LOCOMOTION OF DEFINED LYMPHOCYTE SUBSETS IN RELATION TO INFLAMMATION.

By Ian Newman,

Department of Immunology,

Western Infirmary,

Glasgow.

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ABBREVIATIONS USED IN THE TEXT.

α-	Anti-.
Ab.	Antibody.
APAAP	Alkaline phosphatase anti-alkaline phosphatase.
CD	Cluster determinant.
CD45R	Restricted CD45 gene exon usage.
Ch.	Chapter.
cm	Centimetre.
Con A	Concanavalin A.
DTT	1,4-dithiothreitol.
ed.	Editor(s).
e.s.e.	Estimated standard error of the mean.
EXPT.	Experiment.
FACS	Fluorescence-activated cell sorter.
$F(ab')_2$	Antigen-binding fragment of an Ig following pepsin digestion.
FITC	Fluorescein isothiocyanate.
F_c	Crystallisable fragment of immunoglobulins.
FCS	Foetal bovine calf serum.
g	Gram; or acceleration due to gravity.
HBSS	Hanks' balanced salt solution.
HEPES	N-2-[hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid].
HEV	High endothelial venule.
Hrs	Hours.
HSA	Human serum albumin.
ICAM	Intercellular adhesion molecule.

Ig	Immunoglobulin.
IL	Interleukin.
kdal	Kilodaltons.
l	Litre.
LFA	Lymphocyte function-associated molecule.
MIP	Macrophage inflammatory protein.
μg	Microgram.
μl	Microlitre.
μm	Micrometre.
mg	Milligram.
ml	Millilitre.
mm	Millimetre.
mM	Millimolar.
M	Molar.
MLR	Mixed lymphocyte reaction.
MNC	Mononuclear cells.
MOPS	3-[N-morpholino]propanesulphonic acid.
ng	Nanogram.
NK	Natural killer.
nm	Nanometre.
p	Probability.
PAGE	Polyacrylamide gel electrophoresis.
PBS	Phosphate-buffered saline.
PE	Phycoerythrin.
pH	Negative logarithm of hydrogen ion concentration.
PHA	Phytohaemagglutinin.
PWM	Pokeweed mitogen.
pp	Pages.

PPD	Purified protein derivative of Mycobacterium tuberculosis.
R	Receptor.
RANTES	Regulated upon activation, normal T expressed and presumably secreted.
RMW	Relative molecular weight.
RNA	Ribonucleic acid.
rpm	Revolutions per minute.
RPMI	Roswell Park Memorial Institute, medium number 1640.
SDS	Sodium dodecyl sulphate.
S/N	Supernatant.
suppl.	Supplement.
TEMED	N,N,N',N'-tetramethylethylenediamine.
Tris	Tris(hydroxymethyl)methylamine.
TBS	Tris-buffered saline.
U	Units.
V	Volts.
VCAM	Vascular cell adhesion molecule.
VLA	Very late antigen.
Vol.	Volume.
v/v	Volume per volume.
w/v	Weight per volume.
°C	Degrees centigrade.
>	Greater than.
<	Less than.
%	Percent.
+	Positive.

–	Negative.
±	With or without.

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DECLARATION.

The studies described in this thesis are the original work of the author, and have not been submitted in any form to another University. Use of material provided by others has been acknowledged.

Ian Newman.

SUMMARY.

This project examined whether expression of defined CD45R isoforms was related to the capacity of human blood lymphocytes to show locomotion *in vitro*. In addition the role of IL-2 and IL-8 in stimulating such locomotion was investigated.

Established assays of lymphocyte locomotion were utilised, *i.e.* examination for morphological polarization, assessment of invasion into collagen gels, and measurement of cell size in relation to locomotion. A range of stimulators of locomotion was used, and locomotion of lymphocytes expressing CD45RO or CD45RA was assessed. The locomotion of lymphocytes defined by other surface markers, such as CD29 (the VLA-4 ß chain), CD3, CD4, CD8, CD19, CD22 and CD25 was also examined in conjunction with expression of CD45R isoforms.

Adaptation of methods.

The following methods were developed during the project:- (1) adaptation of a method which enables separation of lymphocytes on the basis of invasion of collagen gels, such that a range of surface markers on the invasive and non-invasive populations could be detected using FACS analysis; (2) the development of a method enabling the expression of surface markers to be detected by modified APAAP staining, in

conjunction with the polarization assay; and (3) the adaptation of both of these methods to enable the size of lymphocytes within defined subsets to be determined using forward scatter values derived from FACS analysis, or cell area measurement in conjunction with the polarization assay.

Results.

In polarization assays a significantly greater proportion of CD45RO+ (and CD45RA-), than of CD45RA+ (and CD45RO-), lymphocytes became polarized in response to all stimuli used. The stimuli used were HBSS, FCS, colchicine, IL-2 and IL-8 for lymphocytes on day 0; HBSS, IL-2 and IL-8 for lymphocytes cultured overnight in FCS; and culture of mononuclear cells for 72 hours in HBSS/HSA, α-CD3, PPD, Con A, allo-MLR, PHA or PWM, in which case lymphocytes respond *in situ* to factors released into the culture supernatant.

In collagen gel invasion assays, a consistently greater proportion of CD45RO+ (and of CD29+) lymphocytes, than of CD45RA+ cells invaded collagen gels incorporating their culture supernatants, after culture of mononuclear cells in PPD or allo-MLR. This was the case for both CD4+ and CD8+ lymphocytes, whether invasion proceeded for 3.5 hours or overnight. However no such differential locomotion of lymphocytes expressing different CD45R isoforms was evident if mononuclear cells were cultured in α-CD3 or Con A.

After culture in α-CD3, but not in PPD, marginally more

CD4+ than CD8+ lymphocytes were found to invade collagen gels incorporating their culture supernatants.

Experiments comparing the response of T and B lymphocytes in collagen gel invasion assays (and in polarization assays) were restricted since it was observed that culture of mononuclear cells in α-CD3 led to down-regulation of surface CD3.

Recombinant human IL-8 was found to stimulate locomotion into collagen gels of lymphocytes cultured in α-CD3. Antibodies to IL-8 blocked the ability of supernatants from mononuclear cells cultured in α-CD3 to stimulate lymphocyte locomotion into collagen gels. These results suggest a pivotal role for IL-8 in the activity of such supernatants. Preliminary experiments indicated preferential invasion by CD45RO+ compared to CD45RA+ lymphocytes of collagen gels containing IL-8, if cells were cultured in PPD or allo-MLR, but not in α-CD3.

Measurement of the cell volume of lymphocytes using forward scatter values derived from FACS analysis showed that CD45RO+ lymphocytes were significantly larger than CD45RA+ cells when freshly isolated, or after culture in HBSS/HSA, α-CD3, PPD, Con A, PHA, PWM or allo-MLR. Using cell area measurement by image processing of round or polarized lymphocytes, CD45RO+ lymphocytes were significantly larger than CD45RA+ cells when freshly-isolated, or after culture in HBSS/HSA, α-CD3, PPD or Con A. When cell areas were measured

by bitpad tracing of cell outlines, similar results were found using all of these stimuli except PPD (in which case the subsets were of similar size), while culture in PHA and PWM resulted in CD45RO+ lymphocytes being of similar size to CD45RA+ cells.

Expression of the activation marker CD25 by lymphocytes cultured in α-CD3 was not greatly enhanced among polarized cells, or among cells which invaded collagen gels incorporating their culture supernatants. The mean forward scatter of lymphocytes which had invaded collagen gels was similar to that of non-invasive lymphocytes.

It was observed that culture of lymphocytes in RPMI/FCS, rather than in the routine medium (HBSS/HSA), altered the proportions of lymphocytes in both polarized, and in blast-like, morphology, as well as expression of CD45RO, CD45RA and CD25 by lymphocytes.

Results are discussed in relation to lymphocyte locomotion and adhesion *in vitro*. Possible extrapolations to observations of lymphocyte distribution *in vivo*, and to lymphocyte functions, are suggested.

CHAPTER 1: INTRODUCTION

SECTION 1A: LYMPHOCYTES, AND ASPECTS OF THEIR FUNCTIONS.

1A.1 Introduction.

The function of the immune system is to provide protection against harmful infection by foreign organisms. Many of the characteristic features of the immune system employed for this function are specific for particular organisms, and adapt once infection has been encountered, giving an enhanced response to subsequent infection. Although many cell types interact to form the immune system, it has become established that the pivotal cell type mediating the specificity and adaptive nature of immunity is the lymphocyte.

Lymphocytes were first described by Hewson in the 1770's (Elves, 1972; McGregor and Mackaness, 1974; Powers, 1989), but their functions and importance remained enigmatic for almost 200 years. Their emergence from this obscurity is widely credited (Ebert and Grant, 1974; Miller, 1977; De Sousa, 1981; Thompson and Proctor, 1984; Roitt, 1991) to have started with the work of Gowans in the late 1950's (reviewed by Gowans and McGregor, 1965). Since then experiments have established critical roles for lymphocytes in antibody production, graft rejection and graft-versus-host reactions, cell mediated cytotoxicity, delayed-type hypersensitivity, cytokine production and the phenomenon of immunological memory (McGregor and Mackaness, 1974; Dvorak, 1973; Ebert and Grant,

1974; Thompson and Proctor, 1984; Roitt, Brostoff and Male, 1989; Roitt, 1991). Lymphocyte infiltration is a hallmark of delayed-type hypersensitivity reactions, graft rejection, contact sensitivity and rheumatoid synovitis (Dienes and Mallory, 1932; Medawar, 1944; Turk, Heather and Diengdoh, 1966; Norton and Ziff, 1966).

Lymphocytes are the predominant cell type in lymph, lymph nodes, thymus, splenic white pulp and in unencapsulated lymphoid tissue in the alimentary tract and lung. They are also found in large numbers in bone marrow and blood, and in smaller numbers in virtually all tissues (McGregor and Mackaness, 1974; Brenner and Hoffbrand, 1992). In adult humans it has been estimated that there are approximately 10^{12} lymphocytes, constituting 0.5-2% of body weight (Roitt, Brostoff and Male, 1989; Brenner and Hoffbrand, 1992). Cells with morphology similar to mammalian lymphocytes are found in all vertebrate species so far examined (Good *et al.*, 1966; Elves, 1972; Roitt, 1991).

Human blood contains red cells, platelets and white cells. Among the white blood cells in health, 20-45% are lymphocytes, the remainder being neutrophils, monocytes, eosinophils and basophils (Dacie & Lewis, 1975; Hoffbrand and Pettit, 1984).

The process of haematopoiesis in humans begins in the yolk sac early in gestation, and lymphocytes have been observed in the circulation of 8 week-old foetuses. Haematopoiesis begins in the liver, and to a lesser extent in the spleen, from approximately 10 weeks of gestation. The

liver then becomes the major site of blood cell production until birth. Haematopoiesis starts in the bone marrow at approximately 12 weeks of gestation, and this is the predominant site of production in the neonate, and beyond into adulthood. Bone marrow-derived precursor cells also seed the thymus, which becomes a major site of lymphocyte production and development. Stimulated secondary lymphoid tissue can also become an important site of lymphocyte production (Hall and Malia, 1984; Clark, 1991; Brenner and Hoffbrand, 1992).

1A.2 Activation of lymphocytes *in vitro*.

Until the discovery that PHA caused mitosis of lymphocytes *in vitro* (Nowell, 1960), it was thought that small lymphocytes were short-lived, and that long-term culture of normal blood lymphocytes was not possible (Nowell, 1976). Subsequently other plant lectins were found to cause mitogenesis of normal blood lymphocytes, including PWM (Farnes *et al.*, 1964) and Con A (reviewed by Powell and Leon, 1970). Mitogenesis is also caused by culture of mononuclear cells in allogenic MLR (Bain, Vas and Lowenstein, 1963), PPD (Pearman, Lycette and Fitzgerald, 1963) or in anti- CD3 (Van Wauwe, De Mey and Goossens, 1980).

Upon activation *in vitro*, resting lymphocytes (*i.e.* in G_o phase of cell cycle) immediately begin to increase in size, and in synthesis of protein and ribonucleic acids, as cells enter cell cycle (Astaldi and Lisiewicz, 1971; Elves, 1972; Roitt, Brostoff and Male, 1989; Roitt, 1991). Synthesis of

deoxyribonucleic acids is delayed for at least 48-72 hours. Lymphocytes may also differentiate, begin to secrete cytokines and undergo cell division.

1A.3 Heterogeneity among lymphocytes.

It is established that efficient control and function of the immune system involves many different cell types, but in order to delineate the mechanisms involved it is necessary to understand which cells are present, and how they function, in particular situations. For many years attempts have been made to use the phenotype of cells as a marker to explain or predict their functions, and how they interact with other cells in *in vitro* or *in vivo* phenomena. The use of cytological staining pioneered by Ehrlich allowed straightforward distinction of lymphocytes from other cell types (Astaldi and Lisiewicz, 1971; Powers, 1989). Sizing of lymphocytes, stained by such methods, was the first classification system used. Using conventional Romanovsky stains approximately 90% of blood lymphocytes appear to be a homogeneous population of relatively small cells (6-10μm in diameter), while larger cells (12-16μm in diameter) constitute approximately 10% of blood lymphocytes (McGregor and Mackaness, 1974; Thompson and Proctor, 1984; Hoffbrand and Pettit, 1984; Powers, 1989).

However, classification of lymphocytes according to size gives only limited information about cell function, particularly in identification of cells responsible for the

humoral, and cell-mediated, arms of the immune system, the relative importances of which were historically the subject of considerable debate (Silverstein, 1989). Clinical observations of diseases such as Di George's syndrome and Bruton's agammaglobulinaemia suggested that deficiencies of cell-mediated and humoral immunity could be delineated (Clark, 1991). A role for the thymus in immunity had been proposed by Beard in 1900 (Miller and Osaba, 1967), and the experiments of Miller (1961) demonstrated its crucial role in cell mediated immunity. The importance of the bursa of Fabricius for antibody production in the chicken (reviewed by Warner and Szenberg, 1964) was first demonstrated by Chang, Glick and Winter (1955). The "bursal equivalent" in mammals appears to be the bone marrow (Roitt, Brostoff and Male, 1989; Roitt, 1991). These studies led to the designation of lymphocytes which have matured in the thymus as T cells, and those produced in the bursal equivalent as B cells (Roitt *et al.*, 1969). This classification broadly defines lymphocytes pivotal for the cell-mediated and humoral arms of the immune system respectively.

1A.4 Surface markers of lymphocyte subsets.

The use of antibodies against cell surface antigens on lymphocytes has proved to be the most useful approach to identify phenotypic markers. Cantor and Boyse (1975), and Kisielow *et al.* (1975) used allo-antisera to define functional subsets among T cells. The use of monoclonal antibodies

(Köhler and Milstein, 1975) is yet more effective, because preparations of identical antibodies can repeatedly be made.

Antibodies can be used to identify, separate, or stimulate cells via their particular ligand, for example in the identification of the lymphocyte molecule responsible for binding to sheep red blood cells (Howard *et al.*, 1981). Rosetting of human lymphocytes with sheep red blood cells (Brain, Gordon and Willetts, 1970; Coombs *et al.*, 1970) enabled separation of T and B cells (Jondal, Holm and Wigzell, 1972; reviewed by Moingeon *et al.*, 1989). Antibodies have been raised which recognise CD2 (the molecule responsible for binding sheep red blood cells), and the use of such antibodies has demonstrated the importance of CD2 in cell/cell adhesion and cell signalling (reviewed by Springer *et al.*, 1987; Clevers *et al.*, 1988; Bierer *et al.*, 1989).

Monoclonal antibodies to many other lymphocyte surface proteins have also proved to be useful as definitive markers for various subsets, particularly if their ligands have important functions in cell signalling. These include CD3, on T cells; CD19 and CD22 on B cells; and CD4 and CD8 within the T cell population. CD3, CD19 and CD22 are crucial in lymphocyte signalling via their specific antigen receptors (reviewed by Clevers *et al.*, 1988; Bierer *et al.*, 1989; Hořejší, 1991; Clark, 1991; Janeway, 1992). CD4 and CD8 are the molecules on T cells responsible for binding to major histocompatibility complex restriction elements crucial for self/non-self discrimination, and expression of CD4 or CD8 broadly defines T cells with helper and cytotoxic function

respectively (reviewed by Springer *et al.*, 1987; Von Boehmer, 1988; Clevers *et al.*, 1988; Von Boehmer *et al.*, 1988; Bierer *et al.*, 1989; Janeway, 1992; Miceli and Parnes, 1993).

As well as T and B cells, blood contains a third major subset of lymphocytes, with natural killer (NK) activity (Heberman *et al.*, 1973; Takasugi, Mickey and Terasaki, 1973; Kiessling, Klein and Wigzell, 1975). Lymphocytes responsible for NK activity were first identified morphologically, as large granular lymphocytes (Timonen, Ortaldo and Heberman, 1981). However not all large granular lymphocytes are NK cells, and monoclonal antibodies recognising surface markers such as CD56 are now used to identify NK cells (Ritz *et al.*, 1988).

In health approximately 14% of human blood lymphocytes are NK cells, 72% are T cells, and 13% are B cells (Hannet *et al.*, 1992).

It has been recognised for many years that there are functional subsets among both CD4+ and CD8+ lymphocytes, but definitive surface phenotypic markers have proved elusive. However differential cytokine secretion has recently been used to discriminate TH1 and TH2 subsets among CD4+ lymphocytes in particular situations *in vitro* and *in vivo* (reviewed by Mosmann and Coffman, 1989).

1A.5 Lymphocyte recirculation.

As emphasised previously, a crucial role of T and B cells is recognition of specific determinants of infectious

organisms or neoplastic cells. This requires contact with surface structures which are unique to each lymphocyte or its progeny. A major feat of the immune system is the ability to survey the entire organism with a pool of lymphocytes of wide enough specificities to mount responses against a vast potential repertoire of organisms which may cause infection or re-infection at any site.

Experiments in the early 1900's demonstrated that cannulation of the thoracic duct of various mammals led to a decrease in numbers of lymphocytes in the thoracic duct lymph, and in the blood (reviewed by Lee, 1922). Gowans (1957) demonstrated that intravenous re-infusion of thoracic duct lymphocytes maintained the numbers of lymphocytes in thoracic duct lymph, and showed that lymphocytes recirculated between the blood and lymphatic system. This provides the major mechanism for surveillance of the body for antigenic insult. Gowans and Knight (1964) identified specialised endothelium-the HEV of post-capillary venules as the major site of entry of small lymphocytes into lymph nodes from blood. HEV-like morphology can also be observed in sites of immune reactions, for example: chronic inflammation (Graham and Shannon, 1972); sites of malignancy (Freemont, 1982); skin graft rejection (Dvorak *et al.*, 1979); and chronic arthropathies (Freemont *et al.*, 1983). The architecture of sites such as chronic granulomata and the rheumatoid synovium resembles that of lymph nodes, and similar trafficking mechanisms may occur (Rannie, Smith and Ford, 1977; Janossy *et al.*, 1981).

The importance of adhesive interactions between vascular

endothelium and leucocytes in the selective localisation of cells from the blood has only become established in the last 10 years (Bevilacqua, 1993). The expression of homing receptors on lymphocytes and their counterstructures (vascular addressins) on HEV (Butcher, 1986) may in part explain the non-random distribution of lymphocyte subsets observed throughout the body (Stevens, Weissman and Butcher, 1982). Similar homing receptors may enable selective traffic of small lymphocytes through sites of chronic inflammation (Jalkanen *et al.*, 1986).

While crossing of endothelium at HEV is undoutedly the major route for small lymphocyte extravasation in normal rodent and human lymph nodes, small lymphocytes can extravasate in the absence of HEV-like structures, for example: in neonatal, or thymectomised, mice (Parrott, DeSousa and East, 1966; Miller, 1969); in the spleens of chickens (Miller, 1969) and mice (Parrott, DeSousa and East, 1966); and in sheep (Hall, 1989), which appear to have no HEV-like structures.

Recirculation between the blood and lymphatics is predominantly a property of small lymphocytes (Gowans and Knight, 1964). Activated lymphocytes appear capable of re-localisation in the tissue in which they were activated (Griscelli, Vassalli and McCluskey, 1969; Rose, Parrott and Bruce, 1976), and in the gut (Gowans and Knight, 1964; Moore and Hall, 1973). There is also considerable evidence that a proportion of lymphocytes activated *in vitro* or *in vivo*, and re-introduced into the bloodstream, localise at sites of

inflammation. This localisation is relatively independant of the specific means of activation, or the nature of the inflammatory stimulus (Prendergrast, 1964; Griscelli, Vassalli and McCluskey, 1969; Asherson, Allwood and Mayhew, 1973; Moore and Hall, 1973; McGregor and Logie, 1974; Rose, Parrott and Bruce, 1976).

Thus lymphocytes are capable of crossing non-specialised endothelium, and the mechanisms governing such interactions are currently being intensively investigated (reviewed by Stoolman and Kaldjian, 1992; Hogg, 1992; Pardi, Inverardi and Bender, 1992; Zimmerman, Prescott and McIntyre, 1992; Bevilacqua, 1993). Lymphocytes can utilise the binding of L-selectin to endothelial selectins, or carbohydrate for initial arrest from the circulation, and the binding of molecules such as LFA-1 or VLA-4 to structures such as ICAM-1, ICAM-2 or VCAM-1 on endothelial cells, to stabilise adhesion. After activation of endothelial cells, expression of ICAM-1 (which binds LFA-1 on lymphocytes) increases rapidly, while expression of VCAM-1 (which binds VLA-4 on lymphocytes) increases more slowly, but is more persistent. Other lymphocyte surface molecules such as CD2, CD4, CD8, CD31, CD44 and CD58 may contribute to the binding of lymphocytes to endothelium. However transmigration of lymphocytes through endothelium does not directly correlate to expression of any of these molecules. Thus other mechanisms also influence the type of leucocyte, or of lymphocyte subsets, which leave the circulation at particular sites.

1A.6 Lymphocyte locomotion *in vitro* .

While leucocyte/endothelial cell interactions, and alterations in local blood flow (Hay *et al.*, 1977) affect the numbers of lymphocytes initially arrested at particular locations, a critical feature of lymphocyte traversal of endothelium, variations in transit times through tissues (Rose, Parrott and Bruce, 1978), and close cell/cell contact, is the process of lymphocyte locomotion. While local proliferation may contribute to an increase in numbers of lymphocytes at a particular site, studies using radiolabelled leucocytes suggest that lymphocytes can rapidly localise from systemic pools to sites of inflammation (Goldman and Walker, 1962; Kosunen, Waksman and Samuelson, 1963; Kosunen *et al.*, 1963; Loewi, 1969).

Study of active locomotion *in vivo* is necessarily very difficult, and most information has been gained from *in vitro* studies, which potentially allow delineation of the processes involved in the more complex situation *in vivo*. However investigation of lymphocyte locomotion has historically been hampered by the fact that few blood lymphocytes appear to be motile *in vitro*, and by the lack of defined stimulators of lymphocyte locomotion.

