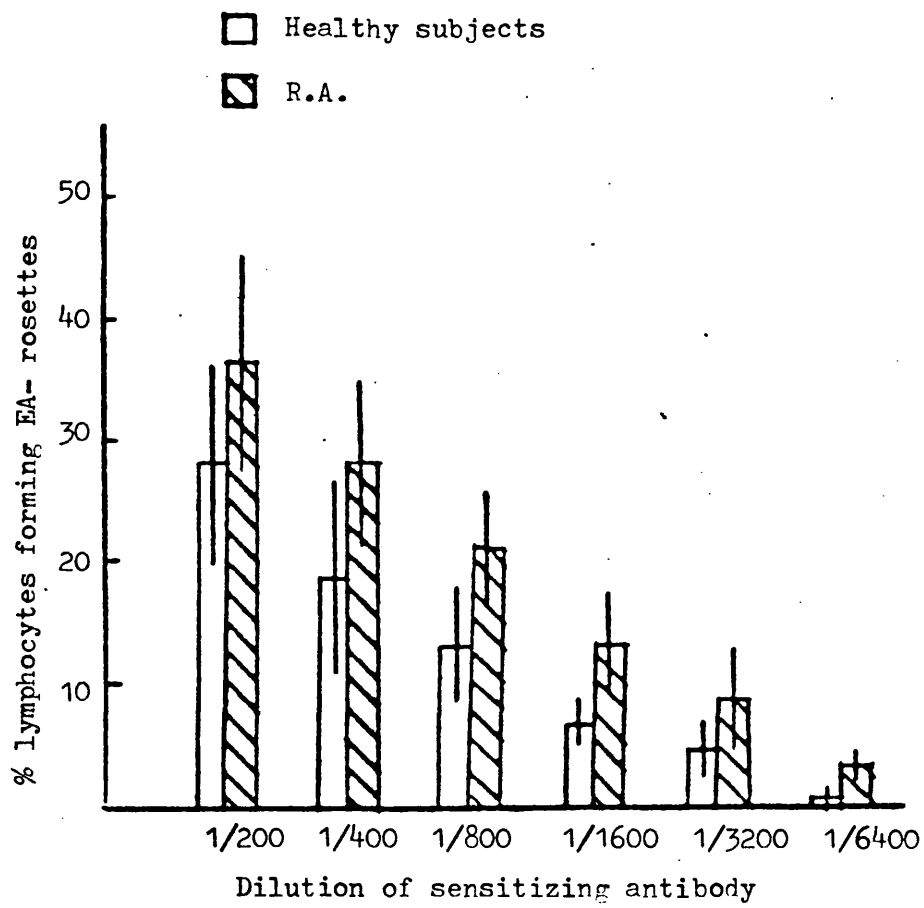
The 1/800 titre was used to detect medium (and high) avidity Fc

receptor binding and the 1/3200 titre was used to detect only

high avidity binding.

The results of the medium avidity analysis of Ficoll gradient

fractions are shown in Fig. 5.7 and summarized in Fig. 5.8.

EA- rosettes were formed at between 28% and 34% for both R.A.

and healthy subjects in fractions B, C, D, E and F. The

differences between the subject groups were not significant in

these fractions, but were in fraction G in which R.A. had significantly

reduced EA--rosettes (p $<$ 0.05). The EA- rosette formation in fraction

G was also reduced relative to other fractions, for both R.A. and

healthy subjects, R.A. patients forming 18% EA- rosettes and healthy

subjects 23%.

Upon relating this percentage to the lymphocyte density

distribution profile the relative numbers of lymphocytes forming

medium avidity EA- rosettes is shown (Fig. 5.9). Healthy subjects

have most of their lymphocytes with medium avidity Fc receptors

in fraction G whereas R.A. patients have relatively more in the

lower density fractions D, E and F. However, R.A. patients

(23.5%) do not form more medium avidity EA- rosettes than healthy

subjects (29.1%).

The distribution of lymphocytes forming high avidity EA-

rosettes is shown in Fig. 5.10 and Fig. 5.11. More EA- rosettes

were formed in the denser lymphocyte fractions. R.A. patients

formed more EA- rosettes than healthy subjects in fractions C,

D, E, F and G, of which C and E are significant (P $<$ 0.05). The

trend shown here is different to that shown for medium avidity

EA- rosettes. Relating these low rosette numbers to the cell

numbers or fractions serves no useful purpose as the numbers are

Fig. 5.7.

5.8.   <u>Fc receptor bearing lymphocytes in Ficoll</u>

5.9.   <u>density gradient fractions. Comparison of</u>

            <u>R.A. and healthy subjects.</u>

30 ml of venous blood was taken and the lymphocytes

prepared (Chapter 2.2.b.ii). Monocytes were depleted (Chapter

3.3.c.) and the lymphocytes separated by density in a Ficoll

gradient (Chapter 3.3.d.). The interfaces were harvested and

the lymphocytes adjusted to $2 \times 10^6 ml^{-1}$. EA- rosettes were

determined using the 1/800 titre of rabbit IgG anti ox red

cell antisera (Chapter 5.2.b.ii). The results (Fig. 5.7) are

expressed as the % EA- rosette forming cells in each fraction and

each point represents the lymphocytes from one patient in each

fraction.

The mean result (I 1 standard deviation) for each fraction

is shown in Fig. 5.8. The only significant difference between

R.A. and healthy subjects is in Fraction G ($p < 0.005$).

The EA- rosetting lymphocytes of each fraction are presented

with the mean total cell proportion in each fraction (Fig. 5.9).

This figure was obtained by multiplying the % incidence of EA-

rosettes by the proportion of total lymphocytes in each fraction.

E. G., in Fraction G for healthy subjects the EA- rosetting cells

are 16 of the 67% (approx 1/4) of the total lymphocytes in the

gradient.

Fig. 5.7.

O Healthy subject

● R.A.