Many definitive studies on the morphology of motile lymphocytes (Lewis and Webster, 1921; McCutcheon, 1924; Lewis 1931a; Lewis, 1931b; De Bruyn, 1944) were carried out long before the important functions of lymphocytes became clear. Indeed locomotor morphology was used as a measure of cell

viability (Gowans, 1957), and to distinguish lymphocytes from other cell types (reviewed by McGregor and Mackaness, 1974). It has been shown that motile lymphocytes initially put out pseudopods. At the bases of pseudopods constriction rings may then be formed, and these appear stationary as the cell body flows forward. Moving lymphocytes adopt a characteristic "pear" or "hand-mirror" shape. Lymphocytes in locomotor morphology have been observed apparently crossing HEV; non-specialised endothelium in sites of nephropathy; the marginal zone of the spleen; and sinusoids of bone marrow (reviewed by Parrott and Wilkinson, 1981). Non-adhesive contacts with three-dimensional matrices may facilitate locomotion (Haston, 1979; Haston, Shields and Wilkinson, 1982). Most of these observations were made using time-lapse cinematography, but other assay systems which have also been widely used are the filter assay, the collagen gel invasion assay and the polarization assay.

1A.6.1 The filter assay.

The filter assay of Boyden (1962) allowed examination of leucocyte migration into cellulose ester filters, and gave impetus to studies of lymphocyte locomotion. This assay, or modifications of it, has been widely-used to investigate various factors which might cause locomotion of human or rodent lymphocytes. Such factors include anti-immunoglobulin (Unanue, Ault and Karnovsky, 1974; Schreiner and Unanue, 1975; Ward *et al.*, 1977); casein (Russell *et al.*, 1975; Moudgil *et*

al., 1977; El Naggar, Van Epps and Williams, 1980; El-Naggar Van Epps and Williams, 1981; Botazzi *et al.*, 1985; Bacon *et al.*, 1988); and IL-2 (Kornfeld *et al.*, 1985; Robbins *et al.*, 1986; Taub *et al.*, 1993a).

The reported activities of C5a (El Naggar, Van Epps and Williams, 1980; El-Naggar, Van Epps and Williams, 1981; Botazzi *et al.*, 1985; Pohajdak *et al.*, 1986); N-formyl-methionine-leucine-phenylalanine (El Naggar, Van Epps and Williams, 1980; El-Naggar, Van Epps and Williams, 1981; Adams *et al.*, 1991); and IL-1 (Miossec, Yu and Ziff, 1984; Bacon *et al.*, 1988; Bacon, Westwick and Camp, 1989) as stimulators of lymphocyte locomotion remain to be established (Wilkinson, 1987).

More recently several other factors have been reported to stimulate lymphocyte locomotion into filters. These include: IL-4 (Clinchy *et al.*, 1991); IL-8 (Larsen *et al.*, 1989a; Bacon, Westwick and Camp, 1989; Leonard *et al.*, 1990; Zachariaè *et al.*, 1992; Sebok *et al.*, 1993); IL-10 (Zachariaè *et al.*, 1992; Jinquan *et al.*, 1993); RANTES (Schall *et al.*, 1990; Taub *et al.*, 1993a; Taub *et al.*, 1993b); transforming growth factor ß (Adams *et al.*, 1991); MIP-1α and -ß (Schall *et al.*, 1993; Taub *et al.*, 1993a); and interferon-inducible protein-10 (Taub *et al.*, 1993b).

Many of these studies examined whether the responses of lymphocytes to test factors were chemotactic. Chemotaxis can be defined as a directional response to a gradient of attractant (Keller *et al.*, 1977; Wilkinson, 1985a), and is one

mechanism by which cells may localise at specific sites of production of a attractant. However since blood lymphocytes represent a non-homogeneous population in locomotor responses, chemotaxis is difficult to assess in preparations of blood lymphocytes (Wilkinson, Haston and Shields, 1982). The first demonstration of chemotaxis by a homogeneous population of lymphocytes in a filter assay was by Russell *et al.* (1975), using lymphoblast cell lines.

1A.6.2 The collagen gel invasion assay.

Collagen gels have been shown to be a useful substratum for the study of lymphocyte locomotion (Haston, Shields and Wilkinson, 1982; Schor, Allen and Winn, 1983). Several factors have been shown to stimulate lymphocyte locomotion into collagen gels. These include colchicine (Wilkinson, 1986); phorbol myristate acetate (Wilkinson *et al.*, 1988); and IL-8 (Wilkinson and Watson, 1990). Unequivocal lymphocyte chemotaxis has been demonstrated using the collagen gel assay (Wilkinson, 1985b). Locomotion of lymphocytes on the surface of two-dimensional collagen films has also been demonstrated (Sundqvist and Otteskog, 1987; Arencibia and Sundqvist, 1989).

1A.6.3 The polarization assay.

The polarization assay (Haston and Shields, 1985), which enables lymphocytes in locomotor (polarized) morphology to be

scored, has been used to demonstrate stimulation of locomotion by several defined factors. These include: colchicine and vinblastine (Wilkinson, 1986); phorbol myristate acetate and phorbol dibutyrate (Wilkinson and Higgins, 1987b; Wilkinson *et al.*, 1988); and IL-8 (Wilkinson and Watson, 1990).

Recently several new substrata for the measurement of lymphocyte locomotion have been reported. These include monolayers of HEV-like cells (Ager and Mistry, 1988; Harris, 1991) or umbilical vein endothelium (Masuyama *et al.*, 1992); and test factors bound to plastic, fibronectin or VCAM-1 (Tanaka *et al.*, 1993).

1A.7 Locomotion of T, B, CD4+ and CD8+ subsets *in vitro*.

Various systems and methods of phenotyping lymphocytes responding in *in vitro* locomotion assays have been reported.

Several such reports have used collagen gel assays to investigate the locomotion of freshly-isolated lymphocytes in response to FCS. The first report of the locomotion of human lymphocyte subsets (Wilkinson, 1985b), used the collagen gel assay, and also demonstrated that similar proportions of T and B cells, but slightly more of the CD4+ than of the CD8+ populations, were motile, using immunofluorescence to identify subsets. Shiu and Schor (1986) found that isolated human T and B cells, and helper and cytotoxic T cell subsets, entered collagen gels in similar proportions. Ratner, Jasti and Heppner (1988) reported that mouse lymphocytes digested from

collagen gels contained similar proportions of T and B cells, and T cell subsets, compared to the starting population.

In polarization assays combined with immunofluorescent staining, predominantly T cells were shown to be responsive *in situ* after culture in α-CD3 (Wilkinson and Higgins, 1987a); while predominantly B cells were found to respond after culture in protein A from Staphylococcus aureus, IL-4 or interferon-γ (Wilkinson and Higgins, 1987b; Wilkinson and Islam, 1989).

Filter assays have been widely used to investigate the responses of lymphocytes to defined factors. The response of rat lymphocytes to anti-immunoglobulin rested chiefly among isolated B cells (Ward *et al.*, 1977). Casein stimulated locomotion in a greater proportion of isolated non-T cells, than of T cells, among human blood lymphocytes (O'Neill and Parrott, 1977), while responses to casein among isolated CD4+ and CD8+ cells were similar (El-Naggar, Van Epps and Williams, 1980). IL-2 stimulated locomotion of isolated mouse NK cells (Natuk and Welsh, 1987); while IL-8 has been shown to stimulate locomotion of isolated human NK cells (Sebok *et al.*, 1990).

The examination of lymphocytes which have migrated to the underside of Boyden-type filters, or through the filter to a lower compartment, has recently been extensively used to investigate the locomotion of subsets of human lymphocytes, generally after overnight culture in FCS. Subsets were isolated first, or were identified by APAAP staining of the filters, or FACS analysis of cells which had migrated into the

lower compartment. A greater proportion of human CD8+ than of CD4+ lymphocytes migrated in response to IL-10 (Zachariaè *et al.*, 1992; Jinquan *et al.*, 1993). Responses to IL-8 on day 0 were found to be similar among CD4+ and CD8+ lymphocytes by Larsen *et al.* (1989a), while Zachariaè *et al.* (1992), and Jinquan *et al.* (1993), found preferential migration of CD4+ lymphocytes compared to CD8+ cells. Schall *et al.* (1990 and 1993) demonstrated that T cells and CD4+ lymphocytes, but not B cells or CD8+ lymphocytes, responded to RANTES, MIP-1ß and MIP-1α (if MIP-1α was used at 10 ng/ml). However if MIP-1α was used at 100 pg/ml, all 4 subsets were found to respond.

Similar methods have suggested that activation of freshly-isolated lymphocytes (by incubating with α-CD3 for 6-8 hours) may affect the responses of particular subsets in a complex manner (Taub *et al.*, 1993a; Taub *et al.*, 1993b). Thus resting or activated T cells, or CD4+ lymphocytes, responded to RANTES; activated (but not resting) T cells responded to MIP-1α or MIP-1ß; activated (but not resting) CD4+ lymphocytes responded to MIP-1ß, but not to MIP-1α; while activated (but not resting) B cells and CD8+ lymphocytes, responded to RANTES, MIP-1α or MIP-1ß.

Such results serve to emphasize that there is no simple pattern of response of T, B, CD4+ or CD8+ lymphocytes in *in vitro* locomotion assays. Variations may occur as a result of different stimulators of locomotion, different assay systems and different methods of isolating or culturing lymphocytes. In addition the activation status of the cells may affect

their responses.

1A.8 Enhancement of locomotion by activation of lymphocytes.

It has long been recognised that both small and large lymphocytes, as well as lymphoblasts, may be motile *in vitro* (Sabin, 1923; McCutcheon,1924; McFarland, Heilman and Moorhead, 1966; Russell *et al.*, 1975; Wilkinson *et al.*, 1976; Wilkinson *et al.*, 1977). As small lymphocytes become activated *in vitro*, there is an increase in cell size and volume (MacKinney, Stohlman and Brecher, 1962). This increased size has been correlated to an enhanced locomotor capacity *in vitro* (Wilkinson, 1986). Suitable activators include polyclonal mitogens such as Con A, α-CD3 and PHA, plus antigens such as PPD and allo-MLR (Wilkinson *et al.*, 1976; Wilkinson, 1986). Thus blood lymphocytes acquire locomotor capacity after culture. This can be assessed by examination of cells *in situ* during culture, or after washing cells after culture and re-stimulating with defined stimuli for short-term responses (Wilkinson and Watson, 1990). It has been demonstrated that the immunosuppressive drugs cyclosporin A and FK506 block the acquisition of locomotor capacity (Wilkinson and Higgins, 1987b; Wilkinson and Watson, 1990), while pertussis toxin inhibits short-term responses of lymphocytes to certain defined stimuli (Spangrude *et al.*, 1985; Wilkinson and Watson, 1990).

The activation-associated enhancement of lymphocyte locomotor capacity *in vitro* has also been related to the

previously-described localisation of activated lymphocytes, re-introduced *in vivo*, at sites of inflammation (Parrott and Wilkinson, 1981).

It has been observed that polarized lymphocytes, and lymphocytes adherent to and crossing endothelial cell monolayers into collagen gels, have increased activation markers and synthesis of RNA and protein, compared to the starting population (Wilkinson, 1986; Masuyama *et al.*, 1992). These studies also suggested that motile lymphocytes contain higher proportions in the G_1 than in G_o phases of cell cycle, although Ratner, Jasti and Heppner (1988) and Ratner (1992), found similar proportions of motile cells in G_o and G_1 phases, while fewer of the motile cells were in G_2 and M phases of cell cycle.

Examination of phenotypic markers of activation on the surface of lymphocytes provides another means of relating activation status to the type of cells which localise in sites of inflammation, as well as giving information about locomotion of lymphocyte subsets *per se*.

One lymphocyte surface glycoprotein which might serve as a candidate marker for the activation status of lymphocytes is CD45, the potential functions of which have recently been extensively studied using a variety of approaches.

1A.9 Characterisation of the CD45 molecule.

CD45 has been designated as the name for the molecule

previously known as Ly-5, T200 and the leucocyte-common antigen (L-CA). The molecule was first recognised using alloantisera in the rat (ART-1; Lubaroff, 1973) and mouse (Ly-5; Komuro *et al.*, 1975). SDS-PAGE analysis of mouse T and B cells (Trowbridge, Ralph and Bevan, 1975), and rat T and B cells, thymocytes and thoracic duct lymphocytes (Standring and Williams, 1977) indicated predominance of relatively few surface glycoproteins, for example T200 in mouse T cells. Rabbit xenoantisera were raised against T200 (Trowbridge and Mazaukas, 1976) and rat thoracic duct lymphocytes (Fabre and Williams, 1978). The latter antibody was found to react with thymocytes, blood lymphocytes and bone marrow cells, and its antigen was termed L-CA. Immunoprecipitation of rat lymphoid cells with another anti-L-CA antibody (Standring *et al.*, 1978) revealed reaction with glycoproteins of differing molecular weights from different cell populations *i.e.* thymocytes expressed predominantly low molecular weight L-CA, B cells expressed predominantly the high molecular weight L-CA, and T cells expressed a mixture of molecular weight forms.

In humans CD45 is found on all cells of haematopoietic origin except mature red blood cells (reviewed by Thomas, 1989). It is one of the most abundant cell surface glycoproteins on leucocytes, and it has been estimated that CD45 might cover 10-27% of the external surface of a lymphocyte (Williams and Barclay, 1986; Thomas, 1989; Barclay *et al.*, 1993). Because of the potential importance of such an abundant surface molecule, a number of studies have attempted to define a function for lymphocyte CD45.

CD45 has been shown to be linked to several important surface molecules on lymphocytes, including CD2, CD3, CD28, and CD19 (Ledbetter *et al.*, 1988; Dianzani *et al.*, 1990; Schraven *et al.*, 1990). CD45 has also been demonstrated in association with fodrin-a component of the membrane-associated cytoskeleton (Bourguignon *et al.*, 1985). Several lectins, for example PHA and Con A, appear to bind to CD45 (Henkart and Fisher, 1975; Sitkovsky *et al.*, 1984). All forms of CD45 so far examined have been shown to bind to CD22 (Stamenkovic *et al.*, 1991; Aruffo *et al.*, 1992; Engel *et al.*, 1993).

The first animal protein tyrosine phosphatase found was shown to have homology to the cytoplasmic domain of CD45 (Charbonneau *et al.*, 1988; Tonks *et al.*, 1990), and CD45 has been demonstrated to have protein tyrosine phosphatase activity (Clark and Ledbetter, 1989; Kiener and Mittler, 1989; Ostergaard *et al.*, 1989; Tonks, Dilz and Fischer, 1990; Streuli *et al.*, 1990). Treatment of cells with anti-CD45 has been reported to increase phosphatase activity, and lead to activation of a T cell clone (Goldman *et al.*, 1992). Expression of CD45 was required for CD4/CD8-mediated cell signalling via the T cell receptor, and it has been suggested that CD45 interacts with tyrosine kinases on CD4 and CD8 (Guttinger *et al.*, 1992; reviewed by Janeway, 1992; Ledbetter *et al.*, 1993). Expression of CD45 appears to be essential for certain cell lines to proliferate in response to antigen *in vitro*, but this may vary among other cell lines (reviewed by Koretsky, 1993).

Treatment of CD45 with blocking antibodies has been shown

to affect several lymphocyte functions. On mouse lymphocytes, the functions blocked included: proliferation in response to Con A; splenic B cell plaque-forming activity; T cell cytotoxicity; antibody-dependent cell cytotoxicity; and NK cell functions (Kasai *et al.*, 1979; Davignon *et al.*, 1981; Seaman *et al.*, 1981; Nakayama, 1982; Yakura *et al.*, 1983; Harp, Davis and Ewald, 1984). Among human lymphocytes, antibodies to CD45 affect T,B and NK cell activation, proliferation and differentiation (LeFrancois and Bevan, 1985; Mittler *et al.*, 1987; Smeland *et al.*, 1990; Kiener and Mittler, 1989; Newman, 1982).

1A.10 Isoforms of CD45.

In humans CD45 has two homologous cytoplasmic domains containing 705 amino acids, and a transmembrane domain of 22 amino acids. The external sequence can contain 391-552 amino acids, depending on expression of various isoforms (reviewed by Thomas, 1989). The variable length of the external domain arises from splicing out of various exon products at the mRNA level (Streuli *et al.*, 1987a). The gene for CD45 has 33 exons (Hall *et al.*, 1988). Three of these exons-numbers 4, 5 and 6 (denoted A, B, or C respectively; Streuli *et al.*, 1987a), may or may not be expressed. 8 potential isoforms can be generated in this manner, most of which have been detected in humans, mice and rats, and some of which may be of similar molecular weight (reviewed by Thomas and LeFancois, 1988;

Thomas, 1989). Forms of CD45 showing restricted exon usage are termed CD45R. Figure 1.1 illustrates how various CD45 exons may be utilised, and the effects on the molecular weights of the products. Figure 1.2 illustrates the predicted structure and size of rat CD45 isoforms.

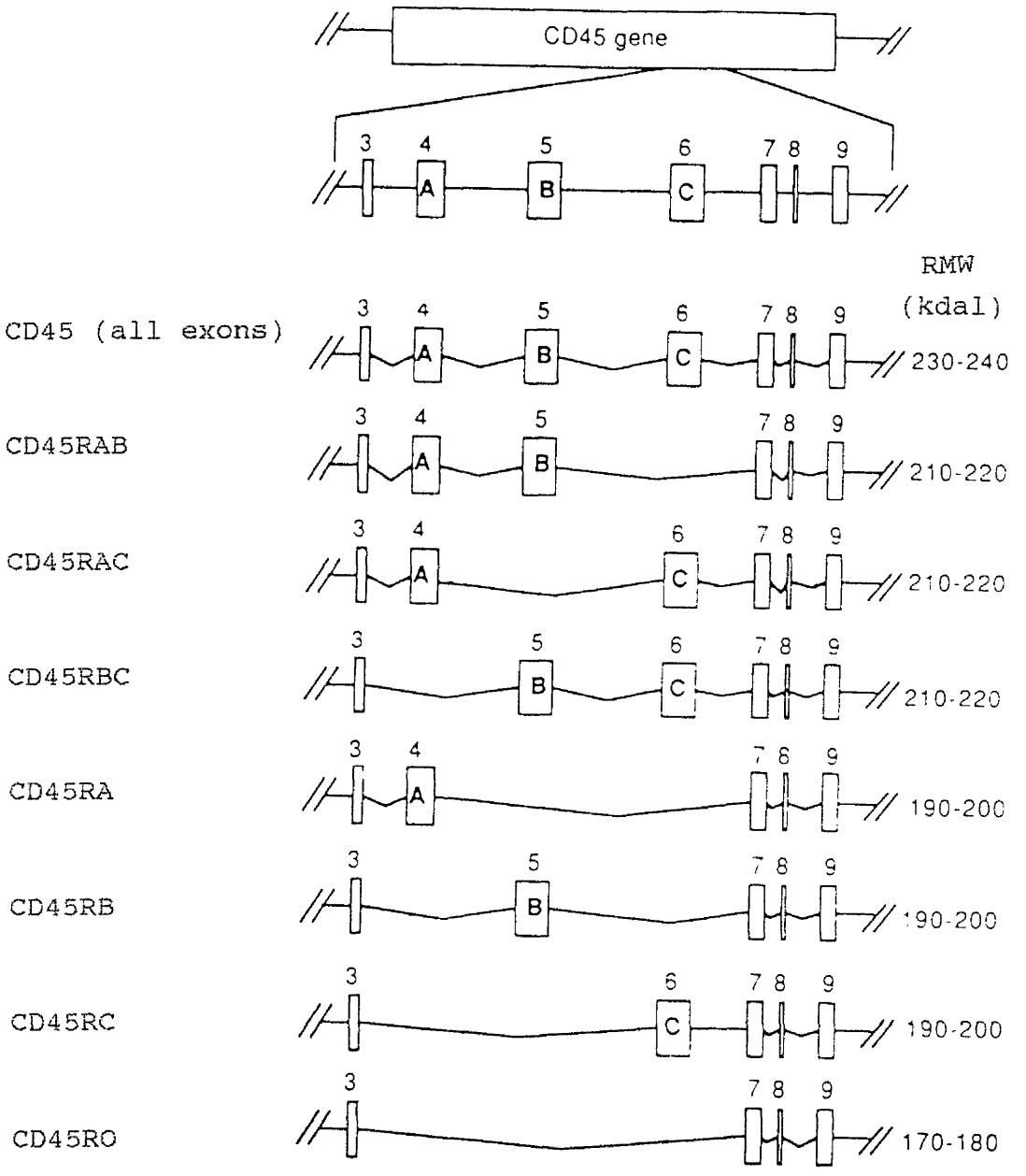
Although different isoforms of CD45 vary considerably in their extracellular sequences, differences in function have proved difficult to demonstrate. All isoforms of mouse CD45 showed phosphatase activity (Ostergaard *et al.*, 1989; Yamada *et al.*, 1990). It has been shown that the CD45 on rat B cells, but not T cells, bound the lectin soybean lentil (Brown and Williams, 1982). CD45RB was demonstrated to associate with CD4 and the T cell receptor to a lesser extent than other isoforms of CD45 on mouse lymphocytes (Dianzani *et al.*, 1990).

Various monoclonal antibodies have been raised that recognise all isoforms of CD45, or which just recognise CD45R isoforms. The widest range of antibodies has been raised against human isoforms. Examples include 2H4 (Morimoto *et al.*, 1985a), which recognises CD45RA (Streuli *et al.*, 1987b); and UCHL1 (Smith *et al.*, 1986), which recognises the junction formed between exons 3 and 7, *i.e.* on CD45RO (Terry, Brown and Beverley, 1988; Thomas, 1989).