Fig. 5.7. Scatter plot of % EA-rosettes in each fraction (B, C, D, E, F, G) for healthy subjects and R.A.
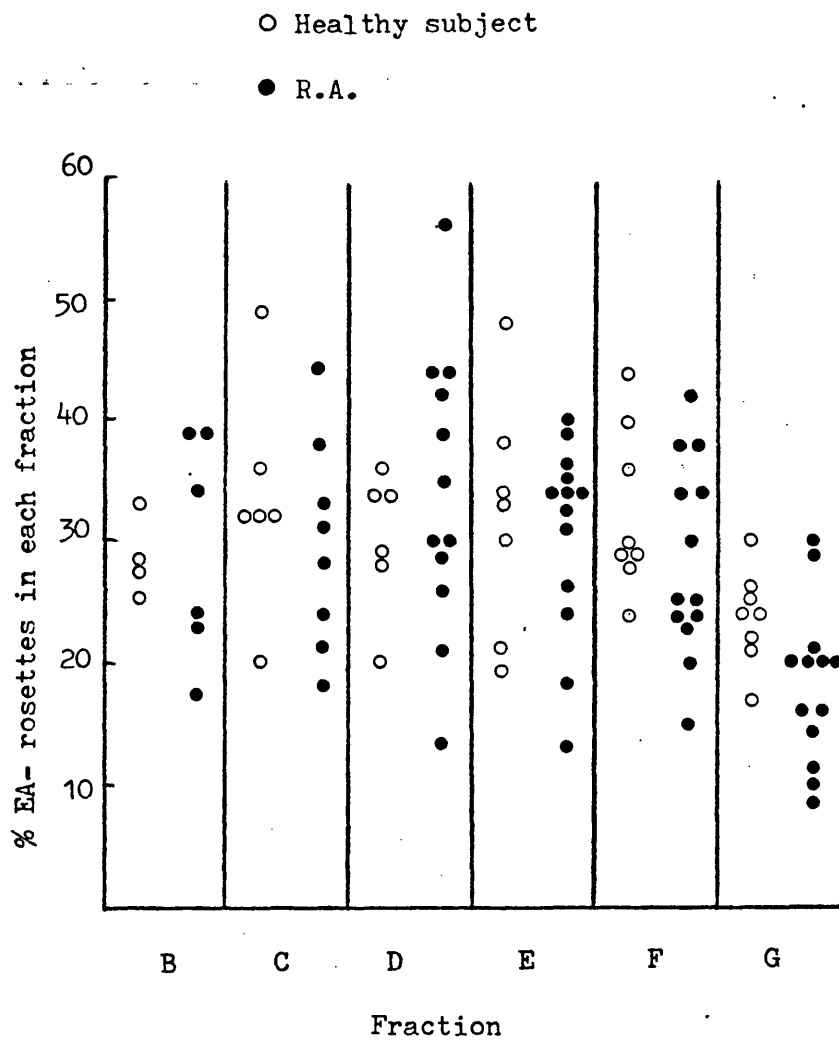
Fig. 5.8.
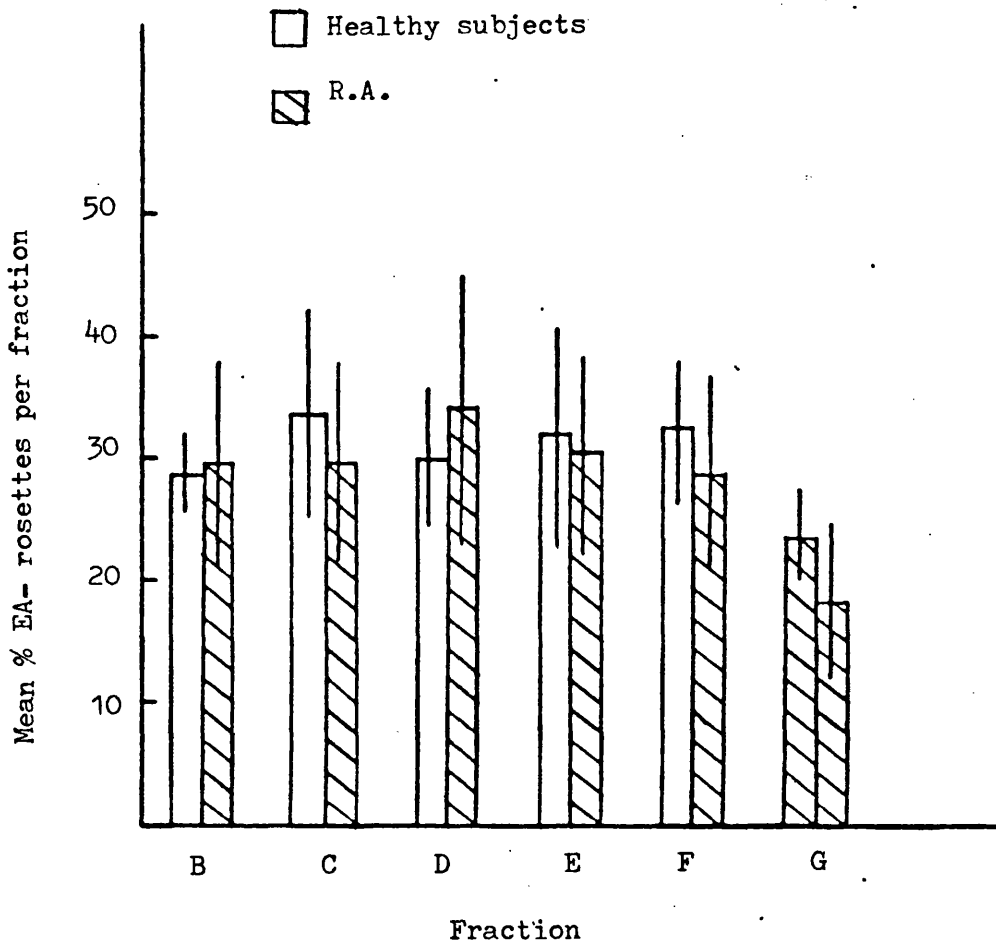
Fig. 5.9.
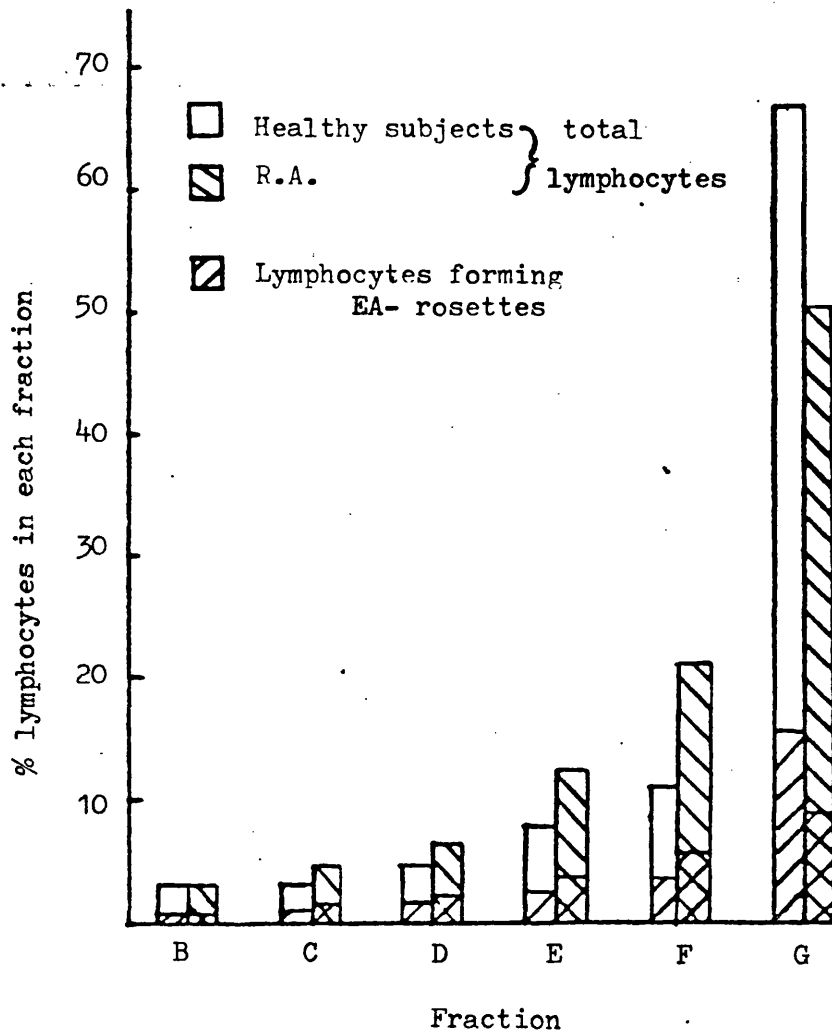
Fraction

Fig. 5.10

High avidity Fc receptor-bearing lymphocytes
in Ficoll density gradient fractions. Comparison
of healthy subjects and R.A.

30 ml of venous blood was taken and the lymphocytes

prepared (Chapter 2.2.b ii). Monocytes were depleted

(Chapter 3.3.c.) and the lymphocytes separated by density

in a Ficoll gradient (Chapter 3.3.d.). The interfaces were

harvested and adjusted to 2 X 10^6 ml^{-1}. EA rosettes were

determined using the 1/3200 titre of rabbit IgG anti-

ox red cell antisera (Chapter 5.2.b.ii). The results

(Fig. 5.10) are expressed as the % EA- rosette forming

cells in each fraction and each point represents the

lymphocyte from one patients in each fraction.

The mean result ($\pm$ 1 standard deviation) for each

fraction is shown in Fig. 5.11. Statistically significant

differences between healthy subjects and R.A. are shown only in

fractions C and D (p < 0.05).

Fig. 5.10.

O Healthy subjects

● R.A.
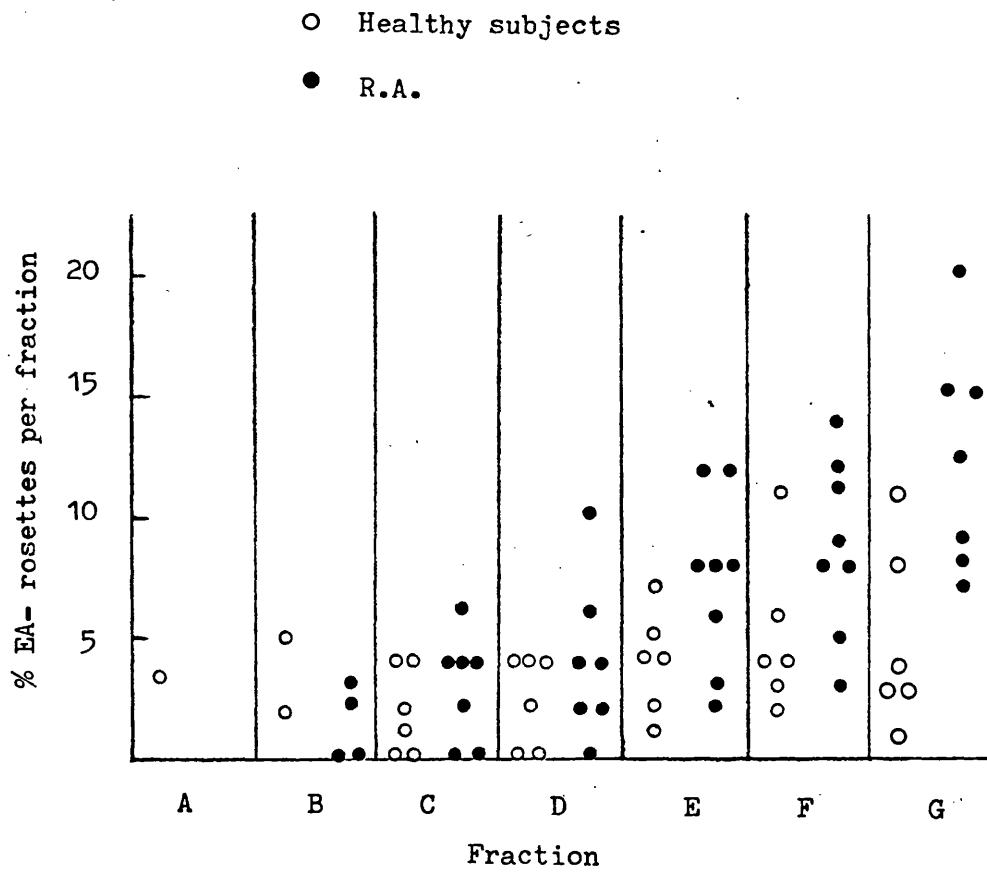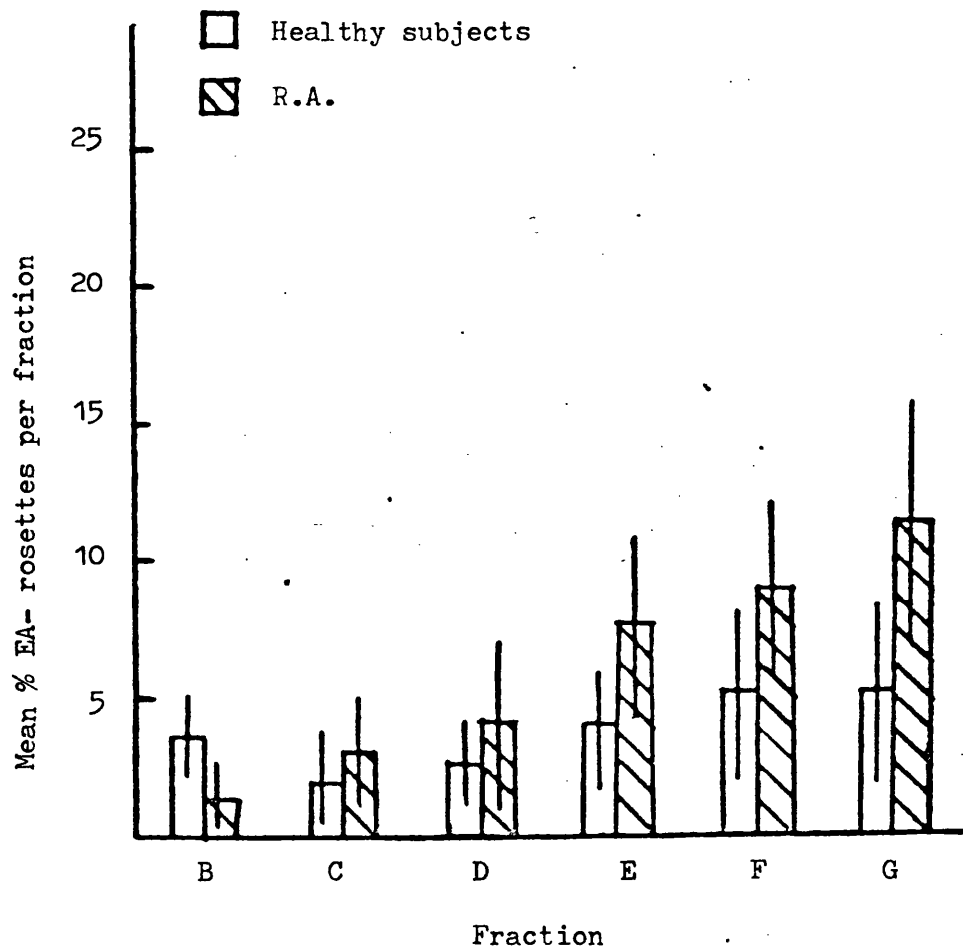
Fig. 5.11.

so low. Medium avidity rosettes also detected the high avidity

Fc receptor bearing population and hence to show the medium

avidity EA- rosette formation the latter set of results may

be subtracted from the former (**Fig.5.12**). This gives a

distribution pattern in which decreased proportions of rosettes

were shown in the denser fractions, particularly in R.A.

iii) SURFACE IMMUNOGLOBULIN

The percent of surface Ig bearing cells in the

Ficoll gradient fraction is shown in Fig. 5.13 and Fig. 5.14.

No statistically different results were recorded for R.A. patients

and healthy subjects. The results show a decrease in percentage

SIg bearing cells in the denser fractions. The dense fraction

(G) had 22% SIg positive cells whereas fraction B had 43% with a

more or less linear fall in numbers between these fractions.

Due to the similarities of these results for R.A. patients

and healthy subjects, relating SIg positive percentages to lymphocyte

density distribution merely becomes a function of the latter (Fig.

5.15).

5.4   DISCUSSION

In this study lymphocytes of varying densities were shown to

include   different proportions of surface markers. Compared with

the high density cells of fraction G, the low density cells were

depleted of T cells, and those cells with high avidity Fc-

receptors. Moreover the less dense cell population was enriched

in B cells and those cells with medium avidity Fc- receptors.

Both E- rosette depletion and direct E-rosette studies on

gradient fractions suggested that low density cells were pre-

dominantly non-T cell. E- rosette depletion was used for

preliminary studies, but discarded due to the loss of lymphocytes

Fig. 5.12

Medium avidity EA- rosette formation

The mean percentages of high avidity EA- rosette

forming cells in Ficoll gradient fractions (Fig. 5.11)

have been subtracted from the medium + high avidity

EA- rosette percentages (Fig. 5.8). The result gives

the calculated number of medium avidity EA- rosette

forming cells in the different fractions.

Fig. 5.12.
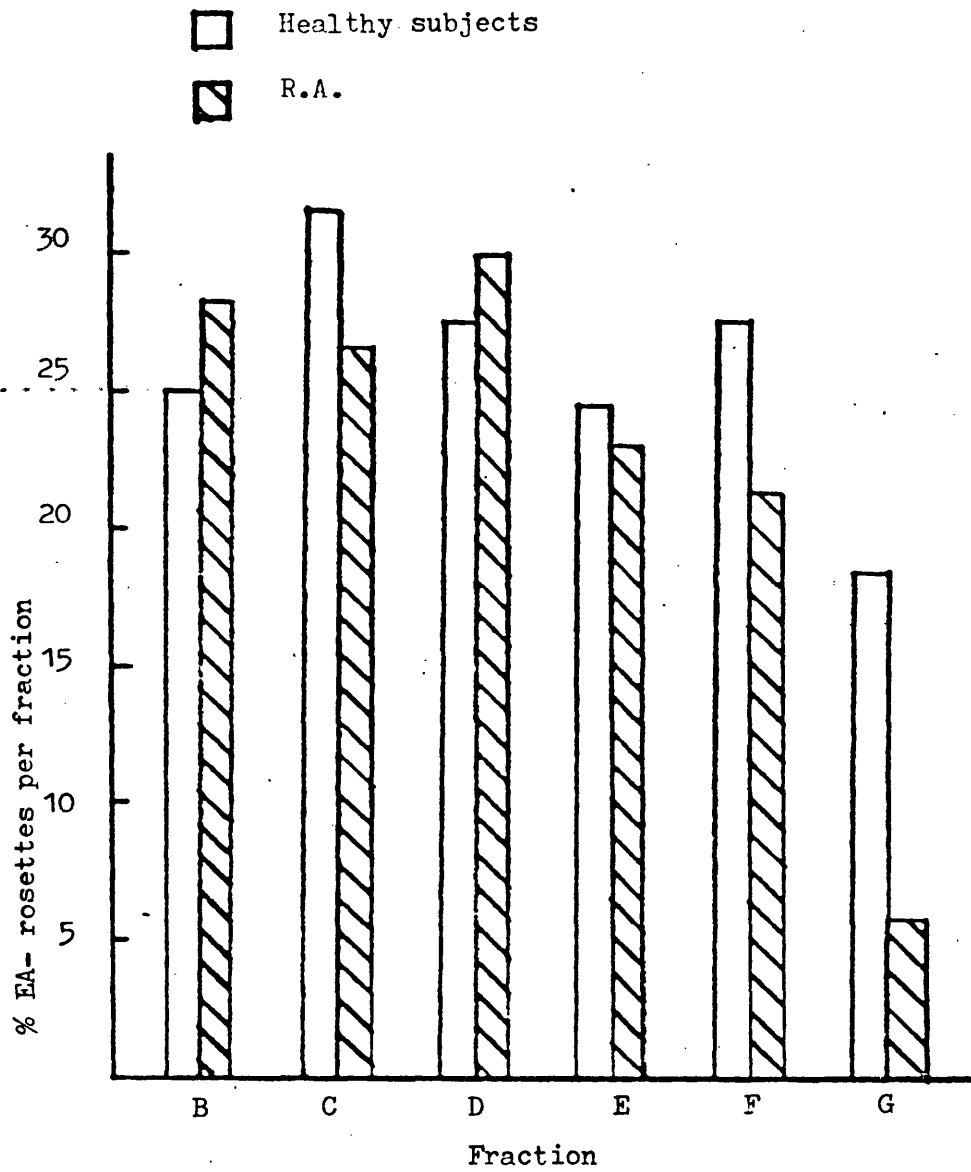
Fig. 5.12.

Fig. 5.13

5.14   <u>Surface Ig bearing cells in Ficoll density</u>

5.15   <u>gradient fractions.   Comparison of R.A.</u>

<u>and healthy subjects.</u>

30 ml of venous blood was taken and the lymphocytes prepared

(Chapter 2.2.b.ii). The lymphocytes were labelled for SIg with 1/5

fluorescein isothiocyonate conjugated to rabbit anti human Ig

(Chapter 2.2.h.). Monocytes were depleted by adherence (Chapter

3.3.) and the lymphocytes separated by density in a Ficoll gradient

(Chapter 3.3.d.). The SIg positive cells were detected under a

U.V. microscope and the results are expressed as percentage

SIg positive cells in each gradient fraction (Fig. 5.13).

Each point represents the lymphocytes from one patient in each

fraction.

The mean result ($\pm$1 standard deviation) is shown in Fig. 5.14.

There was no statistical difference between R.A. and healthy

subjects in any of the fractions. The surface Ig positive cells

of each fraction are presented with the mean lymphocyte proportion

in each fraction (Fig. 5.15). This figure was obtained by multiplying

the % incidence of surface Ig positive cells by the proportion

of total lymphocytes in each fraction, e.g., in Fraction G of

healthy subjects the SIg positive cells are 15 of 67% (approx 1/4)

of the total lymphocytes in the gradient.

Fig. 5.13.

○   Healthy subjects

●   R.A.

Fig. 5.14.

Mean % surface Ig-bearing cells per fraction

Healthy subjects

R.A.