Among human blood lymphocytes, the mean proportions staining positive using these antibodies were reported thus (Morimoto *et al.*, 1985a; Smith *et al.*, 1986; Jensen *et al.*, 1989; Warren and Skipsey, 1991): among T cells, 41% CD45RA+ and 40% CD45RO+; among CD4+ cells, 41% CD45RA+ and 70%

FIGURE 1.1

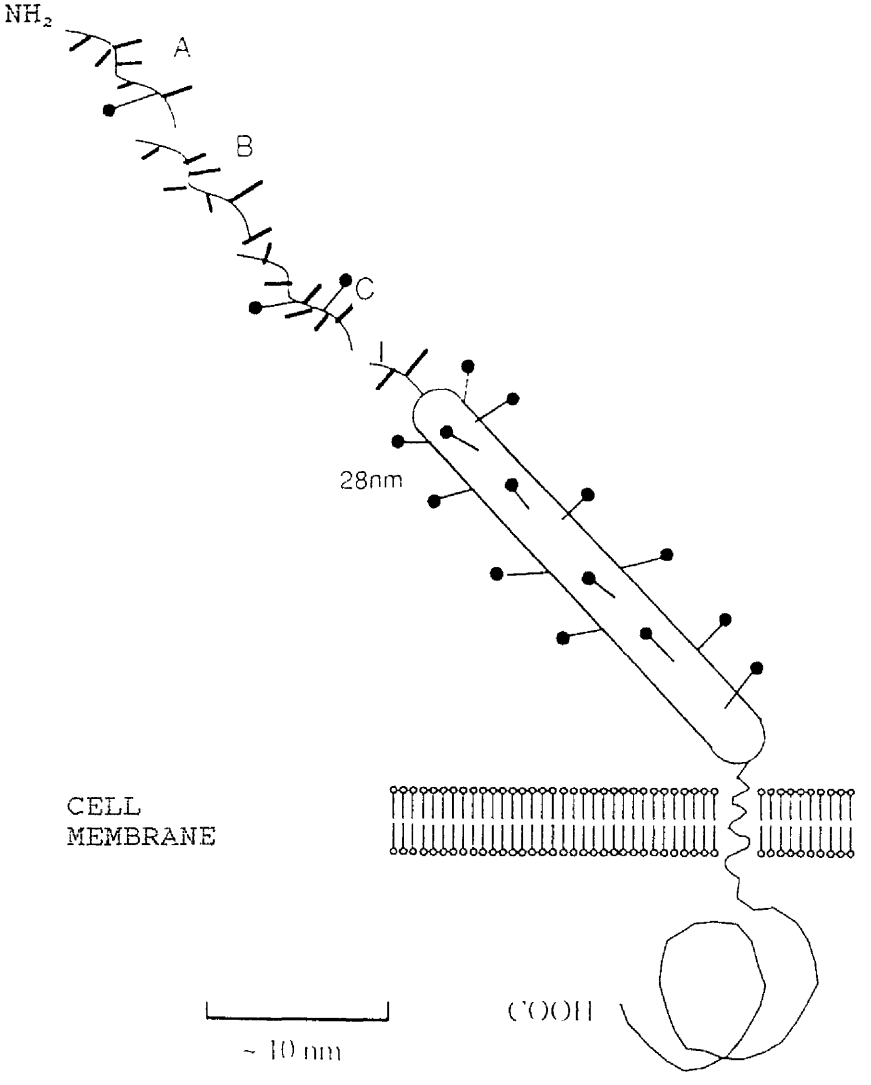
Schematic representation of restricted CD45 exon usage, and the effects on the molecular weight of the proteins generated (from Merkenschlager, 1991).



Combinations of exons 4 (A), 5 (B) or 6 (C) may or may not be expressed in the CD45 gene product. Glycoproteins of various RMW may thus be generated, as indicated.

FIGURE 1.2

Schematic representation of the structure and approximate sizes of rat CD45 isoforms (from McCall, Shotton and Barclay, 1992).



Combinations of exons A, B or C of the CD45 gene may or may not be expressed at the NH_2 terminus of the glycoprotein. Thus the extracellular size of CD45 can vary from approximately 28-51 nm, as indicated.

CD45RO+; among CD8+ cells, 54% CD45RA+ and 35% CD45RO+; among B cells, 92% CD45RA+ and 47% CD45RO+. NK cells were virtually all CD45RA+CD45RO-. Granulocytes and monocytes were virtually all CD45RO+. There were wide variations in both fluorescence intensity and proportions staining positive between the lymphocytes from different individuals, and more sensitive FACS analysers were subsequently able to detect weaker staining, and to demonstrate that among T cells CD45RA and CD45RO were not expressed on reciprocal subsets, as had been reported previously (Richards *et al.*, 1990; Wallace and Beverley, 1990; Mason and Powrie, 1990).

Antibodies to CD45R isoforms have been raised in several other species, including mice (Bottomly *et al.*, 1989), rats (Powrie and Mason, 1988), chickens (Houssaint *et al.*, 1987), sheep (Mackay, Marston and Dudler, 1990) and Guinea pigs (Hart *et al.*, 1992).

The first demonstration of functional differences between lymphocytes expressing different CD45 isoforms was by Spickett *et al.* (1983), using rat cells split according to expression of CD45 isoforms identified by the antibody OX-22, which recognises exon C of rat CD45 (McCall, Shotton and Barclay, 1992). OX-22-, but not OX-22+, T cells showed helper-inducer function *i.e.* enhanced B cell production of antibody *in vitro*. Among human lymphocytes, CD4+CD45RO+ cells showed enhanced helper-inducer function for B cells *in vitro*, compared to CD4+CD45RA+ cells, which suppressed immunoglobulin synthesis (Morimoto *et al.*, 1985a; Morimoto *et al.*, 1985b; Smith *et al.*, 1986). Hence CD4+CD45RO+ cells were

termed helper-inducer cells, and CD4+CD45RA+ cells were termed suppressor-inducer cells.

Among CD4+ lymphocytes, proliferative responses to different mitogens by CD45RO+ or CD45RA+ cells have been shown to vary: both subsets proliferated in response to PHA, Con A and allogeneic MLR (Morimoto *et al.*, 1985a; Morimoto *et al.*, 1985b; Smith *et al.*, 1986; Akbar *et al.*, 1988; Wallace and Beverley, 1990); CD45RA+ cells proliferated better in response to autologous MLR (Morimoto *et al.*, 1985a; Takeuchi *et al.*, 1987); and CD45RO+ cells responded better to anti-CD2 (Wallace and Beverley, 1990). Relative responses to anti-CD3 appeared to vary (reviewed by Beverley, 1990).

Cytokine production by lymphocytes expressing different CD45R isoforms has been studied in humans, rats and mice (reviewed by Sanders, Makgoba and Shaw, 1988; Akbar, Salmon and Janossy, 1991; Bradley, Croft and Swain, 1993). These studies have found that subsets equivalent to human CD45RA+ lymphocytes produce slightly more IL-2, while subsets equivalent to human CD45RO+ lymphocytes produce more IL-3, -4 and -6, and interferon-γ. However, there is no evidence that expression of CD45R isoforms serves as a marker for CD4+ T cell TH1 or TH2 subsets.

Several reports have demonstrated that during activation by mitogens *in vitro*, CD45RA+CD45RO- T, B and NK cells progressively increased CD45RO expression and decreased CD45RA expression; while lymphocytes which were initially CD45RO+CD45RA- did not change in regard to expression of CD45R isoforms (Ledbetter *et al.*, 1985; Akbar *et al.*, 1988; Jensen

et al., 1989; Kristensson *et al.*, 1990; Nesbitt, Jones and Moore, 1990; Rothstein *et al.*, 1990; Warren and Skipsey, 1991). Functionally CD45RA+ cells gained helper-inducer function concomitant with their change to increased expression of CD45RO (Nesbitt, Jones and Moore, 1990). CD45RO+ T cells showed greater proliferation in response to recall antigens such as tetanus toxoid, mumps peptide or PPD, compared to CD45RA+ cells (Morimoto *et al.*, 1985a; Morimoto *et al.*, 1985b; Smith, 1986; Wallace and Beverley, 1990). It was also shown that CD45RO expression on cord blood lymphocytes was very low, but increased progressively during childhood (Sanders *et al.*, 1988; Hayward, Lee and Beverley, 1989). These observations led to the suggestion that CD45RA+ T cells were naive cells and CD45RO+ T cells were memory cells (Beverley, 1987; Sanders, Makgoba and Shaw, 1988).

Subsequently it has been demonstrated that the increased expression of CD45RO on activated lymphocyte populations is not stable among lymphocytes, or their progeny, in long-term culture *in vitro* (Warren and Skipsey, 1991; Yamada and Schlossman, 1991; Sarawar *et al.*, 1993), or in *in vivo* experiments (Bell and Sparshott, 1990; Sparshott, Bell and Sarawar, 1991). This has led to the suggestion that expression of CD45RO defines recently-activated lymphocytes (Bell *et al.*, 1992), rather than defining helper-inducer, or memory, T cells.

The CD45RO+ population of blood lymphocytes expresses high levels of CD29, an antigen identified using the antibody 4B4 (Morimoto *et al.*, 1985b), and which is present at least

weakly on all lymphocytes (Wallace and Beverley, 1990). CD29 is the β_1 chain of the VLA proteins of the integrin family, which are involved in cell adhesion to extracellular matrix, cell/cell adhesion, and possibly in lymphocyte/HEV interaction and cell signalling (reviewed by Hemler, 1990; Bevilacqua, 1993). CD45RO+ lymphocytes showed enhanced adhesion to fibronectin and laminin compared to CD45RA+ cells (Shimizu *et al.*, 1990a; Shimizu *et al.*, 1991a). CD45RO+ lymphocytes also showed increased expression of adhesion molecules such as CD44, CD18/CD11a, CD2, LFA -1, and -3, ICAM-1 and VLA -3, -4, -5 and -6, compared to CD45RA+ cells, (Sanders *et al.*, 1988; Buckle and Hogg, 1990; Shimizu *et al.*, 1990; Horgan *et al.*, 1992). CD45RO+ lymphocytes also showed enhanced adhesion to endothelial cell monolayers *in vitro*, compared to CD45RA+ cells (Pitzalis *et al.*, 1988; Damle and Doyle, 1990; Shimizu *et al.*, 1991a; Shimizu *et al.*, 1991b; Ikuta *et al.*, 1991). A greater proportion of blood CD45RO+ lymphocytes expressed activation markers such as CD25, and CD45RO+ lymphocytes were on average larger than CD45RA+ cells (Maurer, Felzmann and Knapp, 1990; Buckle & Hogg, 1990; Taga *et al.*, 1991; Zola and Flego, 1992). These observations support the suggestion that CD45RO+ lymphocytes are activated compared to CD45RA+ cells, and may be better adapted to adhere to non-specialised endothelium and extracellular matrix components.

In humans it has been observed that CD45RO+CD29+CD45RA- lymphocytes localise non-randomly in tissues, particularly in the gut of healthy subjects, and patients with Crohn's disease or coeliac disease (James *et al.*, 1986; Harvey, Jones and

Wright, 1989; Foster *et al.*, 1990); in the skin (Mackay, Marston and Dudler, 1990); in the lungs (Saltini *et al.*, 1990); in the fluid from sites of chronic arthropathies (Emery *et al.*, 1987; Pitzalis *et al.*, 1987a; Pitzalis *et al.*, 1987b; Kingsley *et al.*, 1988; Silverman *et al.*, 1993), and pleural or peritoneal exudates (Pitzalis *et al.*, 1987a); in sites of tuberculoid leprosy (Modlin *et al.*, 1988); and in sites of chronic dermatoses (Markey *et al.*, 1990). CD29+ lymphocytes have also been demonstrated to adhere more strongly *in vitro* than CD45RA+ cells to skin sections from patients with psoriasis (Chin *et al.*, 1990). Experimental intradermal injection of antigen led to accumulation of CD45RO+CD45RA- lymphocytes relative to the blood within 24-48 hours (Pitzalis *et al.*, 1991; Picker *et al.*, 1993), which suggests that recruitment of lymphocytes from the blood or tissues, rather than activation or proliferation of cells *in situ*, was responsible for relative preponderance of CD45RO+ lymphocytes.

Studies in both humans (Picker *et al.*, 1990; Picker *et al.*, 1991; Shimizu *et al.*, 1991a), and sheep (MacKay *et al.*, 1990; MacKay *et al.*, 1992) suggested that lymphocytes expressing different isoforms of CD45 may follow different recirculation pathways (MacKay, 1991).

Thus blood CD45RO+ lymphocytes, when compared to CD45RA+ cells, appear to have:- enhanced activation status; increased adhesion to non-specialised endothelium *in vitro*; preferential localisation in the gut, and at sites of inflammation *in vivo*; and separate recirculation pathways *in vivo*. As described

previously, many of these features are shared by lymphocytes which show locomotor capacity *in vitro*.

SECTION 1B: AIMS OF THE PROJECT, AND APPROACHES USED.

1B.1 Introduction.

The proposed relation between lymphocytes which exhibit *in vitro* locomotion, and those which localise at sites of inflammation *in vivo*, may have important implications for the understanding of how lymphocytes normally traffic, and how various subsets are recruited from the blood into sites of inflammation. An understanding of these processes might lead to manipulation of processes critical to the function of the immune system in health and disease.

CD45RO+ (or CD29+) lymphocytes isolated from the blood have recently been shown to exhibit enhanced locomotion compared to CD45RA+ cells on endothelial cell monolayers *in vitro* (Masuyama *et al.*, 1992); and in *in vitro* assays of chemotaxis to RANTES and interferon-inducible protein-10 (Schall *et al.*, 1990; Taub *et al.*, 1993a; Taub *et al.*, 1993b). Taub *et al.* (1993a and 1993b) showed that CD29+ lymphocytes showed enhanced locomotion to MIP-1α, and MIP-1ß, although this was not evident when CD45RO+ lymphocytes were compared to CD45RA+ cells by Schall *et al.* (1993).

In view of the documented differences in the *in vitro*

locomotion of lymphocytes expressing defined CD45R isoforms, and the potential implications for lymphocyte locomotion *in vivo*, the general aims of this project were to further investigate the locomotion of human blood CD45R subsets *in vitro*, and to examine some of the defined factors which stimulate lymphocyte locomotion in various *in vitro* assay systems in relation to expression of CD45R isoforms.

1B.2 Preparation of lymphocytes for locomotion assays.

Many different strategies are available for preparing lymphocytes prior to testing in locomotion assays. During this project, two systems were used to prepare lymphocytes:- (1) 24-72 hour-culture of mononuclear cells in mitogens, in which case lymphocytes respond to factors released *in situ* during the course of culture; and (2) use of lymphocytes on day 0 or after overnight culture in FCS, in which case lymphocytes can be tested for response to short-term stimulation by defined factors. The latter approach was used since one drawback of culture in mitogens such as α-CD3 is that lymphocytes subsequently display relatively high levels of spontaneous locomotion, even after washing. Overnight culture in FCS results in a population with low spontaneous locomotor capacity after washing, but which is capable of subsequent response to added stimulators (Wilkinson and Newman, 1992).

1B.3 Stimulators of lymphocyte locomotion.

During the course of this study, the following factors were examined in detail: culture supernatants; IL-2; IL-8; RANTES; colchicine; and FCS.

1B.3.1 Culture supernatants.

Lymphocytes activated by culture for 24-72 hours in mitogens, including α-CD3, Con A, PHA, PPD and allo-MLR, show increased proportions of motile cells *in situ*, responding to factors present in the culture supernatants (Wilkinson *et al.*, 1976; Wilkinson, 1986). Culture supernatants have been used for many years as stimulators of lymphocyte locomotion (Ward, Offen and Montgomery, 1971; Ward *et al.*, 1977; Center and Cruikshank, 1982; Wilkinson, 1986), although the active factors were not clearly defined.

1B.3.2 IL-2.

As described previously, the use of PHA and other mitogens enabled long-term culture of normal blood lymphocytes *in vitro*. PHA-stimulated conditioned medium alone could support lymphocyte growth (Morgan, Ruscetti and Gallo, 1976), and the active factor identified in such media was termed T cell growth factor. The molecule responsible for this activity was found to be identical to that causing activities termed thymocyte stimulating factor, thymocyte mitogenic factor, killer helper factor, secondary cytotoxic T cell

factor and co-stimulator, and these were assigned the name IL-2 (Aarden *et al.*, 1979). IL-2 has also been shown to activate B cells, monocytes and oligodendrocytes (reviewed by Kuziel and Greene, 1991; Minami *et al.*, 1993).

IL-2 is a 15.5 kdal glycoprotein produced by T cells (reviewed by Smith, 1988; Kuziel and Greene, 1991; Waldmann, 1993). NK cells and B cell lines may also be capable of producing IL-2 (Kasahara *et al.*, 1983; Taira *et al.*, 1987).

IL-2 was found to cause chemotaxis of human blood CD4+, but not CD8+, lymphocytes (Kornfeld *et al.*, 1985), who reported that the activity was blocked using α-CD25 (Uchiyama, Broder and Waldmann, 1981), an antibody which binds to the 55 kdal chain of the IL-2 receptor complex (generally referred to as the IL-2Rα chain). There is a second IL-2-binding glycoprotein (IL-2Rß chain; Miyajima *et al.*, 1992) of molecular weight 70-77 kd (Sharon *et al.*, 1986; Tsudo *et al.*, 1986). The IL-2Rß chain can transduce signals to the cell following IL-2 binding (Asao *et al.*, 1990), and allows internalization of the receptor/ligand complex (Robb and Greene, 1987). The ß chain binds to IL-2 with intermediate affinity, but if the ß chain and CD25 bind IL-2 together, the affinity increases (reviewed by Smith, 1987; Waldmann, 1991). Only a small proportion of freshly isolated blood lymphocytes express IL-2R α or ß chains, but this proportion increases after lymphocyte activation (Uchiyama, Broder and Waldmann, 1981; Tsudo, Kitamura and Miyasaka, 1989; Ohashi *et al.*, 1989). A third (γ) chain of the IL-2R complex has also been described , which does not bind IL-2 but is required for

receptor internalisation (Takeshita *et al.*, 1992).

1B.3.3. IL-8.

The name IL-8 was given to the molecule responsible for several activities on leucocytes (Westwick, Li and Camp, 1989), for example:- neutrophil activating protein-1; neutrophil activating factor; granulocyte chemotactic factor; monocyte-derived neutrophil chemotactic factor; lymphocyte-derived neutrophil activating protein; and monocyte-derived neutrophil activating protein. Many of these activities had previously been assigned to IL-1, but could not be reproduced using recombinant IL-1 (Van Damme, 1991). IL-8 is classified as a member of the "CXC", or "α" subfamily of the chemokine family of molecules (reviewed by Wolpe and Cerami, 1989; Oppenheim *et al.*, 1991; Miller and Krangel, 1992), which all appear to bind to heparin and to share a common ancestral gene.

IL-8 is a 6-8 kdal molecule which occurs as a dimer, with some variations in the number of amino acids (Van Damme *et al.*, 1989). It is synthesised by a wide variety of cell types *in vitro* (Yoshimura and Leonard, 1992; Mukaida and Matsushima, 1992).

IL-8 has a wide range of *in vitro* activities on many cell types, particularly neutrophils (reviewed by Oppenheim *et al.*, 1991; Baggiolini, Imboden and Detmers, 1992). IL-8 has been reported to cause *in vitro* locomotion of neutrophils, lymphocytes, basophils, melanoma cells and endothelial cells,

using Boyden chambers (Larsen *et al.*, 1989a; Bacon, Westwick and Camp, 1989; Leonard *et al.*, 1990; Koch *et al.*, 1992). There is evidence from *in vitro* studies that extracellular matrix-bound IL-8 may be crucial for neutrophil transmigration of endothelium (Rot, 1992).

Injection of IL-8 intradermally caused plasma leakage, and infiltration of leucocytes,predominantly neutrophils (Colditz *et al.*, 1989; Foster *et al.*, 1989; Colditz and Watson, 1992). Injection of anti-IL-8 prevented neutrophil infiltration after administration of lipopolysaccharide (Harada *et al.*, 1993). Other reports observed infiltration of lymphocytes (Larsen *et al.*, 1989a; Larsen *et al.*, 1989b; Endo *et al.*, 1991) or eosinophils (Collins *et al.*, 1993), as well as neutrophils.

Other members of the chemokine α subfamily have been shown to cause *in vitro* locomotion of various cell types (reviewed by Oppenheim *et al.*, 1991; Miller and Krangel, 1992): neutrophils are stimulated by platelet factor 4, neutrophil activating protein-2, and proteins termed *gro* and ENA-78; fibroblasts are stimulated by platelet factor 4 and ß-thromboglobulin; and lymphocytes and monocytes are stimulated by interferon-inducible protein-10 (Taub *et al.*, 1993b).

The neutrophil IL-8R has been cloned (Samanta, Oppenheim and Matsushima, 1989; Thomas, Taylor and Navarro, 1991; Holmes *et al.*, 1991; Murphy and Tiffany, 1991; Larosa *et al.*, 1992). Other members of the chemokine family, melanoma growth factor and neutrophil activating protein-2, bind to the IL-8R (Moser

et al., 1991). At least two forms of the IL-8R receptor may exist (Holmes *et al.*, 1991; Murphy and Tiffany, 1991). No neutrophil IL-8R mRNA was detected in PHA-activated T cells, or T cell lines (Holmes *et al.*, 1991; Murphy and Tiffany, 1991), and no surface binding of radiolabelled IL-8 on T or B cell lines was detected by Grob *et al.* (1990). This may reflect that only a sub-population of lymphocytes express this receptor (Oppenheim *et al.*, 1991).

1B.3.4 The ß chemokine subfamily.

RANTES was the first member of the "CC", or "ß" subfamily of the chemokine family of molecules shown to stimulate *in vitro* lymphocyte locomotion (Schall *et al.*, 1990). RANTES was originally isolated as a T cell-specific product (Schall *et al.*, 1988). It appears to have no effect on neutrophils, but was found to stimulate migration of lymphocytes, monocytes, eosinophils and basophils in Boyden chambers (Schall *et al.*, 1990; Rot *et al.*, 1992; Kameoyshi *et al.*, 1992; Taub *et al.*, 1993a; Taub *et al.*, 1993b; Bischoff *et al.*, 1993). A receptor for RANTES has been identified on B, but not T, cells (Gao *et al.*, 1993), despite the finding that RANTES caused chemotaxis of T cells, but not B cells (Schall *et al.*, 1990). Recent reports suggest that there may be several receptors for RANTES, which may also bind MIP-1α (Bischoff *et al.*, 1993). Other members of the ß chemokine subfamily have also been shown to stimulate leucocyte locomotion (Rot *et al.*, 1992; Bischoff *et al.*, 1993; reviewed by Oppenheim *et al.*, 1991;

Miller and Krangel, 1992; Van Damme, 1991):- neutrophils are stimulated by unseparated MIP proteins; lymphocytes are stimulated by MIP-1α and MIP-1ß; monocytes are stimulated by monocyte chemotactic proteins -1, -2 and -3 and the protein I-309; basophils are stimulated by monocyte chemotactic protein-1 and MIP-1α; and eosinophils are stimulated by MIP-1α.

1B.3.5 Colchicine.

Colchicine binds to tubulin (Borisy and Taylor, 1967), and is one of several drugs which block tubulin assembly and which affect the directional locomotion of a wide variety of cell types (reviewed by Mareel and De Mets, 1984). Colchicine has been reported to have other effects on cell metabolism (reviewed by Sartorelli and Creasy, 1969), and the mechanism of action of colchicine on cell locomotion is not clear. Colchicine was found to prevent the inhibition by dibutyryl cyclic adenosine monophosphate of anti-immunoglobulin-stimulated lymphocyte locomotion (Schreiner and Unanue, 1975). Colchicine has been shown to cause locomotion by lymphoblast cell lines in filter assays (Russell *et al.*, 1975), and to cause polarization and invasion of collagen gels by a proportion of lymphocytes freshly isolated from blood (Wilkinson, 1986; Keller, Niggli and Zimmermann, 1989). The importance of microtubule function in lymphocyte locomotion was emphasised by the finding that in the presence of the microtubule stabiliser, deuterium oxide, lymphocyte

polarization in response to FCS was inhibited (Wilkinson, 1986).

1B.3.6 FCS.

Culture of lymphocytes in FCS has been demonstrated to cause a proportion to become motile (Berman, 1966; O'Neill and Parrott, 1977) and FCS stimulated locomotion of freshly-isolated lymphocytes (Wilkinson, 1986). FCS has been reported to be mitogenic (Woodliff and Onesti, 1968), but mitogenic activity is not essential for stimulation of locomotion during culture (Wilkinson, 1986).