Fraction
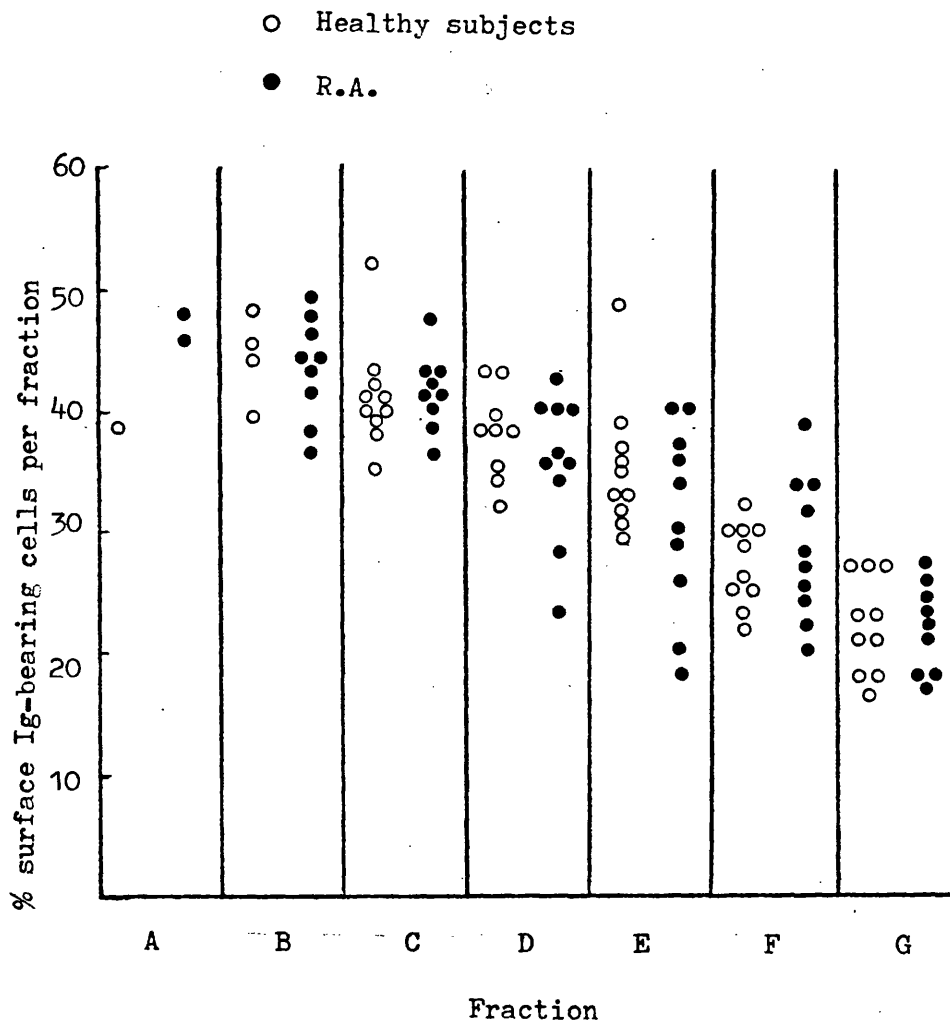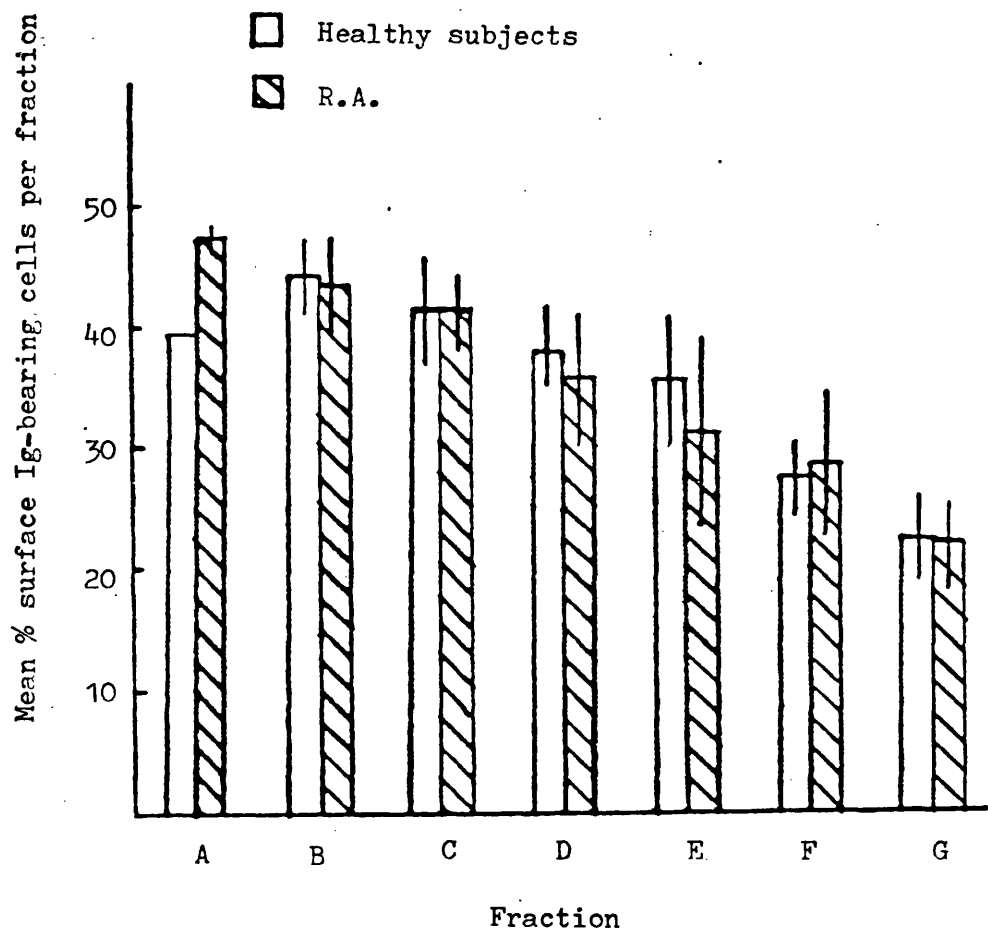
Fig. 5.15.
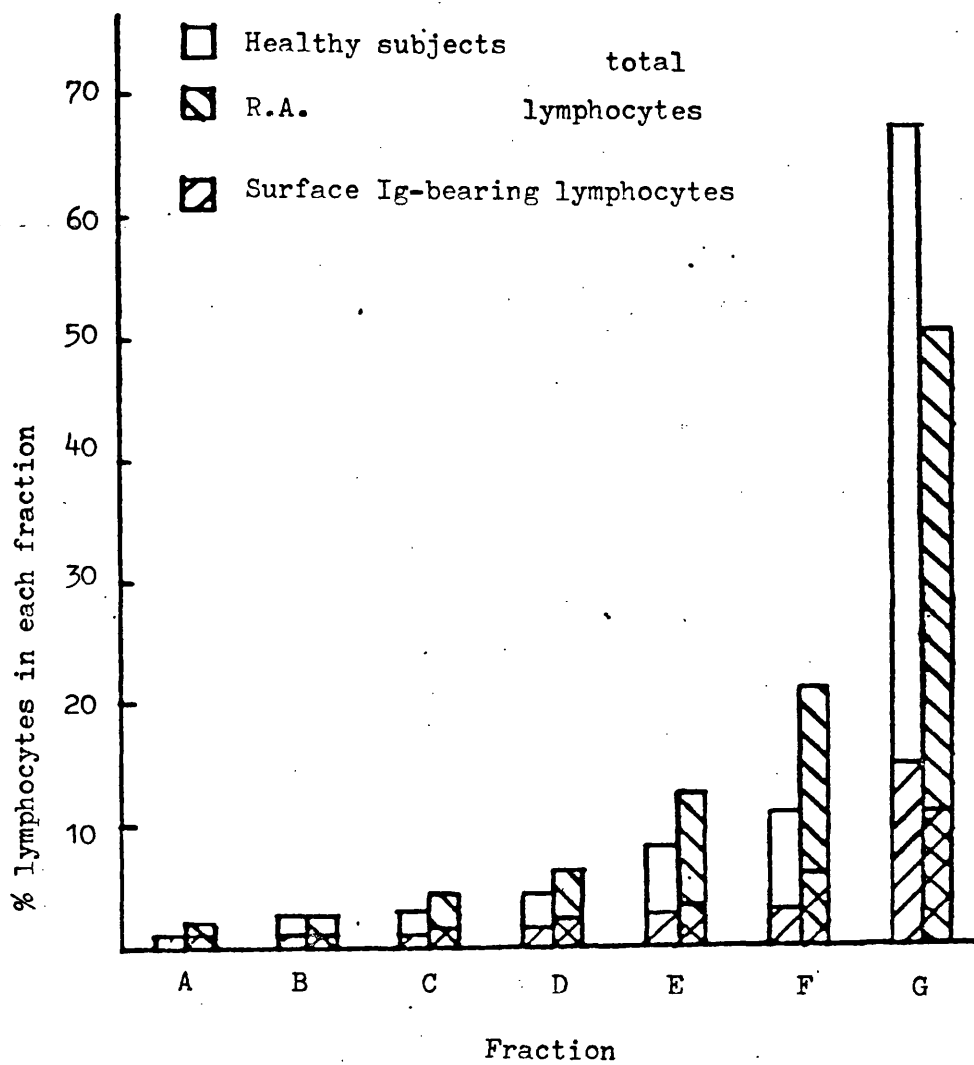
upon using a second Ficoll-Paque separation. This favours the recovery

of the lower density lymphocytes, as shown in Chapter 3.

The conditions used for the determination of E- rosettes

satisfied the criteria suggested by the WHO report (1974).

Divalent cations (Ca^{2+} and Mg^{2+}) were used in the medium, which

included 10% human serum. Higher numbers for E- rosette percentages

were only found by preincubating lymphocytes or SRBC with neuraminidase

(unpublished). This is believed to stabilise the rosettes. The

method employed in this study would also detect "active E rosettes"

(Wybran and Fudenberg, 1973).

The distribution of E- rosettes in the gradient fractions suggests

that the activated lymphocytes, both in R.A. and at low levels in

healthy subjects, are predominantly non T cells. Another possibility

is that T cells have a narrow high density range compared with B

cells, if this were the case a large number would be lost during

the initial isopycnic isolation of lymphocytes from whole blood.

Even if T cells were denser than B cells this would not explain

the increased numbers of low density cells in R.A. as the B cell

numbers in R.A. peripheral blood are considered normal (Brenner,

Scheinberg and Cathcart, 1975; Froland, Natvig and Wisloff, 1975).

The reduced numbers of T cells in the low density fractions could

be explained by T cells losing their receptors for SRBC upon activation

Svedmyr et al (1974) found a high proportion of blasts in mixed

leucocyte cultures lacking any surface marker attributable to B

or T blasts. The authors found such null blasts were produced

from almost pure SRBC - binding lymphocytes and considered the

null blast to be of T origin. However, Jondal (1974, b) found that

PHA and Con A induced T blasts retained their receptors for SRBC.

Moreover, stimulation of purified T cells with allogeneic lymphocytes

produced no change in the detection of receptors for SRBC (Jondal,

1974 b, c). The results of Svedmyr et al (1976) may be contra-
dictory to those of Jondal (1974) because the stimulating cells
used by the former were from a lymphoblastoid cell line.
Thus, E- rosette formation studies of Ficoll gradient fractions
were believed to detect resting and blast transformed T lympho-
cytes. Quantitative antigen-induced T cell proliferation has not
been used to study T marker expression due to the small number
of responding lymphocytes in such an in vitro test system.

The density distribution analysis of peripheral blood
lymphocytes depleted of EAC- rosetting cells suggests that the
low density cells in fractions C and D did not express C3
receptors. This is not conclusive, not just because only one
patient was studied, but because C3 receptor bearing lymphocytes
do not entirely overlap with surface immunoglobulin-positive cells
(Ross et al, 1973). Caution must be taken when using C3 receptors
as B cell markers as this marker is absent on PWM- induced surface
immunoglobulin blasts (Jondal, 1974, b). Hence, the enrichment
of cell numbers in fractions C and D after EAC- rosette depletion
could be due to the presence of either B cells or T cells,
particularly as activated lymphocytes are found in these fractions.
Lymphocytes bearing receptors for C3 were the denser lymphocytes
of fractions F, G and H, suggesting that non-active B cells, or
T cells (Ross et al, 1978), are C3 receptor-positive.

The increased incidence of surface Ig positive cells in the
low density fractions, 35-47% compared with 22% in fraction G,
implies an increased number of B cells in these fractions. This
would be in agreement with the relatively low incidence of T cells
in these fractions. The absolute numbers of surface Ig positive
cells in the fractions illustrates the low number of dense B cells

in R.A., even though the total B cell numbers in fractions B, C

and D are not greater in R.A. than in healthy subjects.

Are the results representative of the B cell numbers

in the density fractions? As the lymphocytes were labelled

prior to gradient separation it is unlikely that the density

equilibrium attained could affect the labelling quality due to

a function of Ficoll concentration. Incubation at $37^{\circ}C$ prior to

Ficoll gradient separation should have removed any passively

bound IgG (Winchester, Hoffman and Kunkel, 1975), and hence

SIg negative cells with receptors for IgG should not be detected.

The distribution of Fc receptor bearing lymphocytes, both medium

and high avidity does not accord with the detection of passively

bound IgG by the usage of FITC-labelled anti human Ig.

B lymphocytes activated in vitro with PWM give rise to B

blasts which are surface Ig-positive (Jondal, 1974, b, c). With

the knowledge that C3 receptors are not found on some activated

B cells it seems that surface Ig detection is a good assay

for B cells. If this holds true for in vivo produced blasts it

may be possible to get information about activation of the immune

system under different clinical conditions. However, there are

difficulties in classifying malignant and non-malignant

lymphoblasts. Acute lymphatic leukaemia is an example in which the

present markers used for T and B cells are unable to classify

the proliferating population (Seligman et al, 1974). Another

example of cells lacking all markers seems to be that of

lymphoblasts induced in vitro by stimulation with lymphoblastoid

cell lines (Svedmyr et al, 1974).

Using SRBC receptors as a criteria for T cells, and surface

immunoglobulin as a B cell criterion, seems to allow classification

of almost all lymphocytes and lymphoblasts with no overlapping

of these markers (Jondal, 1974, b). Recently, Ross et al (1978)

have suggested that the possession of Ia determinants is a better

B cell marker than the presence of surface Ig as it detects the

Ig-negative pre-B cells present in normal bone marrow. However,

some monocytes have surface Ia and B cells ultimately lose

detectable Ia determinants during the differentiation process

into plasma cells. Plasma cells still possess detectable quantities

of surface Ig, some expressing immunoglobulin on the surface of

the same class that they secrete. It is unclear whether this is an

integral membrane component or merely immunoglobulin trapped in the

process of secretion. IgG- and IgA- secreting plasma cells fail

to express surface IgD, but many retain surface IgM, particularly

IgM secretary plasma cells (Halper et al, 1978). It is only

in the terminal stages of B cell development that the surface Ig

pattern changes and if such cells are of a low density the actual

percentage B cells shown in the low density fractions of this study

would be higher than estimated. Conversely, if dense cells, the B

cell numbers shown for fraction G would be an underestimate. Plasma

cells were seen predominantly in fractions C, D and E in this study

(Chapter 3).

The major trend found for Fc receptor expression in the

different gradient fractions was the distribution of lymphocytes

with high avidity receptors. High avidity receptors were found mainly

on denser cells. In R.A. increased numbers of these cells were

found in fractions C, D, E, F and G. The differences in patterns for

medium and high avidity Fc receptor bearing lymphocytes suggests that

these may be detecting different lymphocyte populations. Fc receptors

are found on monocytes, neutrophils, B cells, some T cells (Van

Boxel and Rosenstreich, 1974), L cells (Horwitz and Garrett,

1977) and K cells (Perlmann and Holm, 1969). Sharpin and Wilson

(1977 b) believe the increased avidity of the R.A. lymphocytes

for EA binding is due to increased numbers of K cells in this

disease. K cells are believed to be non-B cell and this could

account for the distribution of the high avidity receptors in the

Ficoll gradient. The distribution of the medium avidity receptor

bearing cells in the gradient is similar to that of B cells,

particularly as this assay will also detect the high avidity

population. Hence the high avidity receptor population are

probably non-B and may be described as L cells, the cytotoxic potential

uncharacterized. The high incidence of medium avidity Fc receptor

bearing lymphocytes in fraction G in health may be related to the

low number of high avidity cells in this fraction in comparison

with R.A.

As T cells were shown predominantly in fraction G there is

the possibility that these possess avid Fc receptors. The possession

of Fc receptors by T cells is well documented (Yoshida and Andersson,

1974; Miyama et al, 1978) but no data on their binding avidity for

IgG has been presented to date. The Fc receptors on T

lymphocytes have been described as being limited to activated cells

which would not normally be found in fraction G. However small

effector cells, resulting from proliferation of a stimulated sub-

population, such as suppressor or cytotoxic T lymphocytes, could be

found in this fraction. Small effector lymphocytes have been demonstrat-

ed with Fc receptors.

The interpretation of the Fc receptor data is open to more

speculation than for the T and B cell markers, mainly due to the

overlap of cell populations resulting from the use of this cell

marker. Certainly the lymphocytes in the low density gradient

fractions in R.A. are more likely to be B cell than T cell,

which composed the major part of the denser population.

CHAPTER SIX

DISCUSSION

6.1 LYMPHOCYTE ACTIVATION IN R.A.

This study describes an attempt to separate circulating lymphocytes by density in health and R.A. and to characterize these cells by surface marker studies. The density separation method is discussed first and the density distribution of R.A. lymphocytes will be related to spontaneous proliferation in vivo. The nature of the different lymphoid populations, as defined by density, is discussed next, with a view to elucidating their role in the pathogenesis of R.A.

In this study, the separation of peripheral blood lymphocytes by density has been successfully achieved in R.A. The discontinuous Ficoll gradient developed for this study has allowed the separation and recovery of lymphocytes of particular densities at high purity as shown by the rebanding of a single peak within a gradient. The lower density cells contain those which are proliferating, as shown by cytological examination and the density distribution of mitogen-stimulated lymphocytes. The lymphocyte density profiles obtained for healthy subjects were shown to be relatively constant and the method repeatable.

The low density fractions also included lymphocytes which morphologically were not activated. Steinman et al, (1978) showed that low density is acquired by lymphocytes soon after activation (18 hours) in vitro with mitogen. These small low density lymphocytes may be either recently activated or 'trapped' by non-specific association with larger cells. However, the rebanding of a peak of cells indicates that the latter is unlikely. To check the former possibility small low density lymphocytes could be prepared and

cultured in vitro and further checks on any density change or morphology performed.

To conclude that low density lymphocytes are activated requires that no other procesries, in vivo or in vitro, affect lymphocyte density. Variations of osmolality and pH are known to change lymphocyte density (Shortman, 1968). Ficoll does not cause any change in toxicity and allows the use of a physiological pH (Gorczynski, Miller and Phillips, 1970) and hence should give a good estimate of the actual in vivo density of a cell. The effect of other factors which may affect lymphocyte density have not been reported. The inumerable factors which a lymphocyte counters in the body may affect density without causing activation. All density distribution data relating to in vivo events must take this proviso into account, particularly when comparing patient sub-groups.