1B.4 Lymphocyte locomotion assays.

During this project invasion of collagen gels and morphological polarization were used to assess lymphocyte locomotor responses.

1B.4.1 The collagen gel invasion assay.

While filter assays are widely used to examine lymphocyte locomotor responses, they utilise a non-physiological matrix, and this makes extrapolation of results to the situation *in vivo* more difficult. Lymphocyte locomotion *in vivo* must inevitably involve interaction with the extracellular matrix, and collagen is the major component of the extracellular matrix of vertebrates (reviewed by Miller, 1976; Kühn and

Glanville, 1980; Linsenmayer, 1981).

Extraction of glues and collagen from animal tissues using hot water has been described since ancient times (Eastoe, 1967), and dilute acids have been used to extract collagen from connective tissue for over 100 years (Piez, 1976). Collagen can be extracted from rat tail tendons using dilute acetic acid (Zachariadès, 1900); can be salt precipitated and redissolved; and will form gels (Nageotte, 1927).

All rat tail tendon collagen is type I (reviewed by Piez, 1976), which is composed of 3 chains - 2 α1(I) chains and 1 α2(I) chain, and which has an overall molecular weight of approximately 280 kdal. (reviewed by Kühn and Glanville, 1980). The isolated tropocollagen monomers (Gross, Highberger and Schmitt, 1954) form fibrils *in vitro* which resemble those observed *in vivo* (Bahr, 1950; Wood and Keech, 1960; Schor and Court, 1979).

Type I collagen gels are suitable for the study of cell behaviour *in vitro* (Elsdale and Bard, 1972), especially since the matrix is translucent, allowing cells to be visualised *in situ* in the gel. Digestion of the collagen gel using collagenase allows cells to be released from the gel matrix (Schor, 1980; Shields, Haston and Wilkinson, 1984; Ratner, Jasti and Heppner, 1988). Collagen is degraded by a variety of enzymes. The collagenase derived from Clostridium histolyticum cleaves the molecule at over 200 sites (reviewed by Stryer, 1981), leaving small peptides (Seifter *et al.*, 1959).

1B.4.2 The polarization assay.

While filter assays and invasion of collagen gels demonstrate active locomotion of cells, it is difficult to assess whether the matrix of the filter, or of collagen, affects locomotor responses of the cells. Several early studies (for example Lewis and Webster, 1921; Lewis, 1931a; Lewis, 1931b; De Bruyn, 1944) reported that lymphocytes show a characteristic pear-shaped, or "handmirror" morphology when moving *in vitro*, in contrast to non-motile cells which remain spherical. Clear distinction of the anterior and posterior edges of motile lymphocytes was termed cell polarization in this context.

The use of glutaraldehyde as a fixative preserves the morphology of living cells (Keller, Niggli and Zimmermann, 1989). The polarization assay of Haston and Shields (1985) uses glutaraldehyde fixation to allow examination of locomotor morphology in lymphocytes (Wilkinson, 1986). The procedure is straightforward, and tests can be done in suspension, so that no interactions with a surface need occur. However factors causing shape changes need not stimulate locomotion (Wilkinson *et al.*, 1988), and motile cells may temporarily revert to spherical morphology.

1B.4.3 Measurement of lymphocyte size.

Since the activation status of lymphocytes can be related to their locomotor capacity (Wilkinson, 1986), the sizes of

lymphocytes responding in collagen gel invasion, or polarization, assays were determined.

Areas of the images of lymphocytes can be measured either by tracing the images manually and measuring the outlines using a bitpad (Wilkinson *et al.*, 1988; Wilkinson and Islam, 1989; Eisele *et al.*, 1991), or by computerised digitation of the image and area measurement (Edwards, Robson and Campbell, 1987).

Measurement of light scatter can be derived using FACS analysis (Mullaney *et al.*, 1969), and it has been shown that the forward scatter of a cell is proportional to its volume (Parks, Lanier and Herzenberg, 1986).

1B.5 The phenotype of lymphocytes responding in locomotion assays.

Contact with monocytes is essential for the enhancement of lymphocyte locomotor responses during culture in mitogens such as α-CD3 (Wilkinson and Higgins, 1987a; Wilkinson, 1990). Consequently during this project the whole mononuclear cell fraction was cultured. This has other advantages compared to culture of isolated subsets *i.e.* the interaction of different subsets can occur, and preparatory steps used to isolate subsets are minimised, reducing the chances of cell activation during such procedures. However a consequence of using mixed populations of cells was that, while methods for the phenotyping of lymphocytes which had migrated to the underside of, within, or through Boyden-type filters have been reported

(Bacon *et al.*, 1988; Schell-Frederick *et al.*, 1991; Zachariaè *et al.*, 1992), novel methods had to be developed to enable the phenotype of lymphocytes responding in assays of polarization, collagen gel invasion and cell sizing.

Thus the objectives of this project were:- firstly to develop methods enabling identification of lymphocytes expressing different CD45R subsets, in conjunction with the assays of locomotion and cell sizing outlined previously; secondly to investigate the locomotion of human blood lymphocytes expressing CD45RO or CD45RA in these assays; and thirdly, to examine the role of defined molecules such as IL-2, IL-8 and RANTES, as stimulators of lymphocyte locomotion either after short-term incubation, or during culture of mononuclear cells in mitogens.

CHAPTER 2: MATERIALS AND METHODS.

2.1 Preparation of media.

The routine medium used throughout this project was HBSS (ICN Flow Ltd., High Wycombe, U.K.), which was obtained as a 10x strength solution, without sodium bicarbonate but with phenol red. This was diluted to 1x strength with distilled water. MOPS (Sigma Chemical Co. Ltd., Poole, U.K.) was used as a buffer for HBSS. 1M MOPS, pH 7.4, was prepared in distilled water, and used at 10 mM. The pH of HBSS was adjusted to 7.4 using 2M NaOH or HCl (BDH Ltd., MacFarlane Robson Ltd., Glasgow, U.K.). For routine cell culture, HSA (Hoechst U.K. Ltd., Twickenham, U.K.) was dissolved at 10mg/ml in HBSS (HBSS/HSA).

Occasionally RPMI/FCS was used as a culture medium. RPMI 1640 (Gibco BRL, Life Technologies Ltd., Paisley, U.K.) was obtained as a 1x strength solution containing sodium bicarbonate. This was supplemented by 10mM HEPES buffer, 2mM L-glutamine, 100U/ml penicillin, and 100μg/ml streptomycin, all obtained as 100x strength concentrates from Gibco. FCS (Biological Industries, Kibbutz Beth Haemek, Israel) was heat inactivated by heating at 56°C for 30 minutes, and used at 10% (v/v).

Cultures were incubated at 37°C in air, which was supplemented with 5% CO_2 for cultures containing bicarbonate.

Saline was prepared by dissolving NaCl (BDH) at 8.5g/l in water. PBS was prepared at 25x strength in distilled water by

dissolving NaCl (200g/l); KCl (BDH; 5g/l); KH_2PO_4 (BDH; 5g/l); and Na_2HPO_4 (BDH; 28.75g/l), and was diluted to 1x strength in distilled water.

2.2 Isolation of lymphocytes from blood.

Human blood was taken by venepuncture and anticoagulated by mixing with heparin (Leo Laboratories Ltd., Princes Risborough, U.K.) at approximately 1-2U per ml blood. 5 ml volumes of whole blood were layered onto 2.5ml lymphocyte separation medium (ICN Flow) or Nycoprep separation medium (Techgen International Ltd., London, U.K.). After centrifugation for 30 minutes at 1100 rpm, mononuclear cells form a layer at the interface with the separation medium, which allows separation from granulocytes (except basophils), red blood cells and most platelets (Böyum, 1968).

Cells were routinely centrifuged at 1100 rpm (approximately 220xg), at room temperature. Sterile, plastic, conical-based tubes (110x16mm; McQuilkin and Co., Glasgow, U.K.) were routinely used. The only exceptions were when siliconised glass tubes were used. These were prepared by filling 4x0.5-inch tubes with "Repelcote" (Hopkin and Williams Ltd., Chadwell Heath, U.K.) for 1 minute. Repelcote was poured out, and tubes were air-dried, rinsed in distilled water, and autoclaved (Islam, 1986).

Mononuclear cells were washed (*i.e.* resuspended in 10ml medium and centrifuged for 5-10 minutes) 2-3 times at 1100 rpm in HBSS. Typically $1-2x10^6$ mononuclear cells were obtained

per ml of blood. Cells were resuspended at 2-4x10^6 cells/ml in culture medium and dispensed in 1ml volumes in 24-well plates (1.6x1.7cm; ICN Flow), or in 10ml volumes in 25cm^2 tissue culture flasks (ICN Flow).

2.3 Preparation of cells prior to further tests.

Only lymphocytes non-adherent to plastic were examined during this project.

Cell viability was confirmed using phase contrast microscopy, in conjunction with trypan blue exclusion, for which cells were mixed with an equal volume of trypan blue (0.4% w/v in saline; ICN Flow), and examined as soon as possible.

To test lymphocytes on the day of blood collection (day 0), MNC in HBSS/HSA were left in culture for at least 1 hour at 37°C, allowing adherence of cells to plastic. Non-adherent cells were removed by gentle pipetting and washed three times in HBSS.

To test lymphocytes after longer periods of culture, MNC were incubated for 24-72 hours in medium (HBSS/HSA) with or without various activators (Wilkinson *et al.*, 1976; Wilkinson, 1986). PHA (Wellcome Diagnostics, Beckenham, U.K.), Con A (Sigma) and PWM (Sigma), were dissolved at 1mg/ml in saline. PHA was used at 1μg/ml, Con A at 5μg/ml, and PWM at 10μg/ml. α-CD3 (Orthodiagnostics, High Wycombe, U.K.) was dissolved at 2μg/ml in HBSS/HSA, dialysed against HBSS to remove sodium azide, and used at 10ng/ml; PPD (Evans Medical Ltd., Horsham,

U.K.) was dialysed against saline to remove phenol, stored at 4°C, and used at a dilution of 1/100 (1000U/ml). Only MNC from subjects known to give vigorous tuberculin reactions were cultured with PPD. Allogeneic-MLR were set up by mixing equal numbers of MNC from unrelated donors. Heat-inactivated FCS was used at 10% (v/v).

Non-adherent lymphocytes obtained after culture in activators were either fixed *in situ* for polarization assays, or washed 2-3 times in HBSS before further testing.

When appropriate, supernatants from these cultures were retained and recentrifuged to ensure removal of cells. They were used at 50% (v/v in HBSS) on the same day, on cells from their own cultures.

2.4 Stimulators of locomotion.

Culture supernatants were prepared as described previously. Other stimuli were stored at -20°C and diluted in medium (routinely HBSS) prior to use. Heat-Inactivated FCS was used at 20% (v/v) (Wilkinson, 1986). Colchicine (Sigma) was dissolved at 10^{-4}M in HBSS, thawed in the dark, and used at 10^{-5}M (Wilkinson, 1986). Recombinant human IL-2 (originally from Glaxo IMB, Geneva, Switzerland) was a gift from Dr. W. Cushley (Dept. of Biochemistry, University of Glasgow, Glasgow, U.K.), or from Dr. D. Stott (Dept. of Immunology, University of Glasgow, Glasgow, U.K.), and was routinely used at 250U/ml. IL-2 from Genzyme Diagnostics (West Malling,

U.K.) was used in some preliminary experiments. There were 4 sources of human IL-8 :- (1) a gift from Dr. A. O. Anderson (Fort Detrick, Frederick, M.D., U.S.A.); (2) a gift from Dr. J. Van Damme (Rega Institute, Leuven, Belgium) ; (3) Peprotech Inc. (Rocky Hill, N.J., U.S.A.); and (4) British Biotechnology Ltd. (Abingdon, U.K.). All were recombinant except that from Dr. J. Van Damme, which was leukocyte-derived, pure, sequence grade IL-8. The commercial preparations were dissolved at 10μg/ml in HBSS/HSA. IL-8 was routinely used at 100ng/ml (Wilkinson and Watson, 1990). RANTES was obtained from Peprotech. It was dissolved in HBSS/HSA at 10μg/ml, and used at various concentrations.

Rabbit anti-IL-8 (Strieter *et al.*, 1989) was a gift from Dr. S. Kellie (Yamanouchi Research Institute, Oxford, U.K.). It has been shown not to cross-react with other members of the ß-thromboglobulin gene family (Huber *et al.*, 1991). Goat anti-IL-8 was a gift from Dr. J. Van Damme. It immunoprecipitates a single protein band, and does not cross-react with ß-thromboglobulin or NAP-2 (Dr. J. Van Damme, personal communication). Both were used at a final dilution of 1/1000.

2.5 Preparation of fixatives.

Cells were routinely fixed by addition of an equal volume of fixative in suspension.

Grade II glutaraldehyde (Sigma; 25% aqueous solution) was used as the routine fixative for polarization assays. A stock

2.5% solution was prepared in saline. This was stored at 4°C. Occasionally this was further diluted in saline. In a few experiments grade I glutaraldehyde (Sigma) was used. This was stored at -20°C until the day of use, then diluted in saline. Paraformaldehyde was the routine fixative for immunofluorescence. A 1% (w/v) solution was prepared by dissolving paraformaldehyde powder (BDH) in PBS (pH 7.4) at 60-70°C for approximately 1 hour. This solution was stored at 4°C. Formaldehyde (4% w/v) was prepared by diluting formalin (40% w/v formaldehyde; R. and J. Wood Laboratories, Paisley, U.K.) in saline, and was stored at 4°C. Methanol/acetone was prepared by mixing equal volumes of methanol (BDH) and acetone (BDH). This was stored at room temperature.

2.6 Primary phenotypic antibodies.

All primary antibodies were monoclonal and of murine origin. α-CD45RO (UCHL1; Smith *et al.*, 1986) was a gift from Professor P.C.L.Beverley (ICRF, London, U.K.) and was used at a final dilution of 1/2. Other primary antibodies were routinely used at a final dilution of 1/10. α-CD45RA (2H4; Morimoto *et al.*, 1985a), and α-CD29 (4B4; Morimoto *et al*, 1985b), were obtained from Coulter Electronics (Luton, U.K.). Unconjugated α-CD3 (Reinherz and Schlossman, 1979); α-CD4 (Reinherz *et al.*, 1979); α-CD8 (Reinherz *et al.*, 1980); and α-CD22 (Dörken *et al.*, 1986) were obtained from SAPU (Carluke, U.K.). Unconjugated α-CD14 (Dimitriu-Bona *et al.*, 1983); α-CD19 (Nadler *et al.*, 1983); and α-CD25 (Uchiyama *et al.*, 1981)

were obtained from Dako Ltd. (High Wycombe, U.K.). The α-IL-2 receptor ß chain antibody Mik-ß1 (Tsudo *et al.*, 1989) was a gift from Dr. M. Tsudo (Dept. of Immunology, Tokyo Metropolitan Institute of Medical Sciences, Tokyo, Japan). Directly phycoerythrin-conjugated α-CD3, α-CD4, and α-CD8 were obtained from Coulter.

As negative control antibodies, unconjugated mouse IgG_1 (Dako) or PE- and FITC-conjugated mouse IgG_1 α-human tissue and serum protein (Coulter) were used.

2.7 Staining of cells by immunofluorescence.

Cells were washed, and antibodies diluted, in PBS containing 0.1% sodium azide. Stock sodium azide (BDH) was dissolved at 10% (w/v) in water. Each staining step was carried out for 30 minutes on ice, and cells were washed twice at 4°C prior to the next step.

After staining with primary unlabelled antibodies the secondary antibody was added. This was FITC-conjugated rabbit anti-mouse immunoglobulin $F(ab')_2$ fragment (Dako), used at a final dilution of 1/200. Cells could then be stained by a directly conjugated antibody for double labelling if required.

Cells were routinely fixed, after staining, for at least 10 minutes in an equal volume of 1% paraformaldehyde. Preparations could be stored for several days at 4°C before examination, visually using a Zeiss Axioskop fluorescence microscope using a x40 phase contrast objective and x10 eyepiece lens; or automatically using a FACS (FACScan; Becton-

Dickinson) using its Consort 30 or Lysis II programmes. 10,000 events were routinely acquired. The machine settings used were determined by staff of the Dept. of Immunology , Western Infirmary, Glasgow, U.K.

2.8 Polarization assays.

The method of Haston and Shields (1985) was used. Cell suspensions were fixed, in conical tubes, in an equal volume of fixative (routinely 2.5% glutaraldehyde), and left for 15-45 minutes at room temperature. Fixed cells were washed at least twice in saline. Usually wet preparations from cell suspensions were examined, using an Olympus BH-2 microscope with a x40 phase contrast objective and a x10 eyepiece lens. Occasionally Nomarski optics were used. Preparations were coded and examined "blind". At least 300 cells of each category were counted.

Lymphocytes were classified as polarized if they showed distinct head-tail polarity; distinct ruffling at one edge; or a clear constriction ring (Haston and Shields, 1984; Wilkinson, 1986). Figure 2.1 shows preparations containing cells in typical polarized and round morphology.

2.9 APAAP staining.

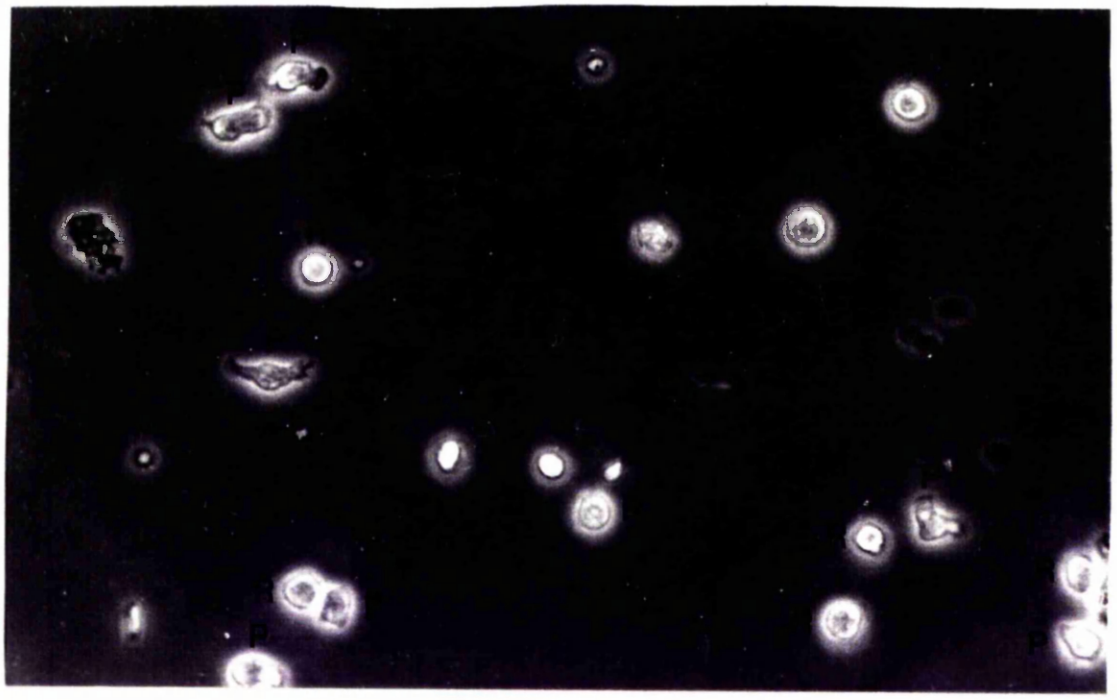
2.9.1 Conventional method.

The method of Cordell *et al.* (1984) was used.

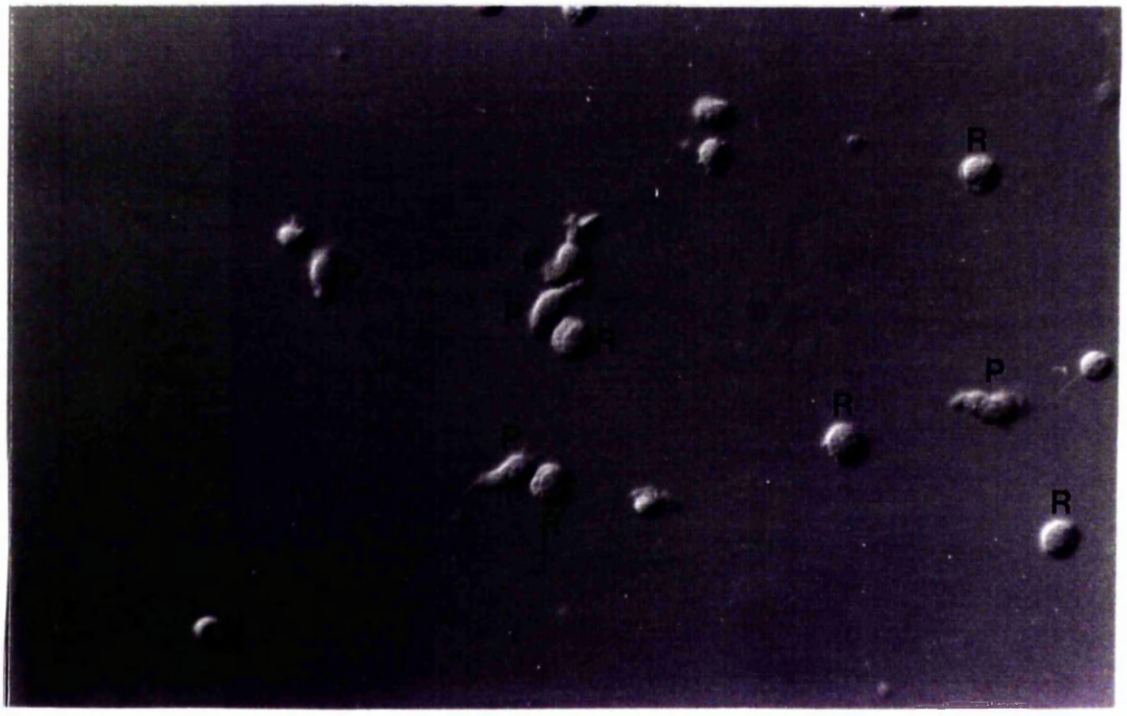
FIGURE 2.1

The morphology of glutaraldehyde-fixed lymphocytes, showing round (R) and polarized (P) cells.

A:- Phase contrast optics.



B:- Nomarski optics.



Staining was done at room temperature, and care taken to ensure that slides did not dry out. TBS (BDH) was used as a diluent, and also for washing slides. This was done by dipping slides in TBS in Coplin jars for 1-2 minutes after each step. Stock 0.5M TRIS was dissolved at pH 7.6 in water. To make TBS this was diluted 1 in 10 in saline, and the pH re-adjusted to 7.6 if required.

Cytocentrifuge preparations of cells were made using a Shandon cytocentrifuge (Shandon Southern Instruments, Sewickley, P.A., U.S.A.). Approximately $5x10^5$ lymphocytes were centrifuged onto methanol-cleaned glass slides at 500 rpm for 5 minutes. Cells were fixed in methanol/acetone for 90 seconds.

Primary antibodies were added and left for at least 30 minutes. Secondary antibody *i.e.* rabbit anti-mouse immunoglobulins (Dako)- was added at 1/25 for at least 30 minutes. APAAP complexes (Dako) were added at 1/50 for at least 30 minutes. Staining by secondary antibody, and APAAP, was repeated, for a minimum of 10 minutes, to enhance the reaction. Filtered substrate was added for 15-20 minutes. To make the substrate, 5.5 ml of water containing 1 mg/ml levamisole (Sigma) and 4 mg/ml fast red (Sigma) was mixed with 0.5 ml of naphthol AS-MX phosphate (0.25% alkaline solution; Sigma). To counterstain, slides were dipped in: haematoxylin (BDH) for 1 minute; Scott's tap water substitute for 30 seconds; and tap water. Scott's tap water substitute contained 3.5 g/l $NaHCO_3$ (BDH), and 20 g/l $MgSO_4$ (BDH), in distilled water. Slides were mounted in an aqueous

glycerol/gelatin mountant (Dako).

2.9.2 Modified APAAP method.

This was developed to enable glutaraldehyde-fixed cells to be phenotyped (Newman and Wilkinson, 1992).

Cells were fixed in suspension with an equal volume of 2.5% glutaraldehyde for 15 minutes at room temperature. Glycine (Sigma) was used to quench free aldehyde groups (Sullivan, Daukas and Zigmond, 1984; Haston, 1987), *i.e.* cells were washed three times, then incubated for 15 minutes at 4°C, in 0.05M glycine, diluted in saline.

Cells were washed in saline and cytocentrifuge preparations made. Addition of antibodies, APAAP complexes and substrate was the same as for the conventional method. Counterstaining was done by dipping slides in freshly-filtered 0.5% (w/v) aqueous methyl green (BDH) for 2 minutes.

Coded APAAP preparations were examined "blind" on an Olympus BH-2 microscope using an oil immersion x100 lens and a x10 eyepiece. For cell counts, at least 300 consecutive, randomly-chosen cells were classified for each category.

2.10 Preparation of collagen.

The method used was based on the original description of Elsdale and Bard (1972), modified by Haston and Wilkinson (1988a) Most steps were carried out at 4°C, and centrifugations were at 3000x g.

Tails from 6-12 adult rats were collected and stored at -20°C. Tails were thawed and the skin stripped. The 4 longitudinal bundles of tendons were stripped and placed in 200ml of acetic acid (BDH), diluted to 3% (v/v) in water. Collagen was allowed to dissolve for 24-48 hours, leaving tropocollagen (Gross *et al.*, 1954) in solution . This was coarse-filtered through 100 μm Nitex gauze (R. Cadisch and sons, London, U.K.) and centrifuged to deposit any debris. NaCl was added to the solution at 10% (w/v). Collagen precipitated overnight and the preparation was centrifuged. Precipitated collagen was removed into a flask, and re-dissolved in 3% acetic acid overnight. The salt precipitation and re-dissolution steps were repeated. The collagen solution was dialysed extensively against distilled water, aliquotted, and stored at -20°C. Two batches of collagen were prepared during this project, and appeared to remain stable over periods of several months.

The concentration of collagen was assessed by comparison with a sample taken from the same batch, freeze-dried, weighed, and re-dissolved at a known concentration in 3% acetic acid. Absorbances of the solutions were measured at 230nm in a Pye Unicam SP 1800 spectrophotometer, using matched cuvettes (Schor, 1980; Haston and Wilkinson, 1988a).

2.11 SDS-PAGE analysis of collagen preparations.

The purity of the collagen preparations used in this study was assessed by SDS-PAGE, using the method of Laemmli

(1970), modified by Beeley *et al.* (1991).

Molecular weight markers of 29, 45, 66, 97.4, 116 and 205 kdal were obtained from Sigma.

Bovine serum fibronectin was a gift from G. Campbell (Dept. of Cell Biology, University of Glasgow, Glasgow, U.K.). It had been purified by affinity chromatography on a gelatin-sepharose column, using the method of Engvall and Ruoslahti (1977), detailed by Brown (1983).

Samples were boiled at 100°C for three minutes in water with: 2% (w/v) SDS (Fisons FSA Lab. supplies, Loughborough, U.K.); 25 mM DTT (Boehringer Mannheim U.K. Ltd., Lewes, U.K.); 10% (v/v) glycerol (Fisons); and 0.001% (w/v) bromophenol blue (BDH).

To form gels, acrylamide and N,N'-methylenebisacrylamide (Fisons) were mixed in a ratio of 30:0.8 in Tris buffer, and de-gassed. 0.1% (w/v) SDS, 0.02% (v/v) TEMED and 0.03% (w/v) ammonium persulphate (BDH) were added. Various strengths of acrylamide were used for stacking and running gels.

Running gels were made up in 375 mM Tris, pH 8.8. Isopropyl alcohol (Sigma) was layered on the surface, and the gel allowed to set. The alcohol was removed and stacking gel, made up in 125 mM Tris, pH 6.8, poured on. Gel dimensions were approximately 150 x 150 x 1.5 mm.

Various amounts of sample were loaded, and gels were run overnight at 60 V on a Protean II rig (Bio-Rad Labs., Hemel Hempstead, U.K.) with a Shandon Vokam 2761 powerpack (Shandon Southern Instruments), at 4°C.

Reservoir buffer was 25 mM Tris, pH 8.3, containing 1.44%

(w/v) glycine and 0.1% (w/v) SDS.

Gels were fixed and stained for three hours in 50% (v/v) ethanol, 10% (v/v) acetone and 0.1% (w/v) Coomassie brilliant blue R-250 (Sigma), in water. Gels were destained in 10% (v/v) acetone for 2-3 days.

2.12 Collagen gel invasion assays.

Stock collagen was thawed, placed on ice, and returned to physiological osmolarity using 10x strength HBSS and water, with MOPS added at 1mM. The pH was adjusted to approximately 7.4. The collagen concentration was adjusted to be twice the required final concentration using water. Equal volumes were mixed with locomotor stimulants. Gels were allowed to form by dispensing 1ml volumes to wells of 24-well plates at room temperature. Gelling occurred after 15-30 minutes. HBSS was then layered onto the gel surface to prevent drying and formation of an impenetrable surface skin (Haston and Wilkinson, 1988). Lymphocytes (0.5-2 x 10^6 per gel) were added in a final volume of 1 ml, and preparations left for 3.5 hours or overnight, allowing lymphocytes to attach and invade the matrix.

Cell invasion of gels was assessed in two ways:-

(1) The overall proportion of cells invading a gel was counted on a Nikon Diaphot-TMD microscope using x400 phase contrast magnification with an eyepiece graticule grid. Cells on the surface of a randomly-chosen field were counted, as were cells which had invaded the gel below that field. These

were visualised by focussing down through the gel. Hence the proportion of cells in that field which had invaded could be derived (Shields, Haston and Wilkinson, 1984). At least 300 cells were counted for each gel.

(2) The surfaces of gels were gently washed three times with HBSS, and the population of cells removed was retained. The gels were then broken up by suction using a Pasteur pipette. The collagen and the population of cells associated with it were transferred to conical tubes and incubated at 37°C for 30 minutes with collagenase (Shields, Haston and Wilkinson, 1984; Ratner, Jasti and Heppner, 1988), at 12.5 U/ml, and cells released were washed and retained. THe collagenase was derived from Clostridium histolyticum (Clostridiopeptidase A; BDH). This was dissolved in HBSS at 1000 U/ml, and stored at -20°C.

Cells washed from the gel surface, or associated with the gel matrix and recovered using collagenase, were counted using improved Neubauer counting chambers. Proportions of cells associated with the gel matrix could thus be derived, and cells could be used for phenotypic analysis (Schor, 1980).

In a preliminary experiment to assess whether contact with collagen and subsequent exposure to collagenase affected surface marker expression, some cells were left in medium (HBSS/HSA), while collagen gels were allowed to form around the others. After overnight culture, collagenase was added to digest the gels. The two populations were washed and stained by immunofluorescence (see section 3.4.2).

2.13 Cell size analysis.

2.13.1 Area measurement by bitpad after tracing of outlines.

Preparations of cells stained using the modified APPAAP method were made. Preparations were examined on a Leitz Ortholux microscope using a x100 oil immersion lens and a x10 eyepiece. Attached to this was a Hitachi VM 173 E/K monitor connected to a Panasonic WV 1850/B television. Cell images were traced using a felt-tip pen onto acetate paper (Eisele *et al.*, 1991). For each preparation the outlines of 50 consecutive round and 50 consecutive polarized, positively-staining lymphocytes were traced. The areas of these outlines were measured by a bitpad connected to a BBC microcomputer using a programme written by Dr. J.M. Lackie, based on that of Drs. G. A. Dunn and A. F. Brown (Dunn and Brown, 1986; Wilkinson *et al.*, 1988; Wilkinson and Islam, 1989). Areas were converted to μm^2 by comparison with the derived value of a $10\mu m$ x $10\mu m$ reference grid.

2.13.2 Area measurement by cell image processing.

APAAP-stained preparations of cultured cells were examined using a x50 objective and x10 eyepiece on a Leitz Ortholux microscope. This was attached to a Hamatsu Vidicon C1000 camera. Cell images were digitised for input to the screen memory of a BBC Archimedes microcomputer using a Data

Harvest video interface. Lymphocytes were randomly selected and the shapes of 50 consecutive round and 50 consecutive polarized, positively-staining cells were measured using a programme written by C. Edwards (Edwards, Robson and Campbell, 1987).

2.13.3 Forward scatter measurement using FACS analysis.

The volume of spherical objects, including cells, can be derived using forward light scatter measurement by flow cytometers (Mullaney *et al.*, 1969) Cells were stained by immunofluorescence, and 10,000 events acquired by the FACS. Mean values of the forward scatter of lymphocytes were then derived using machine software (Buckle and Hogg, 1990), after selecting positively-staining cells if necessary.

CHAPTER 3: DEVELOPMENT AND ASSESSMENT OF METHODS FOR EXAMINING THE PHENOTYPE OF LOCOMOTOR CELLS.

3.1 Introduction.

The study of lymphocyte locomotion *in vitro* has been hampered by the fact that very few lymphocytes direct from blood or from resting lymphoid tissues appear to show locomotor capacity using standard *in vitro* assays, and that cell activation appears to be necessary to induce a locomotor response in a substantial proportion of blood lymphocytes (reviewed by Parrott and Wilkinson, 1981). Furthermore contact with monocytes is essential for lymphocytes to acquire locomotor capacity in response to activators such as anti-CD3 (Wilkinson and Higgins, 1987a). Thus methods were required that would enable culture of the whole mononuclear cell population, followed by testing for locomotor responses, and then phenotyping the locomotor and non-locomotor lymphocytes. Since relating phenotype to locomotion was central to this study, a primary task was to develop such methods.

3.2 Polarization assays.

3.2.1 Preliminary experiments.

Direct visual methods have been shown to be superior to semi-automated cell shape analysis, or differences in light scatter, in assessing lymphocyte polarization (Eisele *et al.*,

1991). Thus attempts were made to examine the phenotype of cells in the visual polarization assay of Haston and Shields (1985).

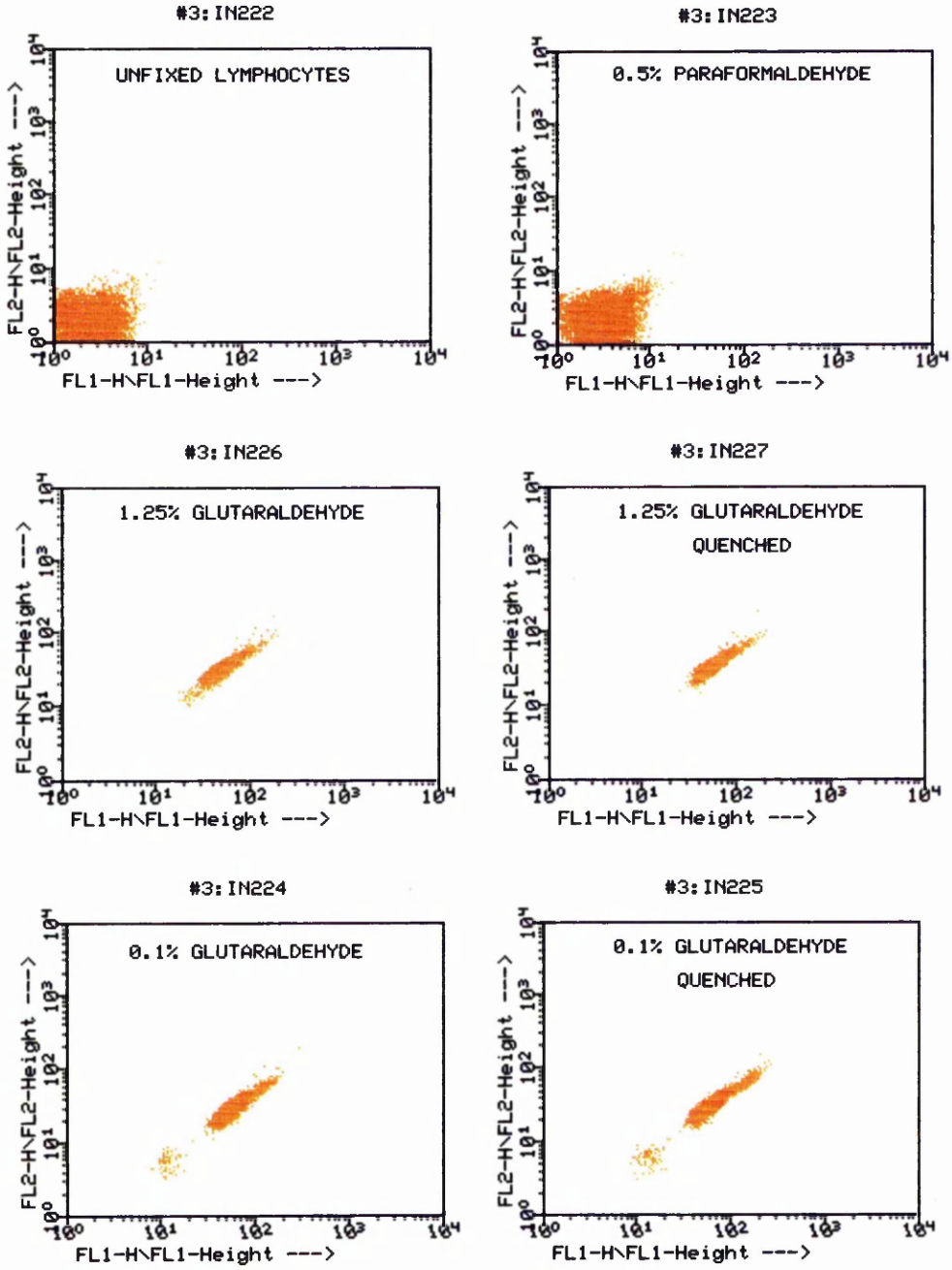
For the conventional polarization assay cells are fixed in a final concentration of 1.25% (w/v) glutaraldehyde, which retains the morphology seen in live lymphocytes (Keller, Niggli and Zimmermann, 1989). However glutaraldehyde causes fluorescence of cells even if no fluorescent probes are added. This was reported to be reduced if the glutaraldehyde concentration was lowered to 0.1%, and free aldehyde groups quenched by washing and incubation in 0.05 M glycine (Haston, 1987; Wilkinson and Islam, 1989). A series of experiments attempted to use this protocol prior to staining by specific fluorescent antibodies.

It was observed visually that very high background fluorescence was seen, before addition of fluorescent antibodies, whatever concentrations of glutaraldehyde were used, and whether cells were incubated in glycine or not. No such background fluorescence was evident in unfixed cells, or cells fixed in 0.5% paraformaldehyde. Samples were acquired on the FACS to illustrate this (figure 3.1).

To attempt to detect phenotypic markers on lymphocytes, lymphocytes were fixed in glutaraldehyde, and incubated in glycine. Staining was performed using primary antibodies against CD3, CD4, CD8, CD19, CD29, CD45RO or CD45RA, and fluorescently-labelled secondary antibodies. However very few cells showed as positive over this background by FACS analysis or visually, and distinction of positive cells visually was

FIGURE 3.1

The effect of glutaraldehyde fixation, and quenching of free aldehyde groups, on background fluorescence of lymphocytes.



Samples from a batch of lymphocytes, treated as indicated, were acquired on a FACS without addition of antibody. Glycine was used to quench aldehyde groups.

Graphs show red fluorescence on the vertical axis, and green fluorescence on the horizontal axis.

extremely difficult, even if secondary antibody concentration was raised to a final dilution of 1/25. A comparison of percentages of positively-staining cells observed using this protocol, with those observed from parallel preparations stained by conventional immuunofluorescence, is shown in table 3.1.

It would appear that only the most strongly-positive staining is discernable using antibodies against CD3, CD8, CD29, CD45RO and CD45RA over the background caused by grade II glutaraldehyde. No positive staining was evident using α-CD4 or α-CD19.

Gillett and Gull (1972) showed that on storage, glutaraldehyde gradually developed impurities (probably glutaraldehyde polymers) which changed its absorbance pattern. This was prevented by storage of highly purified (grade I) glutaraldehyde at -20°C until use. A set of experiments was performed to examine whether the use of grade I glutaraldehyde, as opposed to the grade II glutaraldehyde used previously, would make any difference to background fluorescence.

Experiments such as those illustrated in figure 3.2 indicated that even if grade I glutaraldehyde, freshly diluted from stock stored at -20°C, was used to fix cells prior to quenching of free aldehyde groups, it gave similar background fluorescence to the grade II glutaraldehyde used routinely.

Because of the difficulties encountered due to glutaraldehyde fixation prior to immunofluorescence, 1%

TABLE 3.1

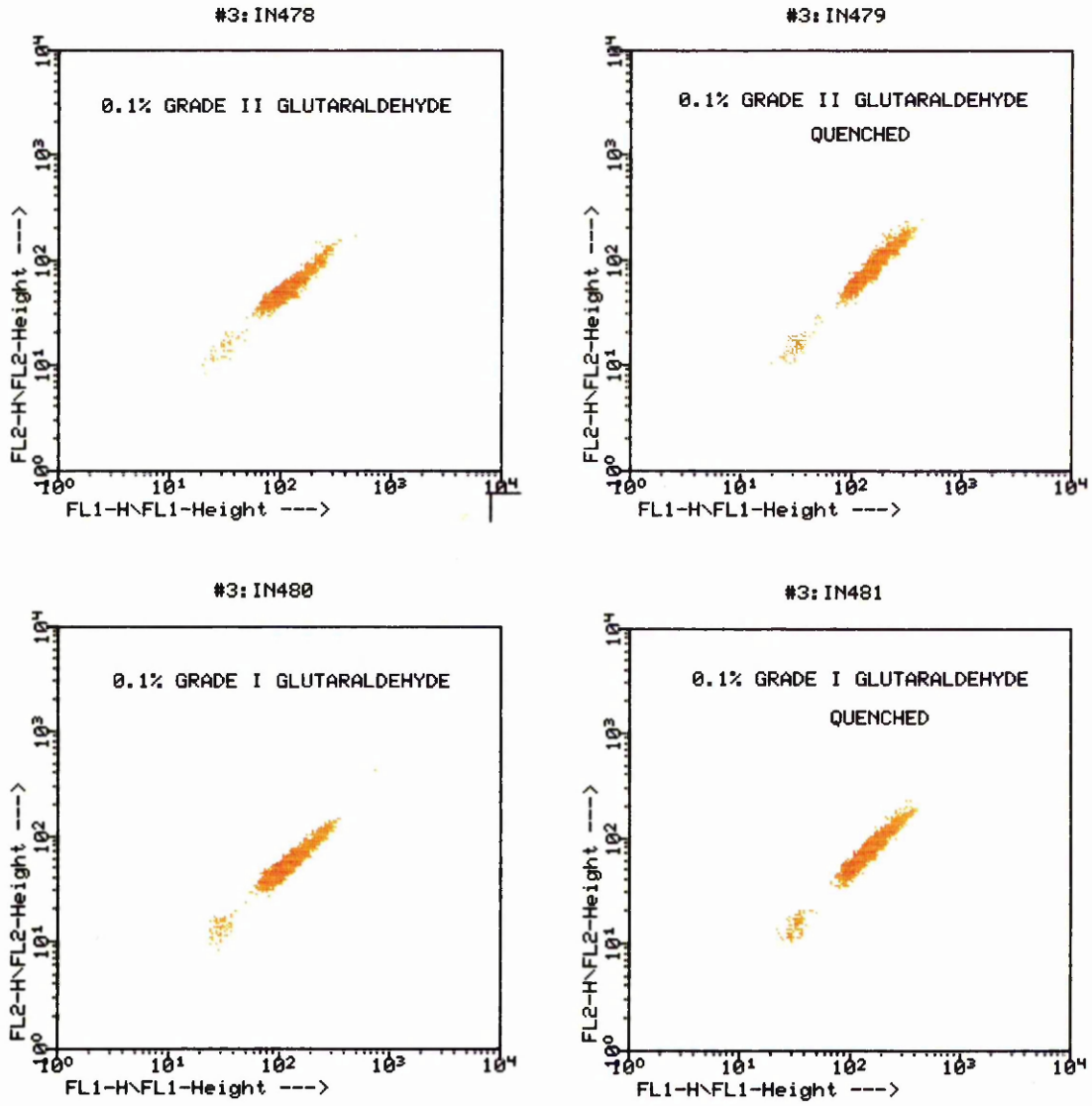
Lymphocyte staining after conventional immunofluorescence, or after cells were pre-fixed in glutaraldehyde.

Primary Ab	Secondary Ab label	Conventional method % +	Glutaraldehyde-fixed % + (above background)
α-CD45RO	FITC	31.7	16.3
	PE	40.0	16.0
α-CD45RA	FITC	71.7	16.0
	PE	69.3	26.7
α-CD29	FITC	55.0	15.7
	PE	59.7	15.0
α-CD3	FITC	83.7	12.7
	PE	71.0	39.7
α-CD3/PE	NONE	83.7	26.3
α-CD4	FITC	53.7	< 0.3
	PE	27.7	< 0.3
α-CD4/PE	NONE	40.0	< 0.3
α-CD8	FITC	25.0	5.3
	PE	33.3	5.0
α-CD8/PE	NONE	23.0	1.3
α-CD19	FITC	6.0	< 0.3
	PE	6.0	< 0.3

Lymphocytes were either stained by the conventional method and fixed in 0.5% paraformaldehyde, or were pre-fixed in 0.1% glutaraldehyde and treated with glycine. Preparations were examined by eye. Numbers in each row refer to preparations from the same donor on the same day.

FIGURE 3.2

The effect of using grade I glutaraldehyde fixation, and quenching of free aldehyde groups, on background fluorescence of lymphocytes.



Samples from a batch of lymphocytes, fixed in grade I or grade II glutaraldehyde, were acquired on a FACS without addition of antibody. Glycine was used to quench aldehyde groups.