In any study in which peripheral blood is sampled it must be remembered that this sample represents one point in time. Activated lymphocytes are programmed to a set of functions and during the course of their development the nature of these cells will change. Steinman et al (1978) has demonstrated that recently activated cells acquire a low density, but has not commented on their density changes during development to blasts and to effector cells and/or memory cells. Plasma cells have a lower density than resting lymphocytes (Haskill, Legge and Shortman, 1969) but this has not been quantified with respect to other lymphoid cells. Dense lymphocytes will include small resting cells and recent progeny from activation. The lowest density cell will be the largest cell, the blast, due to its high cytoplasm/nuclear ratio. Plasma cells will have a density intermediate between blasts and small lymphocytes and hence will be found in the

same fractions as recently activated lymphocytes. It thus cannot

be accurately predicted from a single sample whether an intermediate

density cell is getting larger or smaller or is recently activated

or maturing. Unfortunately there is no simple answer to this problem

since serial assays of the different functions has not been attempted

here but it is known that activated less dense cells do not survive

well in culture (Moore and Hall, 1970).

The increased number of circulating immunoblasts found in

R. A. is in general agreement with the findings of other authors

(Bacon, Crowther and Sewell, 1975; Delbarre, Le Go and Kahan, 1975;

Lalla et al, 1973). However, the large size reported by these

authors was not confirmed. Certainly, cells up to a **diameter**

of 30 µm, as recorded by Delbarre et al (1975), were not observed.

This is probably because of technical details. The numbers of

immunoblasts, as recorded in this study, were related to disease

activity. Raised numbers of immunoblasts were found particularly, if

not exclusively, in patients with clinically active R.A. The

presence of increased numbers of immunoblasts in the peripheral blood

has been suggested as a useful indicator of increased intrinsic

disease activity (Bacon et al, 1975). By comparison, the healthy

subjects had low numbers of immunoblasts. This agrees with the findings

of a number of authors (Bond et al, 1959; Crowther, Fairley and Sewell,

1969a; Bacon et al, 1975; Delbarre et al, 1975). The percentage

of immunoblasts is often increased in pathological situations where

inflammatory and immune reactions occur together, such as acute viral

infections and connective tissue diseases. In acute viral infections

this increase is transient, going on for 10-15 days, while in connective

tissue diseases the immunoblast level may be high for long periods

and grossly reflect the level of clinical activity. Inflammation

alone does not sufficiently explain these results, since patients

suffering from psoriatic arthritis, even if associated with acute

polyarthritis, have normal immunoblast levels (Delbarre et al, 1975).

Gout patients also have low immunoblast levels (Eghtedari et al,

1976).

Thus the occurrence of immunoblasts in the peripheral blood

is assumed to be related more to immune reactions than to inflammation

and this hypothesis is supported by a number of investigations.

The immunoblasts resemble the large, rapidly dividing hyper-

basophilic blasts which are released into the efferent lymph by

antigenically stimulated lymph nodes. (Hall, and Morris, 1963, 1965;

Delbarre et al, 1969; Sprent and Miller, 1972b). Circulating

immunoblasts are capable of producing immunoglobulins (Birbeck

and Hall, 1967); they synthesize DNA as they circulate in the blood

(Bond et al, 1959; Carter, 1965). Crowther, Fairley and Sewell

(1969b) showed raised levels of immunoblasts during infection and

immunization.

Evidence for the role of lymphocytes in the pathogenesis of

R.A. is strengthened by the thoracic duct drainage studies of Paulus

et al (1977). Prolonged continuous removal of thoracic duct

lymphocytes through a surgical fistula resulted in significant

clinical improvement of disease activity in patients with severe

R.A. Cessation of lymph drainage, as well as reinfusion of auto-

logous lymphocytes, resulted in exacerbation of disease activity.

The increased number of large lymphocytes recorded in this study

in R.A. patients is supported by Whaley et al (1971) and was also found

in juvenile R.A. by Brungard, Moser and Athreya (1975). The inference

is that the increase is due to lymphocyte activation in vivo. However,

the size of large lymphocytes in this study was not so great as that

recorded by other investigators (Bacon et al, 1975; Delbarre et al, 1975).

The reported physical susceptibility of large lymphocytes (Hall

and Morris, 1965) may help to explain this discrepancy. As

immunoblasts do not recirculate from blood to lymph (Hall

et al, 1967) and hence only exist in the circulating blood for a

matter of hours, they must be produced in relatively large amounts

by comparison to the production of small lymphocytes which do

recirculate and have a life span measured in months or years.

As they do not recirculate the immunoblasts are either sequestered

in the tissues, divide to produce small lymphocytes in the circulation

or die. The large number of activated lymphocytes in the rheumatoid

synovium could explain either the source or the fate of the circulating

immunoblasts.

The increased number of low density lymphocytes found in

R.A., particularly in clinically active patients, is consistent with

the activation of lymphocytes in vivo in this disease. The increased

number of immunoblasts was also increased in active R.A. although

no study was undertaken to compare lymphocyte density profiles and

immunoblast levels sequentially in the same patients. The density

profiles of R.A. lymphocytes were strikingly different to healthy

subjects, in contrast to the small differences in lymphocyte

diameter. Possibly, as suggested by Steinman et al (1978) this is a

very sensitive assay for lymphocyte activation.

Yu, Peter, et al (1973), in their studies of prolonged thoracic

duct drainage of R.A. patients, showed that during drainage the

number of peripheral blood low density lymphocytes increased as the

disease activity regressed. However, this is almost certainly

due to an increased bone marrow output of lymphocyte

precursors to compensate for the loss of lymphocyte during drainage,

confirming the idea that less dense cells are recently activated ones.

Increased numbers of low density lymphocytes were found in the

peripheral blood of clinically active SLE patients, whereas patients

with inactive disease had cell density distributions similar to

healthy controls. (Glinski et al, 1976). These investigators

also found increased spontaneous transformation in SLE patients

but upon investigating spontaneous ^{3}H-thymidine incorporation

found maximum incorporation in the denser gradient fractions.

However, the lymphocytes had been cultured in vitro for 72 hours

prior to the adding of the radiolabelled DNA precursor and most large

lymphocytes do not survive well in such culture conditions.

Hence the low density lymphocytes would have disappeared from the

cultures before spontaneous transformation was determined. Other-

wise, one would expect the low density lymphocytes to have the highest

spontaneous proliferation, as found in this study.

It is easy to envisage the majority of the low density

cells as precursors of morphological immunoblasts; the large number

relative to morphological immunoblasts representing the long time

span of the G1 and G2 phases of the cell cycle which culminate in the

short lived blast cell which rapidly divides to produce memory or

effector cells. However, as stressed earlier, this was a single

point in time rather than a sequential study.

Most of the patients in this study were taking analgesics or

non-steroid anti-inflammatory drugs. The effects of these drugs on

lymphocyte function in vivo are largely unknown, there is no recorded

evidence of lymphocyte activation by these agents. However, there is always

the possibility that they may alter lymphocyte density in vivo.

The low number of immunoblasts recorded in other inflammatory

arthritides, where the patients were receiving a range of drug

therapies (Delbarre, et al, 1975) indicates that this drug-induced

activation is unlikely. A number of 'second-line' drugs are known
to affect lymphocyte function in vitro (Renoux et al, 1976; Markham
et al, 1978), but not by acting as antigens. In this study the
R.A. patients receiving gold therapy had a decreased number of low
density lymphocytes relative to the patients with active disease.
Yet the patients receiving the gold were doing so for previously active
disease not controlled by NSAIDs. There are a number of possible
explanations for these results. The gold could be lymphocytotoxic
and be removing the cells with the greatest turnover i.e, activated
lymphocytes. Secondly, the gold could cause a remission of disease
activity by some other means which itself caused a decrease in
spontaneous lymphocyte activation.

The widespread effects of corticosteroids and cytostatic
drugs make their mechanism difficult to study. A longitudinal study
of patients receiving various forms of these therapies is needed
to examine changes in the density distribution pattern. This would
help increase our knowledge as to the role of these cells in the
disease state and aid in the search for effector mechanisms of these
drugs.

The studies on cell markers showed that the low density cells
had low numbers of T cells, were enriched with B cells and had few
cells which bound IgG avidly. As to whether all the low density cells
were in various stages of activation can be only conjecture due to
the problems of morphological comparison. This must be borne in mind
when ascribing numbers to the surface markers present in each fraction.

As B cells retain surface Ig upon activation and T cells retain
their receptors for SRBC the use of these markers for B and T cells
respectively, is justified for analysis of all cell fractions. Upon
examination of the cell marker data for healthy subjects the
immediate impression is that T cells are denser than B cells. For

this to be so the reduction of fraction G in R.A. would require a

drastic T lymphopenia, particularly in the active patients. This

does not appear to happen in R.A. (Brenner et al, 1975; Froland, Natvig

and Wisloff, 1975). Hence, in R.A. more B cells than T cells have a

low density. The low density B cells in healthy controls are

probably part of the 'background immune response' which results in

the low numbers of immunoblasts. These have been shown in rats to

be part of gut defence, probably against bacteria, by the ability of

syngeneic blasts to home to the gut of antigen-free neonates and many

are precursors of IgA secreting cells. (Hall and Smith, 1970).

The increase in B cells in R.A. is unlikely to be due to an increase

in gut defence mechanisms. Patients receiving analgesics or

NSAIDs often suffer from gastro-intestinal bleeding, but as these

materials are not antigenic it is unlikely that a rise in the rate

of B blasts migrating to the gut would arise.