Graphs show red fluorescence on the vertical axis, and green fluorescence on the horizontal axis.

paraformaldehyde (Wilkinson and Higgins, 1987a) or 2% formaldehyde (Haston and Shields, 1984) were investigated as potential fixatives for phenotyping polarized cells. Lymphocytes fixed in formaldehyde or paraformaldehyde showed distortions on cell outlines with the appearance of "haloes" on the cell membranes (figure 3.3). Nevertheless attempts were made to stain paraformaldehyde- or formaldehyde- fixed cells by immunofluorescence.

Unlike glutaraldehyde-fixed cells, there was no background staining prior to addition of fluorescently-labelled antibody. However when staining with fluorescently-labelled antibody was attempted, a large amount of apparently non-specific staining was observed visually.

To illustrate this observation, unfixed, paraformaldehyde-fixed, or formaldehyde-fixed cells, were stained using PE- and FITC-labelled negative control antibody, and washed thrice. Samples were acquired by the FACS (figure 3.4). A large amount of non-specific uptake of antibody by pre-fixed cells was apparent. This non-specific uptake would make any phenotypic analysis invalid.

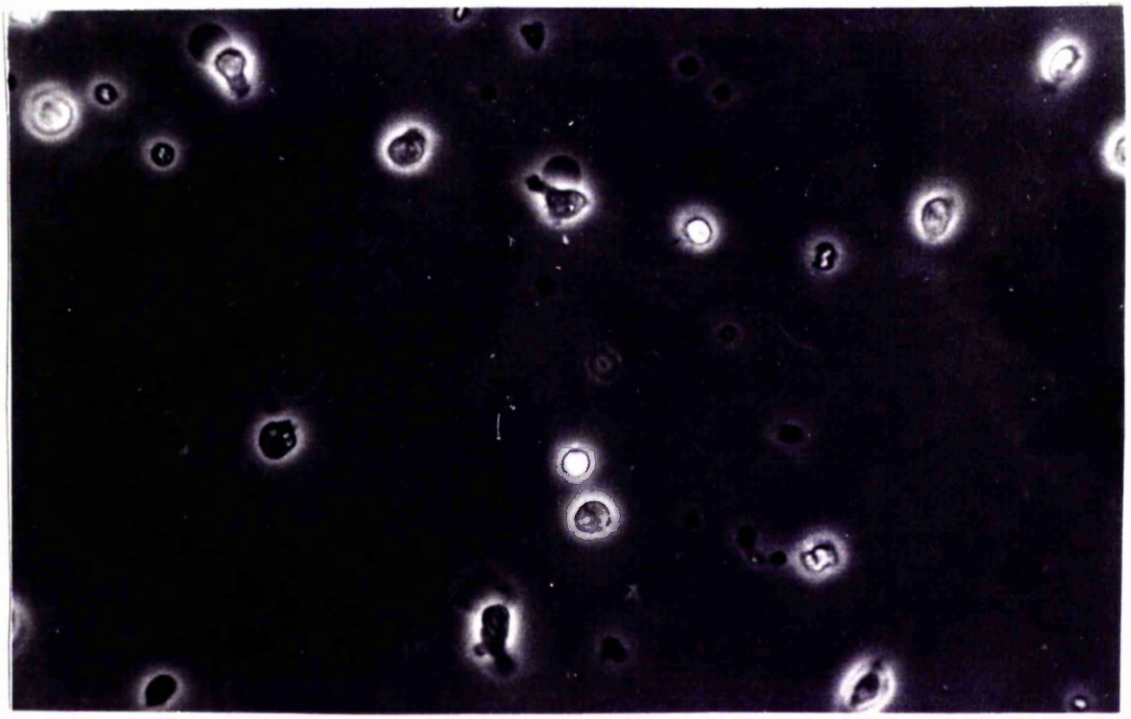
3.2.2 Development of a modified APAAP method.

Because of the methodological problems encountered in attempts to stain pre-fixed cells by immunofluorescence, a non-fluorescent label, APAAP (Cordell *et al.*, 1984), was evaluated. Conventionally, cells are fixed in methanol/acetone after cytocentrifuge preparations are made.

FIGURE 3.3

The morphology of paraformaldehyde-, and formaldehyde-fixed lymphocytes (phase contrast optics).

A:- Cells fixed in 0.5% paraformaldehyde.



B:- Cells fixed in 2% formaldehyde.

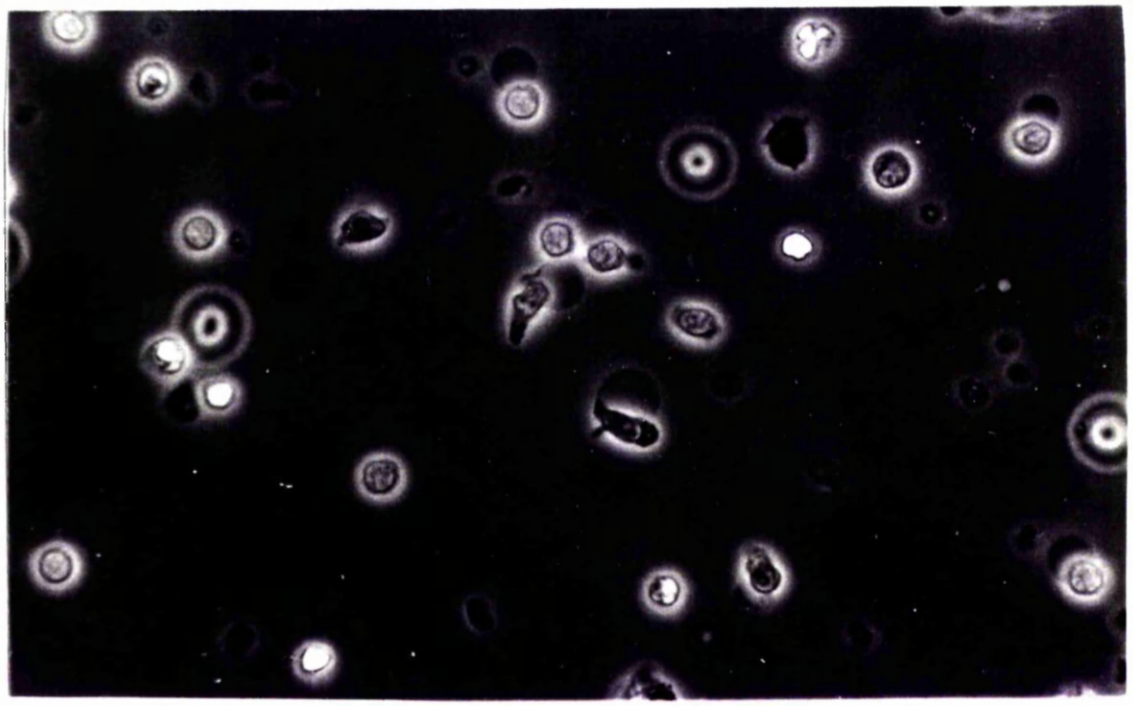
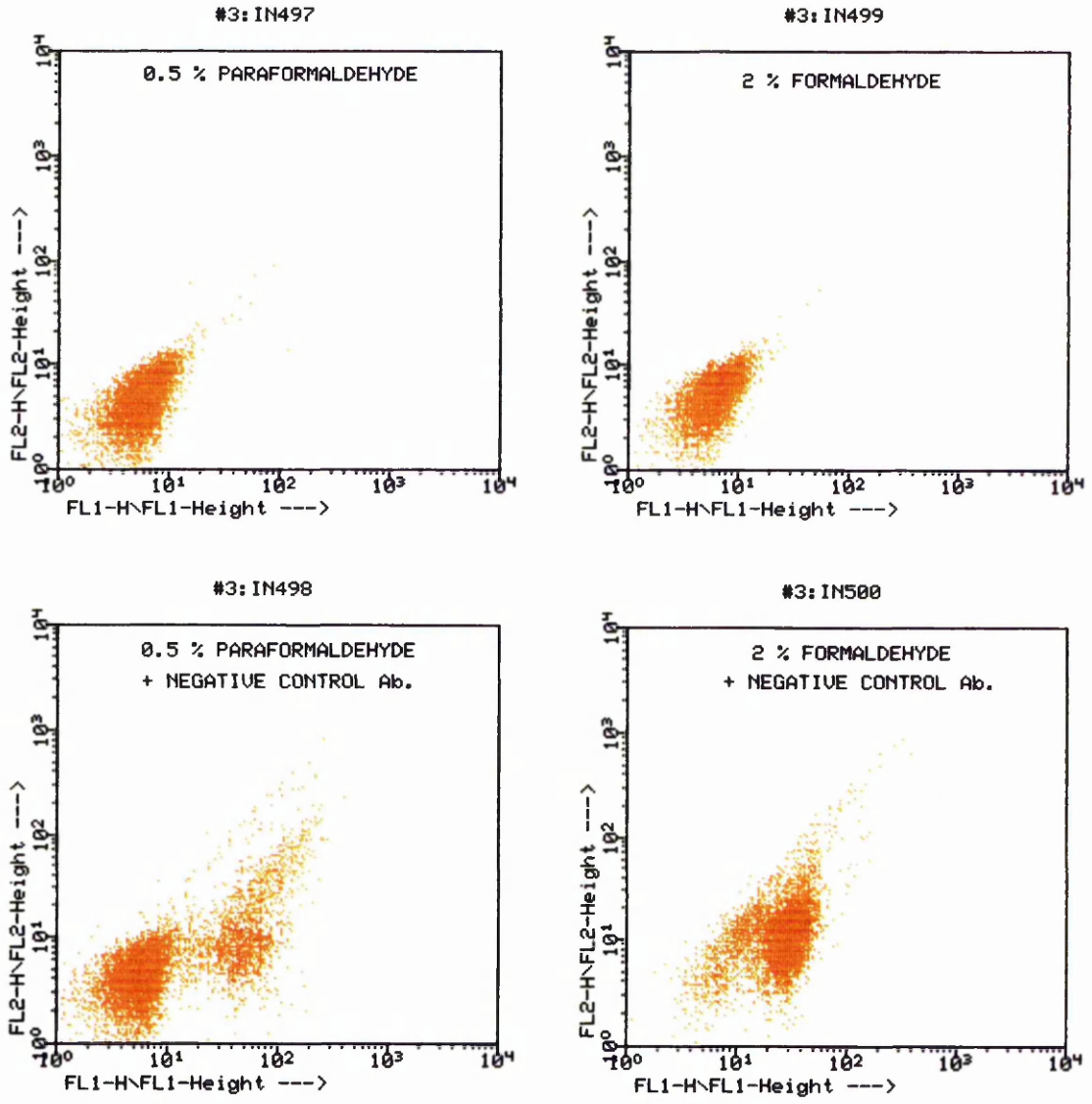


FIGURE 3.4

The effect of fixation of lymphocytes in paraformaldehyde or formaldehyde on subsequent staining using immunofluorescently-labelled antibodies.



Samples from a batch of lymphocytes were fixed in paraformaldehyde or formaldehyde. Cells were either left unstained, or stained using the FITC- and PE-labelled negative control antibody.

Graphs show red fluorescence on the vertical axis, and green fluorescence on the horizontal axis.

It was observed that using this protocol caused loss of polarized morphology in lymphocytes. Consequently 1.25% glutaraldehyde was reverted to as a fixative and subsequent APAAP staining evaluated.

Conventional APAAP staining cannot be done using cells in suspension, while to preserve morphology in conventional polarization assays, cells are usually fixed in suspension. Preliminary experiments were undertaken to assess whether the proportion of polarized cells changed when cytocentrifuge preparations were made from cell suspensions. MNC were therefore cultured for 24 hours in αCD3 and fixed in 1.25% glutaraldehyde. Cells were examined by phase contrast microscopy, in suspension or after cytocentrifugation. Results are shown in table 3.2. The proportion of polarized lymphocytes did not change after cytocentrifugation in these experiments, nor in a range of subsequent experiments. This indicates that the process of cytocentrifugation did not affect lymphocyte morphology.

Various cell preparations were then stained after fixation in glutaraldehyde, but otherwise using the conventional APAAP method. It was found that haematoxylin, which is used for the conventional method, was unsuitable as a counterstain since it gave poor colour contrast. 0.5% methyl green was found to be suitable. Thus the only modifications to the conventional method, which were required to enable locomotor morphology to be retained, were changes in the fixative and counterstain.

Various cell preparations were stained by the modified

TABLE 3.2

Comparison of the proportions of polarized lymphocytes in wet preparations, or in cytocentrifuge preparations from the same cell suspensions.

EXPERIMENT NUMBER	STIMULUS	% LYMPHOCYTES POLARIZED (WET PREPARATION)	% LYMPHOCYTES POLARIZED (CYTOCENTRIFUGE PREPARATION)
1	MEDIUM	14.0	15.0
	α-CD3	44.7	43.0
2	MEDIUM	3.0	5.3
	α-CD3	28.3	28.3
3	MEDIUM	5.7	7.0
	α-CD3	37.7	40.3

Lymphocytes were cultured for 24 hours in medium ± α-CD3. The proportions of polarized lymphocytes in wet preparations, or in cytocentrifuge preparations from the same cell suspensions, were determined using phase-contrast microscopy.

method for surface markers CD3, CD4, CD8, CD14, CD19, CD22, CD25, CD29, CD45RA or CD45RO.

Typical staining for CD45RO by the conventional, or modified, APAAP procedures is shown in figure 3.5, with positive cells staining red against the haematoxylin (blue), or methyl green, counterstains.

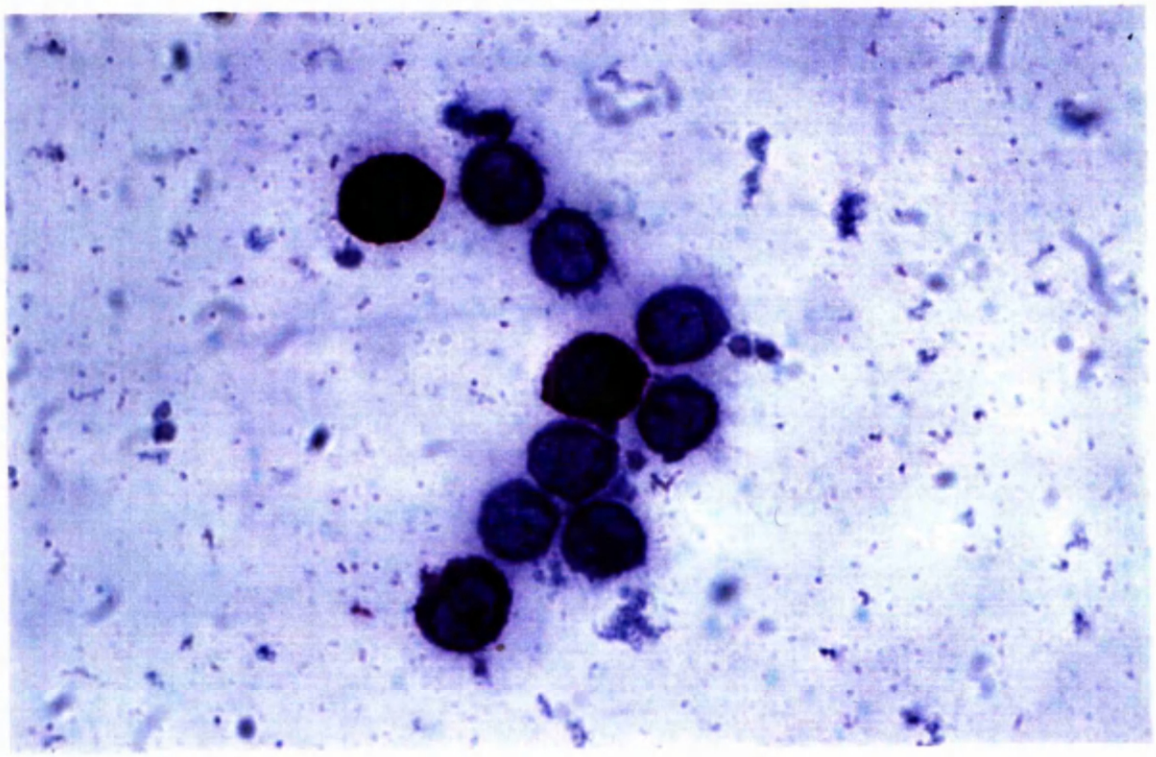
The modified technique showed staining for all antibodies tested, except CD4, CD14, CD19, and anti-IL2 receptor ß chain. No staining was seen even if these antibodies were used neat. The α-CD4, α-CD14 and α-CD19 antibodies worked using conventional APAAP (and immunofluorescent) staining. The anti-IL-2 receptor ß chain antibody was found to work using immunofluorescence, but not using conventional APAAP staining.

Possible reasons for the failure of the α-CD4 to react with glutaraldehyde-fixed cells were investigated further. The isotype of the α-CD4 was IgG1. This was the same as all of the other antibodies used except α-CD14, α-CD45RO and α-IL-2 receptor ß chain (which were IgG2a), so the secondary antibody should have combined with bound α-CD4 equally well. Preliminary experiments showed that α-CD4 stained lymphocytes pre-fixed in paraformaldehyde, then stained by APAAP, although cell morphology was very poor. Together with the lack of any staining by immunofluorescence after glutaraldehyde fixation (see table 3.1), these results suggest that the antigenic determinants of CD4 may be modified or destroyed by glutaraldehyde, preventing detectable α-CD4 binding. Alteration of antigens by glutaraldehyde may be a limitation of the modified APAAP technique. Even the conventional

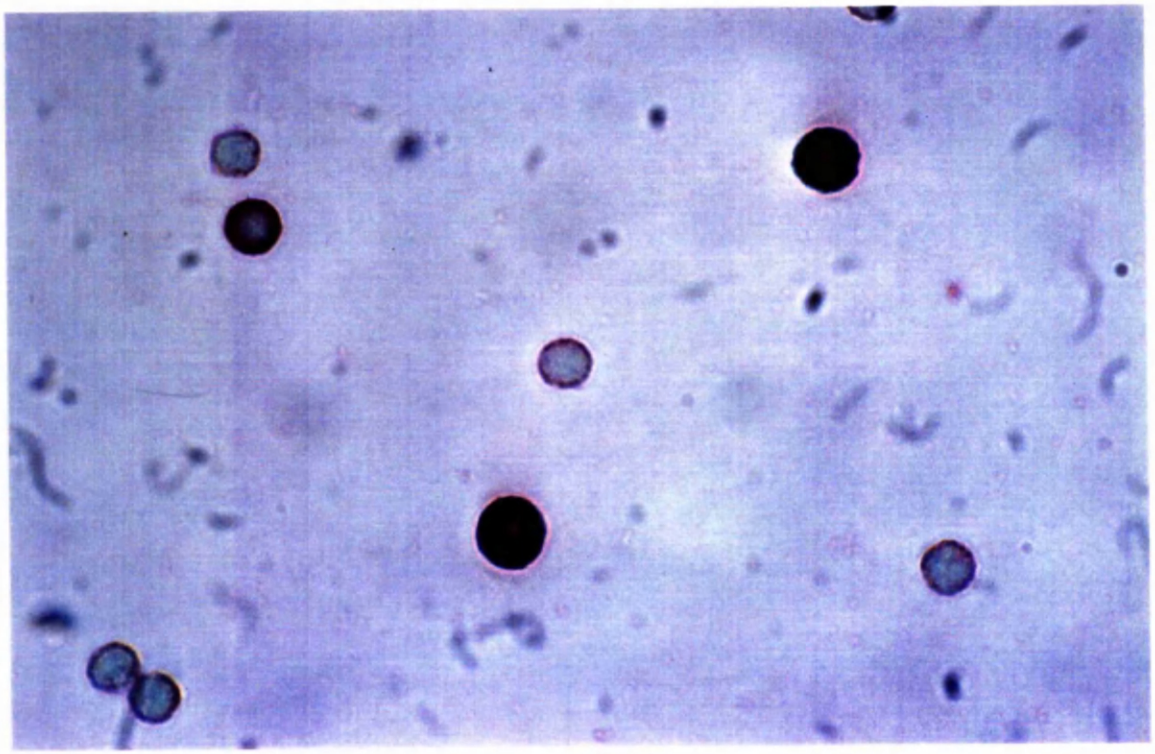
FIGURE 3.5

The appearance of lymphocytes stained for CD45RO by conventional or modified APAAP methods.

A:- Conventional method.



B:- Modified method.



technique may not be suitable for all antibodies, for example the anti-IL-2 receptor ß chain antibody did not work at dilutions which were suitable for other antibodies, although the antibody did work using immunofluorescence.

When staining for CD29 by the modified APAAP method was attempted, weak staining was evident on virtually all lymphocytes, and it was impossible to confidently discern positive staining. CD29 is present on virtually all lymphocytes (Wallace and Beverley, 1990), but while strongly positive cells can also be distinguished using immunofluorescence and FACS analysis, this was not clearly evident using the modified APAAP method.

In an attempt to detect any effects of pre-fixation and cytocentrifugation on staining of lymphocytes, cell preparations were stained for CD3, CD8, CD22, CD25, CD45RA or CD45RO, using the modified APAAP method in parallel with conventional immunofluorescence and FACS analysis, to quantify the proportions staining positive. Results are shown in table 3.3, and indicate that the proportions of cells staining positive for these markers were similar using both methods. Thus specific staining appears to be preserved in the modified method using these antibodies.

3.2.3 Conclusions from attempts to phenotype polarized cells.

Attempts to use immunofluorescence to phenotype polarized cells were unsuccessful because of the need to pre-fix cells

TABLE 3.3

Comparison of lymphocyte staining for various markers after immunofluorescence, or modified APAAP, methods.

EXPT. NUMBER	METHOD USED	CD45RO+	CD45RA+	CD3+	CD8+	CD22+	CD25+
1	FACS	21.0	55.1	52.2	35.3	14.3	26.2
	APAAP	23.0	60.3	55.3	31.3	17.7	30.7
2	FACS	26.3	73.8	58.2	19.8	11.9	53.5
	APAAP	30.3	67.7	52.7	15.0	15.3	53.7
3	FACS	29.8	61.0	56.5	21.8	10.6	49.1
	APAAP	28.7	54.7	51.3	21.7	11.0	50.7
4	FACS	23.7	61.0	NT	22.3	NT	NT
	APAAP	21.7	58.0	NT	21.7	NT	NT

Proportions of lymphocytes positive for various markers were determined by FACS analysis after conventional immunofluorescence, or by microscopy after staining by the modified APAAP method.

To obtain samples for CD25 staining, mononuclear cells were first cultured in α-CD3 for 24-48 hours to increase CD25 expression.

The table summarises 3-4 experiments for each marker, done at various times. Each pair of FACS and APAAP results refers to cells from the same sample.

NT = Not Tested.

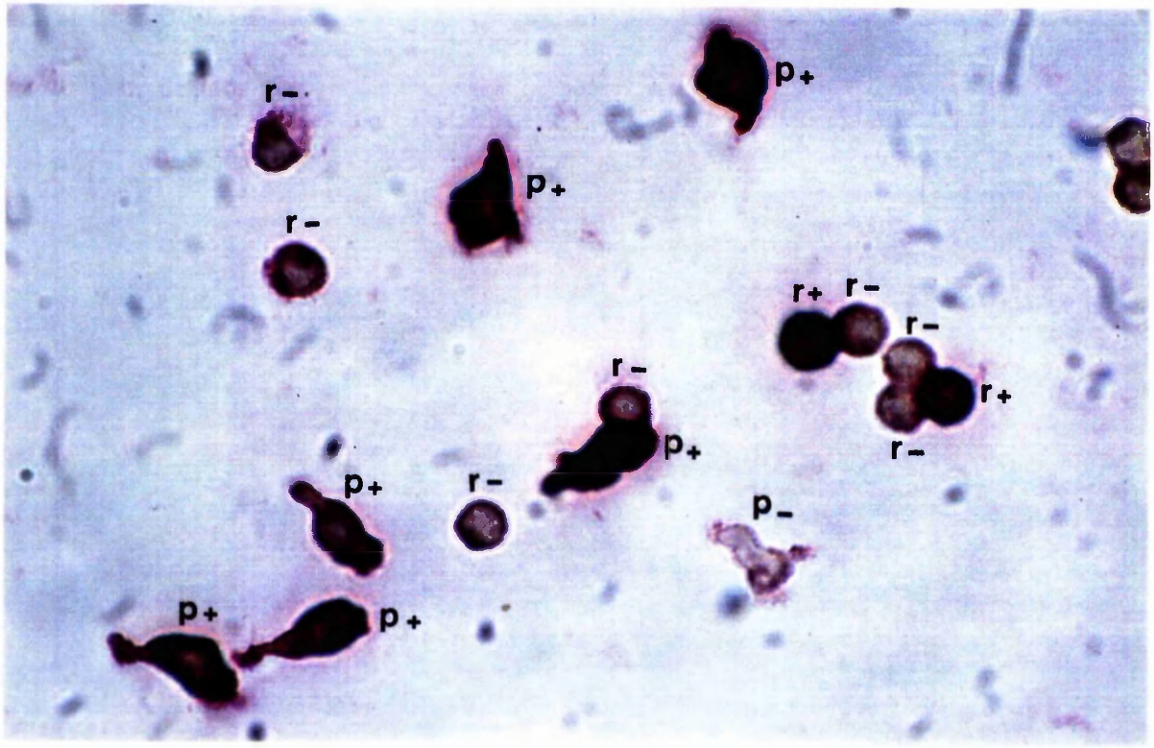
to retain morphology. Glutaraldehyde caused high background fluorescence, while formaldehyde and paraformaldehyde caused non-specific uptake of antibody. The reasons for these findings are not known. Aldehydes appear to immobilise surface proteins-glutaraldehyde directly cross-links surface proteins and polyhydroxyl compounds, while formaldehyde and paraformaldehyde can cause protein cross-links via a two-step reaction (Sabatini, Bensch and Barrnett, 1963; Veis, 1967). Why this would cause non-specific fluorescence, but apparently no non-specific staining using the modified APAAP method, is not clear. The extent of cross-linking differs between mono- and di-aldehydes, and varies with different proteins, which may partially explain the differences between glutaraldehyde and formaldehyde or paraformaldehyde, and the apparent modification of surface CD4, but not other lymphocyte surface molecules.

Some modifications were required to conventional APAAP staining, but these appeared to result in a reliable method for detection of the CD45R isoforms to be examined in this project, plus other lymphocyte surface markers. Typical preparations of cells cultured in α-CD3, fixed in glutaraldehyde, and stained for CD45RA or CD45RO by the modified APAAP procedure are shown in figure 3.6. Both cell surface marker staining and morphology were readily assessed, enabling simultaneous phenotyping and classification of cells as round or polarized. Preparations appear to be stable over a period of at least a year.

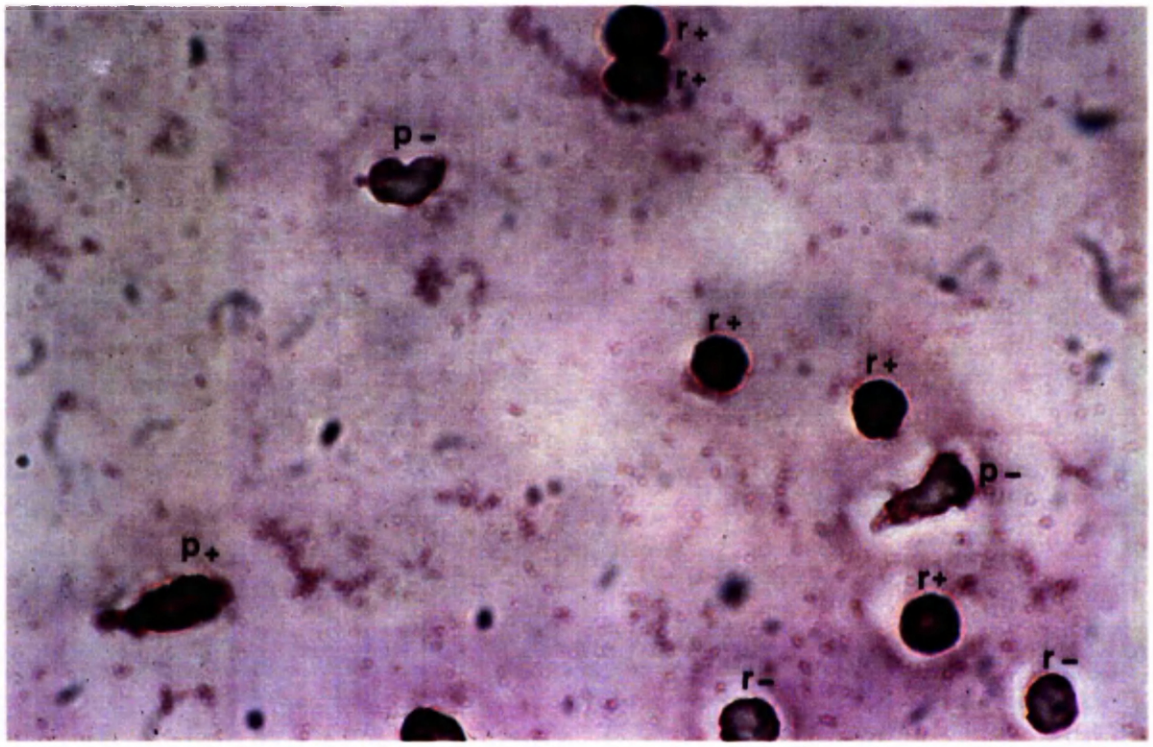
FIGURE 3.6

Modified APAAP staining showing round (r); polarized (p); positive (+); and negative (-) lymphocytes.

A:- Cells stained for CD45RO.



B:- Cells stained for CD45RA.



3.3 Assessment of proportions of monocytes present in cell preparations.

Since only lymphocytes were to be examined during this project, and since monocytes are virtually all CD45RO+ (Smith *et al.*, 1986), any contaminating monocytes present in lymphocyte preparations might affect the results obtained, in particular when comparing CD45RO+ and CD45RO- cell populations.

To exclude monocytes during FACS analysis, "leucogate" (Becton Dickinson, Cowley, U.K.) was used. This contains PE-conjugated anti-CD14 (Dimitriu-Bona *et al.*, 1983), which stains monocytes, and FITC-conjugated anti-CD45, which stains all blood leukocytes (Dalchau, Kirkley and Fabre, 1980). The monoclonal anti-CD14 was stated by the manufacturers not to react with lymphocytes, although certain anti-CD14 monoclonal antibodies react with B cells (Labeta *et al.*, 1991). Using leucogate staining, CD45+CD14+ monocytes could be identified by fluorescence, and hence their location in forward/side scatter profiles determined. They were then excluded from further analysis using gating.

For lymphocytes which were to be tested in polarization assays, preliminary attempts were made to stain glutaraldehyde-fixed MNC preparations. The following stains were used:- May-Grünwald/Giemsa, or Leishman's, stains (Dacie and Lewis, 1975); alpha-naphthyl acetate esterase staining (Mueller *et al.*, 1975; Islam, 1986); and using the modified APAAP method to detect CD14. However none of these approaches

allowed monocytes to be identified. The proportion of monocytes in wet preparations was therefore determined using phase contrast microscopy, which was found to be adequate for identification of unfixed, or glutaraldehyde-fixed monocytes using morphological criteria. This procedure was carried out routinely, and it was found that <3% of non-adherent MNC appeared to be monocytes using phase contrast microscopy.

Further preliminary experiments examined whether monocytes might be enriched after the cytocentrifugation of MNC prior to APPAP staining by the modified method. The morphological distinction of lymphocytes and monocytes was found not to be clear in such preparations after the staining procedure, and monocyte contamination might thus remain undetected. To examine whether enrichment of monocytes would occur, MNC were isolated, washed in siliconised glass tubes to retain monocytes, and fixed in 1.25% glutaraldehyde. Wet preparations of such cells were examined by phase contrast microscopy, in suspension or after cytocentrifugation. Results are shown in table 3.4. Proportions of monocytes remained unchanged. This indicates that the process of cytocentrifugation did not lead to enrichment of monocytes, and thus the proportions counted routinely in suspension prior to cytocentrifugation were a valid assessment of monocyte contamination.

3.4 Assessment of the purity of collagen preparations.

While lymphocyte invasion of collagen gels appears not to

TABLE 3.4

Comparison of the proportions of monocytes in wet preparations, or in cytocentrifuge preparations from the same cell suspensions.

EXPERIMENT NUMBER	% MONOCYTES (WET PREPARATION)	% MONOCYTES (CYTOCENTRIFUGE PREPARATION)
1	21.7	21.0
2	26.7	26.3
3	8.0	7.0

Mononuclear cells were collected, and washed, in siliconised glass tubes. Cell suspensions were fixed in 1.25% glutaraldehyde. The proportions of monocytes in wet preparations, or in cytocentrifuge preparations from the same cell suspensions, were determined using phase-contrast microscopy.

depend on adhesive interactions with the gel matrix (Haston, Shields and Wilkinson, 1982), lymphocytes express receptors for collagen and other extracellular matrix components (reviewed by Hemler, 1990). Lymphocytes, and in particular CD45RO+ cells, have been shown to adhere to components such as fibronectin and laminin (Arencibia and Sunqvist, 1989; Shimizu *et al.*, 1990a; Shimizu *et al.*, 1991a).

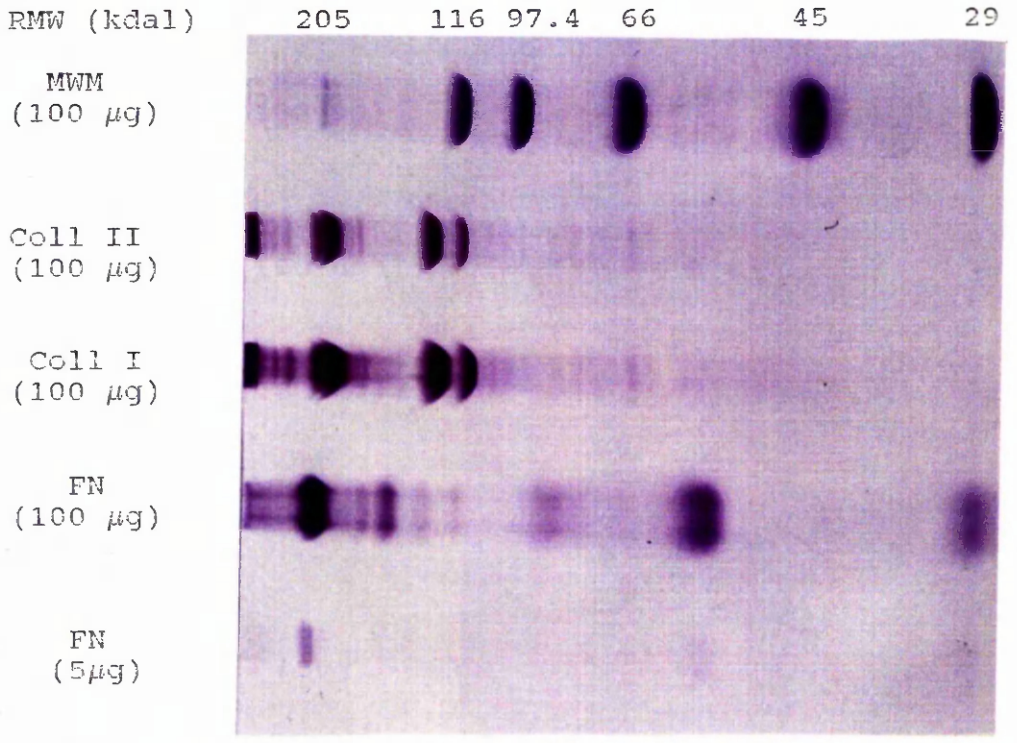
To ensure that the interactions tested in the collagen gel invasion assay were with collagen alone, and not with a mixture of collagen and other contaminants, the two batches of collagen used during this study were assessed for the presence of fibronectin using a strategy similar to that of Brown (1983). SDS-PAGE was used to determine the molecular weights of the components of the collagen preparations. These were compared to those of bovine plasma fibronectin.

Photographs of typical gels are shown in figure 3.7. The gels illustrated in figure 3.7a compared markers of known molecular weight to the bands from 100μg of the two batches of collagen used during the project. These appear to correspond to collagen α1(I) and α2(I) chain monomers and dimers, plus higher molecular weight trimers (Linsenmayer, 1991). No bands corresponding to those seen after loading 100μg of fibronectin were detected. As little as 5μg of fibronectin was visible, showing the expected 2 bands of approximately 260 kdal. (Weinstein, Legas and Hildebrant, 1974), although other bands were detected if 100μg of fibronectin was loaded. These were probably impurities (Dr. J.G.Edwards, personal communication). The results suggest that there was less than 5% (w/w) of

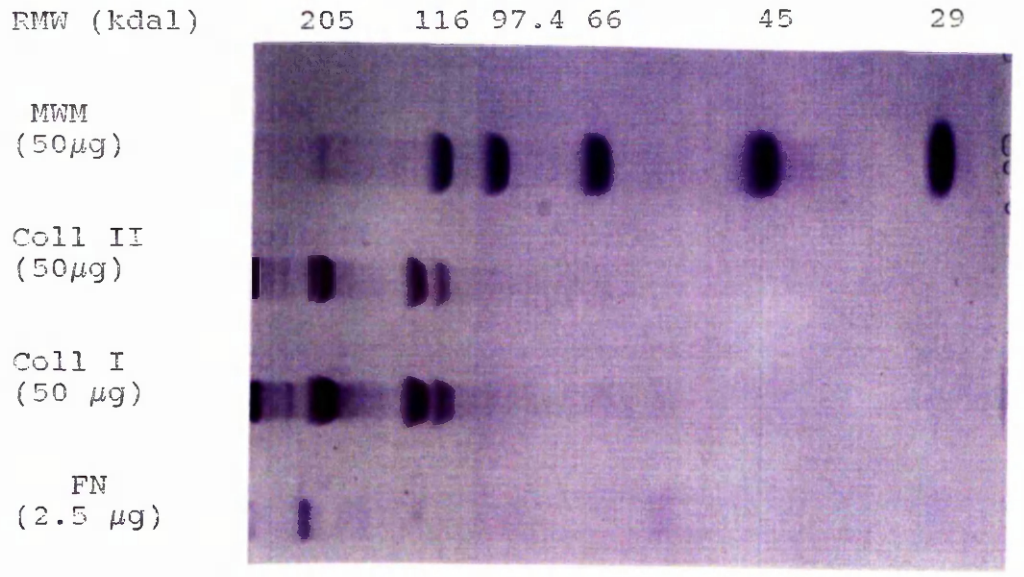
FIGURE 3.7

SDS-PAGE analysis of the two batches of collagen used to study lymphocyte locomotion.

a:-Stacking gel 5%; running gel 12.5%



b:-Stacking gel 2%; running gel 5%



Various protein loads of the two batches of collagen (Coll I & Coll II), fibronectin (FN) and molecular weight markers (MWM) were examined. Gels with different concentrations of acrylamide for stacking, and running, gels were used in figure 3.7a and 3.7b.

Gels were stained using Coomassie brilliant blue R-250.

fibronectin present in the collagen preparations.

In gels illustrated in figure 3.7b, the protein loads from the collagen batches were decreased to 50μg, and that of fibronectin to 2.5μg. The concentration of acrylamide in the gels was decreased in this experiment. These changes resulted in better resolution of the protein bands.

The staining and destaining procedures used for the gels cause proline-rich proteins to stain a pink-violet colour, while other proteins stain blue (Beeley *et al.*, 1991). Collagen is rich in proline and hydroxyproline (Linsenmayer, 1991). Pink-violet staining was evident in all of the bands from the collagen preparations. Contaminating, non-proline-rich proteins were not detected in the collagen preparations. This procedure thus appears to offer a useful way of assessing the purity of the kind of collagen preparations used during this project.

3.5 Collagen gel invasion assays.

3.5.1 Introduction.

As well as a method for phenotyping polarized cells, a method was sought to examine the phenotype of cells invading collagen gels. The method used was based on that of Shields, Haston and Wilkinson (1984) and Ratner, Jasti and Heppner (1988). Lymphocytes were overlaid on 3-dimensional collagen gels and allowed to invade the matrix either for 3.5 hours or overnight. Proportions of cells which had entered the gel

were counted *in situ* using an inverted microcope, and cells washed from the gel surface (non-adherent cells) or digested from the collagen matrix (adherent and invasive cells) were collected. These populations could be counted to derive proportions adherent and invasive, and then used for phenotyping. Preliminary experiments were carried out to examine whether surface marker expression was affected by exposure to collagen and collagenase; and to try to relate the proportions invading gels to the proportions polarized in the initial cultures.

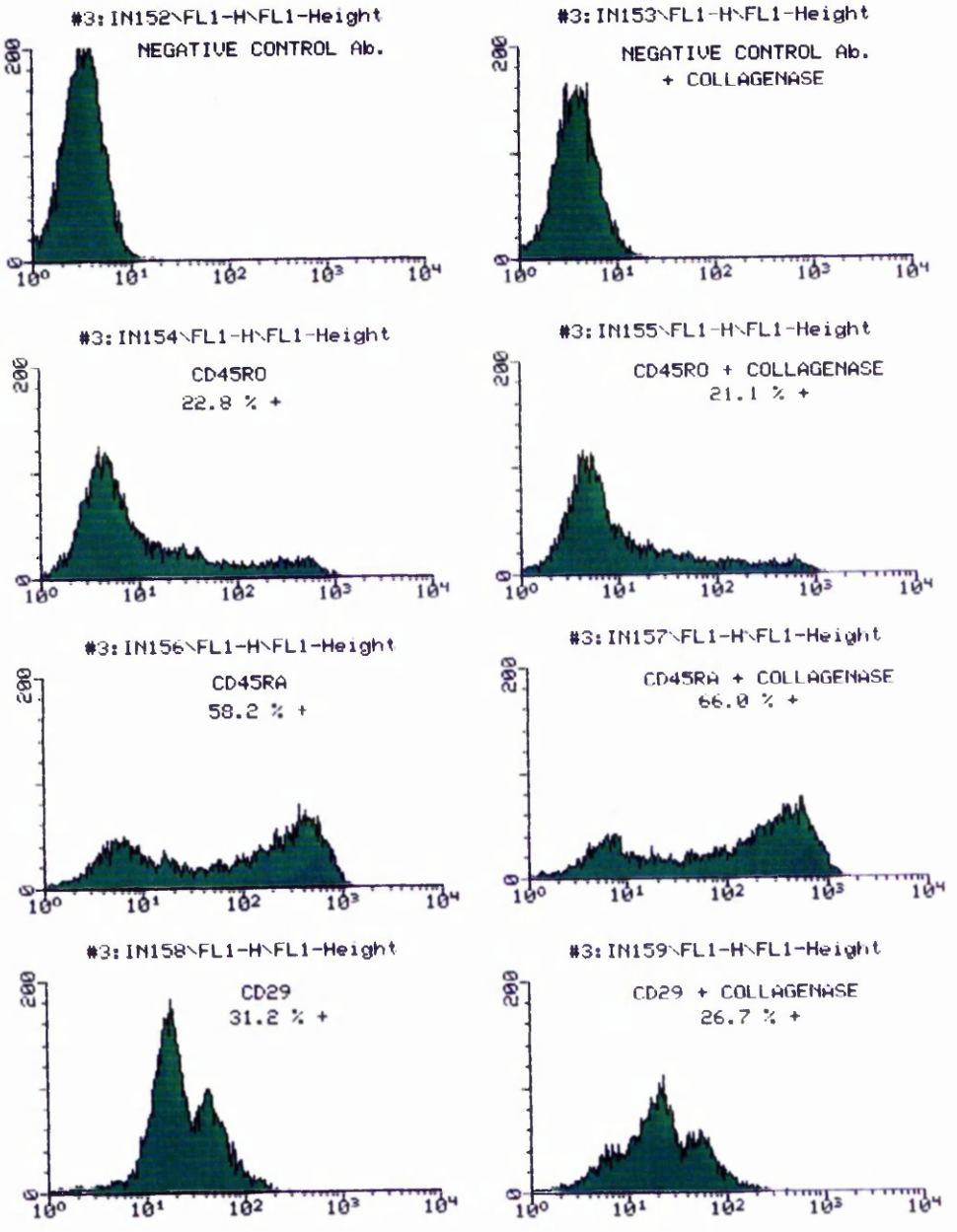
3.5.2 Effects of collagen and collagenase on surface markers.

To assess whether contact with the collagen, and subsequent treatment of the gels with collagenase, affected lymphocyte surface marker expression, mononuclear cells were cultured for 48 hours in α-CD3. A collagen gel was allowed to form around some of the lymphocytes, while the others were left in medium. Both preparations were left overnight at 37°C. Cells in contact with the collagen were then liberated from it using collagenase, and washed. The two populations were stained by immunofluorescence for CD3, CD4, CD8, CD19, CD29, CD45RA or CD45RO, and samples acquired on the FACS.

The results are shown in figures 3.8 and 3.9, and indicate that neither the proportion staining positive, nor the pattern of fluorescence, are affected by exposure to collagen and collagenase. The proportion of lymphocytes which stained for CD3 in this experiment was lower than might be

FIGURE 3.8

The effect of exposure of lymphocytes to collagen, then collagenase, on expression of CD45RO, CD45RA and CD29.