Lymphocytes in the peripheral blood and synovial fluid of R.A.

patients have been characterized by surface markers in a number

of studies (Brenner et al, 1975; Froland, Natvig and Wisloff,

1975; Utsinger, 1975; Holborow, Sheldon and Papamichai, 1975;

Van de Putte et al, 1976). Comparisons of these reports are difficult

due to the different T and B cell assays employed. The methodology

for T cell determinations used in this study was recommended by the

WHO/IARC Workshop on human B and T cells (1974), but differences

in results can easily occur in the different methods of resuspension

of the cell pellet prior to staining the rosettes. In this study

standardization of the resuspension was attained by using a rotary

turntable. The variations in B lymphocyte percentages could be due

to a number of variables. Incubation at $37^{\circ}C$ for 30 minutes has

been used to remove cytophilic antibody from lymphocytes with Fc

receptors (Lobo, Westervelt and Horwitz, 1975). Immunoglobulin

capping, endocytosis and loss of surface marker occur at

$37^\circ C$ (Ross et al, 1978). A depression of synovial fluid B

lymphocytes has been shown by EAC rosette formation (Mellbye

et al, 1972). However, this work has not taken into account the

loss of C3 receptors by B lymphocytes upon activation (Jondal,

1974b). The study of C3 receptors in low density fractions

in R.A. was touched on in this study, showing their relative

absence in one case. This would support Jondal's work, but needs

further experimentation to be conclusive.

The distribution of cells with Fc receptors showed that cells

with high avidity receptors were predominantly dense cells. The

results for medium avidity receptors will also include the

detection of high avidity receptors and the subtraction of the

two sets of results would indicate increased medium avidity receptor

bearing lymphocytes in the low density fractions. Antibody dependent

lymphocyte toxicity is mediated by a sub-population of lymphocytes

which express Fc receptors but are neither B nor T lymphocytes,

as defined by integral surface Ig or SRBC receptors respectively.

These K cells (Perlmann and Holm, 1969) have high avidity Fc receptors,

by comparison with B cells (Wooley and Panayi, 1978). Hence,

the increased number of high avidity Fc receptor bearing lymphocytes

in fraction G may reflect K cell activity and the medium avidity

Fc receptor bearing lymphocytes, found mainly in low density fractions,

may be the B cells. This hypothesis is consistent with the B cell

distribution as determined by integral surface IgG. Monocytes also have

Fc receptors, but were excluded from the sample prior to density gradient

separation. The problems of a single time point study are illustrated

again here, since the surface markers expressed by lymphocytes are, in

some instances, dependent upon the stage of the cell cycle and this

is reflected by the difference of Fc receptor expression of lymphocytes

in the density gradient. If any of the low density T cells are

activated cells, they are not possessors of high avidity Fc receptors.

Hence the possession of high avidity Fc receptors is limited to

small lymphocytes, be they B, T, L or K sub-populations.

Upon accumulating the results for the Fc receptor positive

populations in the gradient fractions it may be seen that the

healthy subjects show a higher total EA- rosette formation than R.A.

patients. This is contradictory to the results for the whole

lymphocyte population data and could be due to two reasons.

Firstly, the lymphocytes lost during the density gradient separation

in R.A. may be mainly the Fc receptor positive cells. This

preferential loss in R.A. would suggest that more of the Fc receptor

positive cells in R.A. were activated than in healthy subjects.

This would be in agreement with the physical susceptibility

found for low density cells in this study. The other alternative

is that the expression of Fc receptors by lymphocytes is altered

by the passage through the Ficoll gradient. There is no evidence

of this from other authors. The expression of integral surface

Ig and receptors for SRBC does not appear to be so affected.

6.2   SIGNIFICANCE OF LYMPHOCYTE ACTIVATION IN R.A.

Having established the presence of both increased percentages

of immunoblasts and low density lymphocytes in R.A., both in this

study and in relation to other authors findings, I now turn to their

possible significance in R.A. The role of activated lymphocytes

in R.A. is discussed, particularly in the propogation of rheumatoid

disease. The activation of lymphocyte sub-populations is particularly

considered to highlight the roles of both cellular and humoral immunity in the disease pathogenesis.

The raised numbers of immunoblasts and low density cells in clinically active arthritis suggests the importance of activated lymphocytes in the disease state. Immunological findings are not often shown to be temporally related to the clinical state of R.A. and the big question is whether blasts are a primary cause of increased disease activity or a secondary phenomenon. The possibility that they are bone marrow stem cells produced to compensate for a leucopenia must always be considered (Barnes and Loutit, 1967). However, an increase of R.A. disease activity is not always reflected by an increased severity of leucopenia. Secondly, the increased number of plasma cells is unlikely to be a result of increased bone marrow output.

The main possibility for the presence of these cells is antigen activation of circulating small lymphocytes. Generally, the search for a specific antigen in rheumatoid synovial tissue has proved unsuccessful (Runge, 1976; Morgan, 1978), although some success has been claimed (Bacon et al, 1973). However, one cannot exclude the existence of a putative antigen and the evidence for an infectious aetiology of R.A. has been extensively reviewed by Dumonde (1976). The possibility of antigens being sequestered in other tissues must always be considered particularly in view of the apparent systemic nature of the disease, which this study must support.

Antigens are often sequestered in lymphoid tissue, where they are exposed to a maximal number of circulating lymphocytes, and if this is the case in R.A. it may explain the lack of success at finding a synovial antigen. However, the rheumatoid synovium may

easily be considered a lymphoid organ, particularly with regard to
the dense, follicular lymphoid infiltration.

Antigen stimulation of rat lymph nodes results in the appearance
of large numbers of immunoblasts in the efferent lymph (Birbeck
and Hall, 1967). The high percentage of these blasts which have
been shown to migrate to the gut probably do so because of the variety
of antigenic stimuli there (Parrott and Ferguson, 1974), although
antigen is not necessary for migration to the gut (Moore and Hall,
1972). This may be related to a change in receptors for GALT expressed
by immunoblasts, as suggested by the change in surface markers
of activated cells (Jondal, 1974b). A change in the endothelial
receptors on activated lymphocytes could explain their inability to
recirculate from blood to lymph. Extending this theory to the
immunoblasts in R.A. could account for the lymphocyte rich accum-
ulations in the rheumatoid synovial membrane.

The investigations of Hall have centred largely on B blasts,
but Jacobsson and Blomgren (1973) have produced evidence for a loss of
recirculating capacity of T cells after antigenic stimulation.
A large number of the lymphocytes in the rheumatoid synovium
are T cells (Bankhurst, Husby and Williams, 1976) and may be a
result of selective migration of T blasts to this tissue
(Loewi, Lance and Reynolds, 1975). The mechanism of T cell
attraction to the chronically inflamed synovium may be viewed
in the light of the work of Asherson and co-workers (for review
see Asherson , 1974). He showed that T cells obtained from
lymph nodes draining sites of immunization in rats, or re-injection
into other rats, moved to sites of inflammation in a non-antigen
specific manner. Earlier North (1973) had shown that activated
T cells appeared in the spleen after immunization with Listeria

monocytogenes, and these were considered to be the cells responsible

for the passive transfer of community to Listeria. These cells have

similarly been shown to accumulate at sites of inflammation such

as an oil induced peritoneal exudate. Asherson states that such cells

may be produced by procedures which give rise to cell-mediated

immunity such as allogenic skin grafts, the injection of Freunds

complete adjuvant, and skin painting with contact sensitizing

agents.

In recent work, McGregor and Logie (1974) have shown that rats

infected with L. monocytogenes contain immunoblasts in the thoracic

duct which have the property of homing to peritoneal exudates induced

either by injection of the same or another organism such as Salmonella

typhii. This is most readily explicable in terms of a non-specific

attraction by the endothelial cells of capillaries of inflamed tissues

causing adherence of blast cells followed by their passage through

the capillary wall into the inflammatory site. The blast cells may

develop locally into T lymphocytes and they are able to arm locally

accumulated macrophages, thus rendering them protective (Koster,

McGregor and Mackaness, 1971). It is, of course, an open question

whether a similar mechanism occurs in the rheumatoid synovial membrane

or whether the lymphoid as well as other events are of a more local

nature in the synovium.

Recently, Dumonde, Kelly and Morley (1976) have discussed the

rheumatoid synovium as an ectopic lymphodi organ and suggested that

alteration of lymphoid microvascular endothelia may result in

abnormal lymphocyte function in R.A. Envisaging the rheumatoid

synovium as a lymphoid organ could explain the lymphocyte activation

and accumulation in this tissue. However, the alteration in lymph-

ocyte function could still be ascribed to sequestration of an

antigen(s) within this lymphoid tissue.

There are, as yet, no data on the nature of the cell traffic

between the systemic pool and the local accumulation. Such

data may be obtainable by suitable labelling techniques in rheumatoid

patients. The traffic of small and large lymphocytes could be studied

by labelling the various lymphoid populations from a Ficoll

gradient and monitoring their localisation in the syngeneic

host.

The intense infiltration of the synovium by lymphocytes includes

many aggregates or follicles of plasma cells, which have been shown

to contain deposits of immunoglobulin (Fish et al, 1966). The local

synthesis of large amounts of immunoglobulins, both IgM and

IgG, by rheumatoid synovium has been demonstrated (Sliwinski and

Zvaifler, 1970) and Natvig and Munthe (1975) have interpreted the

role of the many of the plasma cells to be the production of an

IgG rheumatoid factor which combines with similar IgG molecules

("self-associating IgG") within the cell. However, only

20-60% of the IgG plasma cells showed anti-gammaglobulin fixing

activity. The role of the remaining 40-80% and the IgM plasma

cells requires better understanding, particularly as Ig M

is found in lower amounts than IgG in the rheumatoid synovium and

is not identified with complement (Zvaifler, 1973). The

activated B cells demonstrated in this study could be related

to this anti-immunoglobulin synthesis, and hence may be involved

in propogating the disease in the body in this manner. Such an

explanation could result in the sudden onset of polyarthritis noted

clinically in some cases, by the propagation of activated B cells

from regional lymphoid tissues which are sequestered in synovial

tissue in many joints and become RF plasma cells secreting altered

IgG or antiglobulin (IgG or IgM).

The thoracic duct drainage studies of Paulus et al (1977)
suggest that lymphocytes leave the synovial space via lymphatics and
the thoracic duct, rather than by directly entering the blood.
This was determined by following the distribution of reinfused
^{51}Cr-labelled, living autologous lymphocytes. The disappearance
of radioactivity was slower from arthritic joints, which had
preferentially taken up some of the lymphocytes, than from the liver
or spleen, indicating a prolonged retention of some lymphocytes
in the inflamed joint. Support for this hypothesis comes from
Whitehouse et al (1969) who reported that the removal of thoracic
duct lymphocytes from rats that had been injected with Freunds
complete adjuvant tended to prevent the subsequent development of
adjuvant arthritis. However, if the drained lymphocytes were
infused into untreated syngeneic littermates the recipient rats
developed adjuvant arthritis, confirming the suggestion that thoracic
duct lymphocytes were mediators of the disease.

The activated lymphocytes found in the synovial membrane in R.A. may
give rise to the activated lymphocytes demonstrated by microscopy by
Traycoff, Pascual and Schumacher (1976) in the synovial fluid. Again
their presence was restricted to rheumatoid arthritis, being largely
absent in gout, pseudogout or infective arthritis. Sebok, Talerman
and Wouters (1977) found increased lymphocyte activation in R.A.
synovial fluid by comparison with osteoarthritis and gout by the
measurement of increased lymphocyte intra-nuclear birefringence.
The authors interpreted their findings as being due to activation
in the synovial membrane. The nature of these activated cells has not
been determined. Similarly, Eghtedari et al (in press) found immuno-
blasts in the synovial fluid in R.A. as a higher percentage of
lymphoid cells than in peripheral blood and the authors interpreted
their presence as being a result of activation within the joint.

Studies on the T and B cell percentages in the synovial

fluid of R.A. have yielded conflicting results. Utsinger

(1975) reported normal T and B cell percentages in

R.A. synovial fluid and was supported by Brenner et al (1975).

Winchester et al (1973) reported an increased percentage of

E- rosetting cells in R.A. synovial fluid compared with peripheral

blood which Van de Putte et al (1976) explained as a general feature

of inflammatory exudates. The T cells in synovial fluid have been

shown to have impaired blastogenic responses to PHA (Ivanyi,

Lehner and Burrey, 1973; Hepburn, McDuffie and Ritts, 1976)

which may be interpreted as a prior committment of lymphocytes

due to activation in **vivo**.

Analysis of the mitogen and antigen responses of peripheral

blood lymphocytes in R.A. has again produced equivocal results.

The majority of data suggest that the response of R.A lymphocytes

to PHA is identical to control lymphocytes (Georgescu et al.,

1975; Silverman et al, 1976). However, diminished responses have been

recorded (Lockshin et al, 1975) and considered to be due to

the blocking of PHA receptors by a factor present in autologous

serum (Rawson, 1975). Thus the activation of peripheral blood T

lymphocytes in vivo is not suggested. Accordingly R.A. lymphocytes

respond to and stimulate **normally** with healthy lymphocytes in the

MLC reaction (Stastny, 1974).

The range of T lymphocyte found in this study is similar to

that found by other authors; this is not difficult as percentages have

been reported between 30% (Keith and Currey, 1973) and 92%

(Winchester et al, 1973) in the peripheral blood of R.A. patients.

T and B cell percentages are generally reported as being normal

in R.A. (Brenner et al, 1975). This is reasonable since the markers

employed, surface Ig and SRBC receptors are retained by lymphocytes[1]

even upon activation. Thus, enumeration of T and B cell ratios

in peripheral blood and synovial fluid shows no drastic changes in

R.A. and mitogen studies of T cell activity suggest there

may be some in vivo cell activation.

The B cell activation in R.A. intimated in this study

is difficult to relate to previous studies but is not surprising

in view of the hypergammaglobulinaemia and circulating immune

complexes found in R.A. Such increases have also been noted in

other diseases, indeed, serum rheumatoid factor has been detected

in sub-acute bacterial endocarditis and syphilis, indicating the

non-specific nature of the classification or IgM anti-IgG anti-

globulin. The study of PWM responses of peripheral blood lymph-

ocytes in R.A. has shown no differences compared to controls

(Rawson, 1975; Clot, Mathieu, Sany, Serre, 1975; Silverman et al, 1976).

However, PWM activates both human B and T lymphocytes independently

(Janossy and Greaves, 1971) and thus gives no clear indication

of B cell function. Antigen responses by B cells result in the

activation of so few cells that comparisons between R.A. and controls

are difficult.

The presence of rheumatoid factor secreting plasma cells in the

circulation and non-synovial tissues has been demonstrated in certain

R.A. patients by a haemolytic plaque-forming cell assay (Moore

et al, 1978). It has been shown recently (Male, Roitt, and Hay,

1978) that the immune complexes present in the synovial fluid

in R.A. consist almost entirely of gammaglobulins which establish

the importance of the IgG as an auto-immunogen.

Agglutination tests for IgM rheumatoid factor are positive

in 60-70% of patients with R.A. The large variation in titre

does not necessarily reflect disease activity accurately.

The appearance of rheumatoid factor can precede clinical signs

of disease or occur subsequently (Maini, 1977). As previously

discussed (chapter 1) rheumatoid factors have been implicated

as an important component of immune complexes in the synovial

fluid of R.A.,and it has been suggested that both IgG and IgM

classes of rheumatoid factor are involved in activation of

complement enhanced phagocytosis and consequent increased production

and release of lysosomal enzymes.

The activated B cells in peripheral blood are probably precursors

of plasma cells, although a memory function for at least some seems

likely. An increased number of plasma cells would account for

some of the B cell hyperreactivity (Barden et al, 1967; Bandilla

et al, 1970). However, this may represent the non-specific

stimulatory effect of chronic inflammation rather than

specific hyperreactivity of the B cell system. The disturbed

control of B cell function in R.A. may be due to a malfunction of

immune regulation by T cell sub-sets. Certainly a deficiency of

suppressor T cells could explain this. Patients with systemic

lupus erythematosus have been demonstarted with a quantitative

defect in suppressor T cells (Fauci et al, 1978). This disease

is also characterized by a B cell hyperreactivity resulting in a

humoral auto-immune state characterized by hypergammaglobulinaemia

and auto-antibodies against a variety of substances, particularly

nucleoproteins and lymphocytes. T suppressor dysfunction has

also been detected in the NZB/NZW mouse model which is felt to be

the rodent equivalent of SLE (Krakuer, Waldman and Strober, 1970).

It is of interest that Moretta et al (1978) recently demonstrated

that human T lymphocytes with IgG receptors exposed in vitro

to immune complexes irreversibly lost this Fc receptor. T lympho-

cytes with Ig G receptors have been shown to be T suppressors

cells while those with IgM receptors were shown to be T helper

cells. (Moretta et al, 1976). The immune complexes found in the

synovial fluid in R.A. may cause the irreversible loss of T suppressor

cell Fc receptors and ultimate depletion of these cells from the

circulation. It is quite possible that T cell modulation of

B cell function could be affected in this way, both in the joint

and the peripheral blood. This could help to explain the chronicity

of the disease, particularly as IgM immune complexes are more readily

phagocytosed by polymorphs (Hurd et al, 1970). Thus the sero-

positivity of the patient, as assayed by classical IgM rheumatoid

factor would not necessarily reflect the chronicity of the disease,

IgG rheumatoid factor possibly accounting for this by the alteration

of immune regulation. However, as IgM complexes are phagocytosed

more avidly than IgG complexes these wil cause much of the

lysosomal enzyme release. Thus, both types of plasma cell play

a role in the disease pathogenesis. Increased number of circulating

B blasts would be necessary for B cell hyperreactivity in the blood

and the production of IgG and IgM as antiglobulins and IgG as antigen

in the circulation and joint.

Envisaging rheumatoid synovitis as an extravascular immune complex

disease is not difficult (Zvaifler, 1974) and the question must be

asked; do the immune complexes activate the lymphocytes? Hall (1978)

has shown specific migration inhibition in R.A. by immune complexes

and shown (Hall, in preparation) specific binding by rheumatoid

lymphocytes of an altered IgG structure (Facb). The inference

is that not only is the autoimmunogen IgG altered but so are the

lymphocyte receptors. This may be due to the increased avidity

of Fc receptors recorded for R.A. in this study and these cells are

predominantly small lymphocytes. Hence the activated population

may result from IgG activation of small B cells with altered

receptors which upon activation lose .these high avidity

receptors.

This study has shown increased numbers of activated lympho-

cytes in the peripheral blood in R.A. These were shown to be

predominantly B cell, although some T cell activation was noted.

In all of the mechanisms discussed activation of peripheral

blood lymphocytes is likely or indeed an essential component.

It is the immunoblasts which appear to have the ability of progogate

an immune response most effectively. Also it has been shown that

spreading of inflammation to non-inflamed sites may be mediated

by these cells. These properties could be most important in the

immunopathogenesis of R.A.

The presence of a putative antigen, in the joint or

sequestered in lymphoid tissue, must remain a strong possibility.

Doubtless the role of circulating lymphocytes is complex; almost

certainly both T and B populations are involved and activation,

in the joint and in the circulation, a contributing part. Once we

have found out the function of the activated population we will

have isolated a major part of the altered immune mechanisms in R.A.

The increase in knowledge of immunogenetics in recent years

may help us unravel some problems by finding the susceptible subjects.

For now we must content ourselves with the knowledge that many

of the findings in R.A.- lymphocyte infiltration immune complex

formation, autoantibodies, and impaired immuno-regulation-one

common to a number of chronic diseases where an etiologic agent

has been identified. Based on present information I propose that

it will be shown that rheumatoid arthritis is caused by an infectious

agent (or agents) and limited to individuals with a particular genetic

constitution.

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