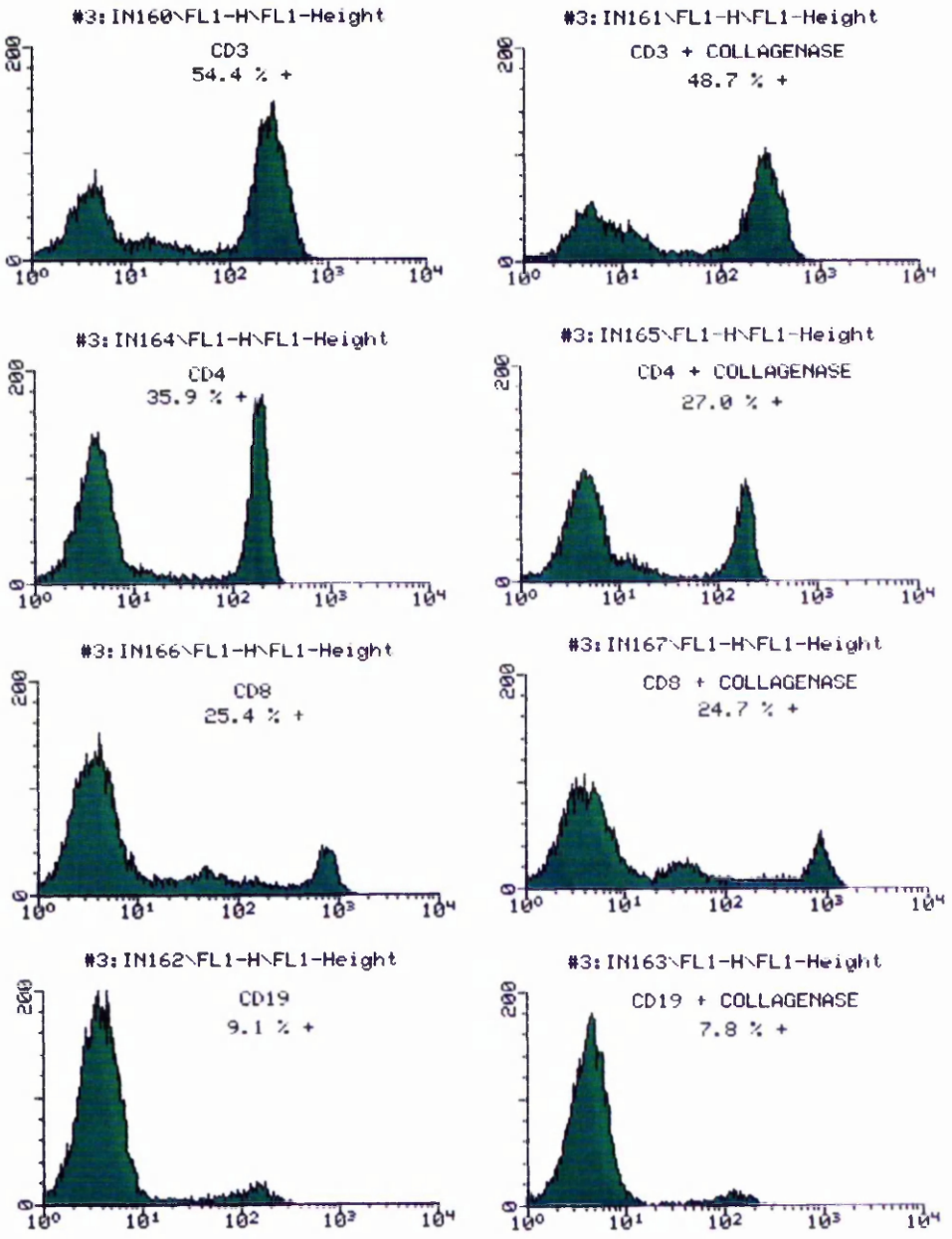


Samples from a batch of lymphocytes were cultured overnight in medium (graphs on the left of the page), or in contact with collagen (graphs on the right of the page). Collagen was digested using collagenase. Samples were then stained by immunofluorescence.

Graphs show relative cell numbers on the vertical axis and intensity of green fluorescence on the horizontal axis.

FICURE 3.9

The effect of exposure of lymphocytes to collagen, then collagenase, on expression of CD3, CD4, CD8 and CD19.



Samples from a batch of lymphocytes were cultured overnight in medium (graphs on the left of the page), or in contact with collagen (graphs on the right of the page). Collagen was digested using collagenase. Samples were then stained by immunofluorescence.

Graphs show relative cell numbers on the vertical axis and intensity of green fluorescence on the horizontal axis.

expected (Hannet *et al.*, 1992), but the reasons for this are not known.

3.5.3 Relating collagen gel invasion and polarization.

A series of counts on cells cultured in various stimulants was performed to examine the relationship between the proportion of cells polarized, the proportion entering gels, and the proportion of cells recovered from the gels by first washing, then digesting, the gels.

The proportion entering gels *in situ* was determined by counting the cells on an area of the gel surface, and cells within the gel below that area. The proportion recovered from the gel matrix was determined by relating counts of cells washed from the gel surface to those of cells digested from the gel matrix. Results from a range of experiments, in which MNC were cultured in a variety of stimuli, are summarised in table 3.5.

If invasion proceeded overnight it was observed that the proportion of cells recovered was similar to the proportion counted within the gels *in situ*. It was also found that the proportion recovered from, and the proportion entering, gels were usually greater than the proportion which were polarized in the original culture, although it should be noted that cells were sampled for polarization assays at an earlier time point, prior to setting up the gel invasions.

TABLE 3.5

Comparison of the proportion of lymphocytes polarized in culture with the proportions subsequently entering collagen gels, and with the proportions recovered from those gels after collagenase digestion.

	NUMBER OF TESTS	% POLARIZED IN CULTURE	% ENTERING GELS	% RECOVERED FROM GELS
OVERNIGHT INVASION	28	34.3 ± 1.9	48.5 ± 3.0	49.6 ± 3.4
3.5 HOUR INVASION	9	25.7 ± 4.0	27.1 ± 5.8	43.2 ± 3.4

This table summarises a number of counts obtained during collagen gel invasion assays (means ± e.s.e.). Mononuclear cells were cultured in various stimuli (*i.e.* α-CD3, PPD, Con A or allo-MLR) for 48-72 hours. The proportion of polarized cells was determined.

Cells were allowed to invade collagen gels for 3.5 hours, or overnight. The proportion of cells entering the gels was counted *in situ*.

Cells were washed from the gel surface, or recovered from the gels using collagenase. Cell counts of these populations were used to determine the proportion of cells recovered from the gel matrix.

If lymphocytes invaded gels for 3.5 hours, the proportion counted entering *in situ* was consistently similar to the proportion polarized, as reported previously (Wilkinson and Higgins, 1987a). Under these conditions, a greater proportion of cells were usually recovered than were counted entering *in situ*. This was presumably due to cells adhering to the gel surface and thus being retained along with cells which had invaded.

It was observed that after invasion of collagen by lymphocytes for 3.5 hours or overnight, a proportion of cells present on the gel surface were in polarized morphology, and a proportion of cells present within the gel matrix had returned to spherical morphology.

Thus the relationship between the proportion polarized in the original cultures, the proportion counted entering *in situ*, and the proportion retained by the gel may not be a simple one. Contact with the gel surface may also affect cell morphology.

Polarized morphology has been well documented in motile lymphocytes filmed using time-lapse photography since the experiments of Lewis (1921). Lymphocytes do not need to adhere to collagen in order to move (Haston, Shields and Wilkinson, 1982), but they do possess receptors for extracellular matrix components (Hemler, 1988), and collagen itself stimulates lymphocyte locomotion (Arencibia and Sundqvist, 1989). Hence the interactions between lymphocyte locomotion and adhesion in collagen gels are probably complex, whereas polarization in culture may involve different

interactions and responses.

3.5.4 Conclusions from the assessment of the methods used to phenotype cells invading collagen gels.

Results of these experiments indicate that the method used to isolate lymphocyte populations washed from gel surfaces, or digested from the gel matrix, did not affect surface marker expression. Hence phenotypic analysis was possible.

The population of cells retained by, and digested from, the collagen gel matrix appears to contain most of the motile population, as well as cells adherent to the collagen which may not be motile, particularly if invasion proceeds for only 3.5 hours. This population will subsequently be referred to as the invasive population in this project, since some level of interaction with the 3-dimensional matrix is presumably required to prevent cells being washed from the surface. This population may be regarded as enriched for motile cells, but non-motile cells may also be present.

CHAPTER 4: THE RESPONSE OF CD45RO+ AND CD45RA+ LYMPHOCYTES IN POLARIZATION ASSAYS.

4.1 Factors which cause polarization of lymphocytes after short-term stimulation.

4.1.1 Preliminary experiments.

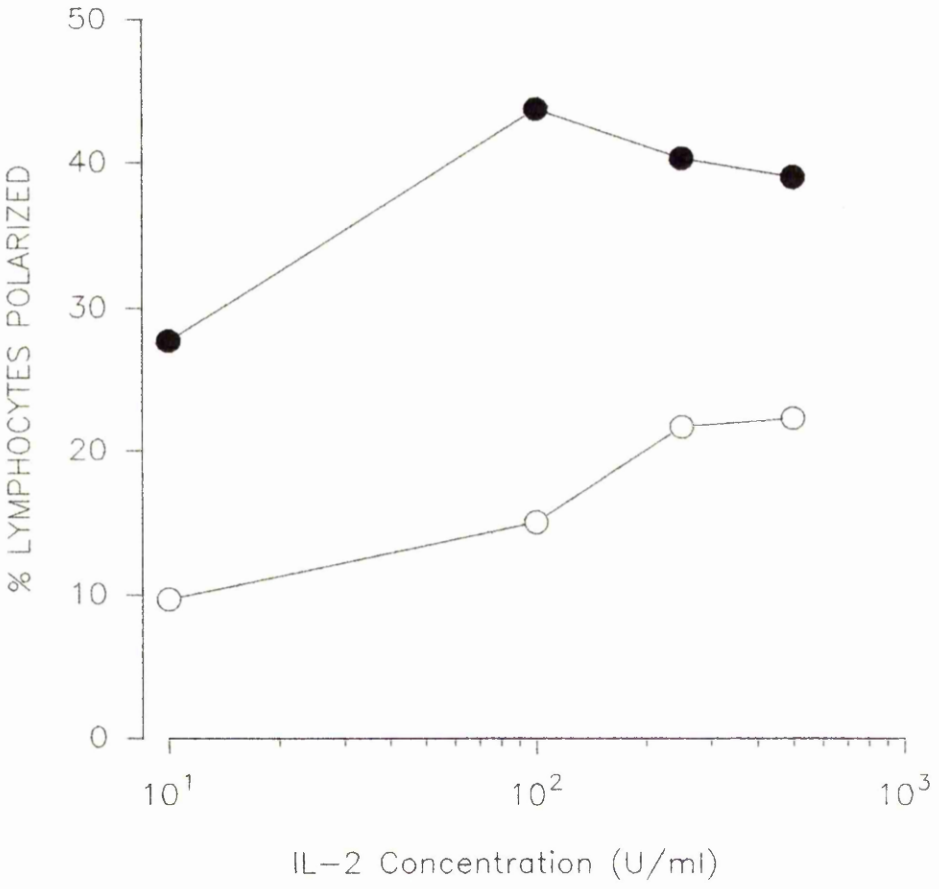
Only a small proportion of freshly isolated lymphocytes respond in polarization assays in suspension (Wilkinson, 1987), and few defined factors stimulating polarization have been identified. To investigate the effects of some defined factors on CD45R subsets, lymphocytes were exposed to factors for a short term (1 hour), and fixed for polarization assays. Experiments were performed on day 0, or after overnight culture in FCS, which is a means of increasing the proportion of lymphocytes with locomotor capacity, while leaving fairly low background responses (Wilkinson and Newman, 1992). Factors tested were IL-2, IL-8 and RANTES.

4.1.2 IL-2.

Figure 4.1 shows the results of a preliminary dose/response experiment testing IL-2 on lymphocytes on day 0, or after overnight culture in FCS. Good responses were seen on day 0, and after overnight culture in FCS, to doses of IL-2 greater than 250 U/ml (approximately 100 ng/ml), and after overnight culture in FCS, to doses of greater than 100 U/ml

FIGURE 4.1

Lymphocyte polarization in response to various doses of Glaxo IL-2.



Lymphocytes were tested on day 0 (open symbols), or after overnight culture in FCS (solid symbols). After washing cells, IL-2 was added at 10, 100, 250 or 500 U/ml for 30 minutes, and proportions polarized were determined.
CONTROLS: Day 0 FCS (+) 26.3; HBSS/HSA (-) 13.0
Day 1 FCS (+) 43.7; HBSS/HSA (-) 26.3

(approximately 40 ng/ml). The IL-2 was obtained from Glaxo, and its activity was in contrast to that of IL-2 obtained from Genzyme, which failed to induce lymphocyte polarization at similar doses.

Optimal doses of IL-2 stimulated as large a population to polarize as FCS, which was used as a positive control. The active agents in FCS are not known, but the results suggest that IL-2 is potentially capable of stimulating almost maximal polarization under these conditions. Stimulation of cells with mixtures of FCS and IL-2 might show whether different populations of lymphocytes respond, and measurement of levels of IL-2 in FCS might show whether sufficient IL-2 is present to stimulate lymphocyte polarization.

To attempt to relate response of lymphocytes to IL-2 to expression of IL-2 receptor, CD25 expression was examined. CD25 is one of the IL-2 receptor chains, and is usually termed the α chain. Lymphocytes were stained by immunofluorescence on day 0, and after culture in FCS (overnight), or α-CD3 (over 72 hours). Results summarised in table 4.1 show that while CD25 expression increases during culture in α-CD3, very few lymphocytes direct from blood, or cultured overnight in FCS, express CD25. This suggests that expression of CD25 is not required for response to IL-2, and the paucity of CD25+ among cells freshly isolated from blood makes study of such cells difficult. This suggests that the cells responding to IL-2 in polarization assays as described previously must bind IL-2 via a different surface receptor, so various experiments were attempted to examine the role of the IL-2 receptor ß chain.

TABLE 4.1

Proportions of CD25+ lymphocytes during culture.

CULTURE MEDIUM	% CD25 + (DAY 0)	% CD25 + (24 Hr.)	% CD25 + (48 Hr.)	% CD25 + (72 Hr.)
FCS (n=9)	4.9 ± 0.5	4.9 ± 0.6	NT	NT
α-CD3 (n=3)	3.0 ± 1.0	26.5 ± 4.7	38.7 ± 6.8	47.1 ± 7.1

Mononuclear cells were tested on day 0, and after culture in FCS (for 24 Hr.), or α-CD3 (for 24, 48 and 72 Hr.). Lymphocytes were stained for CD25 by immunofluorescence, and samples analysed by a FACS.

The table shows the means ± e.s.e. of n experiments.

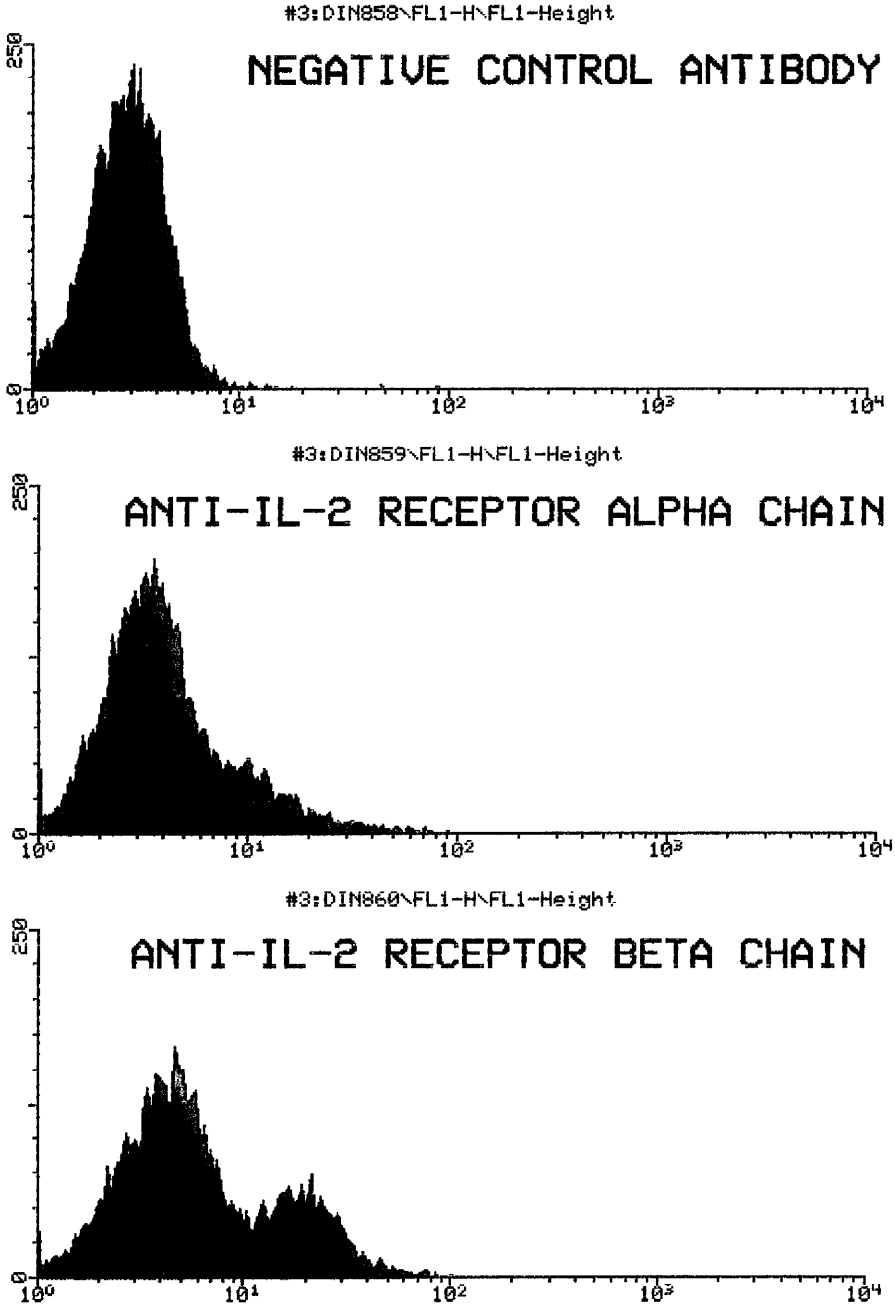
NT = not tested.

Figure 4.2 shows typical expression of the IL-2 receptor ß chain on lymphocytes freshly isolated from blood. In a series (n=5) of comparisons of such cells it was consistently found that more cells expressed the ß chain than the α chain. This supports the findings of Tsudo, Kitamura and Miyasaka (1989), who also found that the population of lymphocytes freshly isolated from blood expressing the ß chain was distinct from that expressing the α chain.

Antibodies to the IL-2 receptor ß chain block the response of lymphocytes, freshly-isolated from blood, to IL-2 (P.C. Wilkinson, personal communication). Anti-CD25 had little effect in similar experiments. Thus binding of IL-2 to the IL-2 receptor ß chain may be crucial for lymphocyte polarization. Receptor internalisation following binding of ligand to the IL-2 receptor ß chain (Robb and Greene, 1987; Takeshita *et al.*, 1992) might make delineation of expression of IL-2 receptors on cells which have responded to IL-2 difficult, as would the observation (discussed previously) that modified APAAP staining does not work using the anti-IL-2 receptor ß chain antibody. It has been reported that among blood lymphocytes, natural killer and CD8+ cells, but not CD4+, or B cells express the IL-2 receptor ß chain (Tsudo *et al.*, 1989; Ohashi *et al.*, 1989). It might be instructive to examine whether the motile population responding to IL-2 was enriched for natural killer, or CD8+, lymphocytes.

FIGURE 4.2

Expression of IL-2 receptor chains α (CD25) and ß on lymphocytes freshly isolated from blood.



Lymphocytes freshly isolated from blood were stained for IL-2 receptor chains α (CD25) or ß by immunofluorescence, and analysed using a FACS.

Graphs show relative fluorescence on the horizontal axis, and relative cell numbers on the vertical axis.

4.1.3 IL-8.

Figure 4.3 shows the results of preliminary dose/response experiments testing IL-8 from Peprotech or British Biotechnology on lymphocytes on day 0, or after overnight culture in FCS. However in these experiments, no response to IL-8 from either source was evident, for reasons which are unknown.

4.1.4 RANTES.

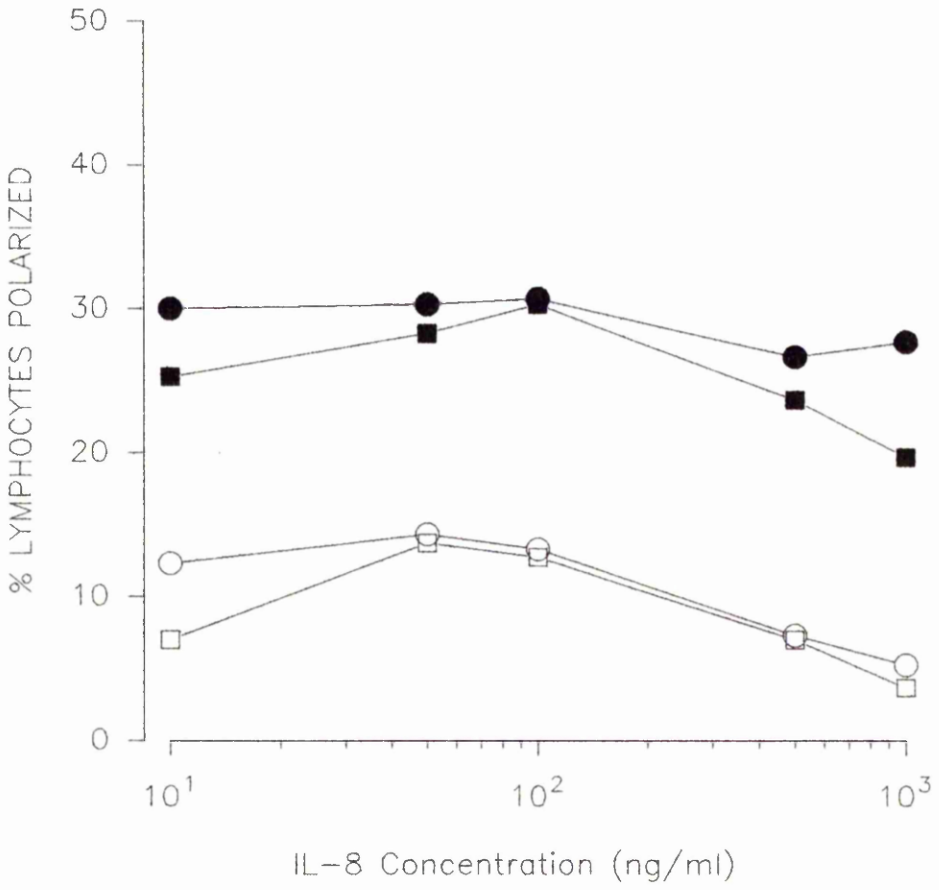
Figure 4.4 shows the results of a preliminary dose/response experiment testing RANTES. The shape of the curves is similar to that reported by Schall *et al.* (1990), who used a culture supernatant containing an undefined amount of RANTES.

Further experiments were performed testing the response of lymphocytes to RANTES at 100 or 500 ng/ml. Generally little response was evident, and if cells from individuals found to respond were re-tested on different occasions, they showed no response. Without a consistent pattern of response it was felt that attempts to phenotype the cells would not be worthwhile.

The same batch of RANTES was consistently active on blood monocytes in polarization assays (P.C. Wilkinson, personal communication), so the reasons for the variable responses by lymphocytes in polarization assays are not clear. Lymphocytes from individuals tested at different times may vary in expression of a receptor for RANTES, or in some other

FIGURE 4.3

Lymphocyte polarization in response to various doses of IL-8, from Peprotech or British Biotechnology.

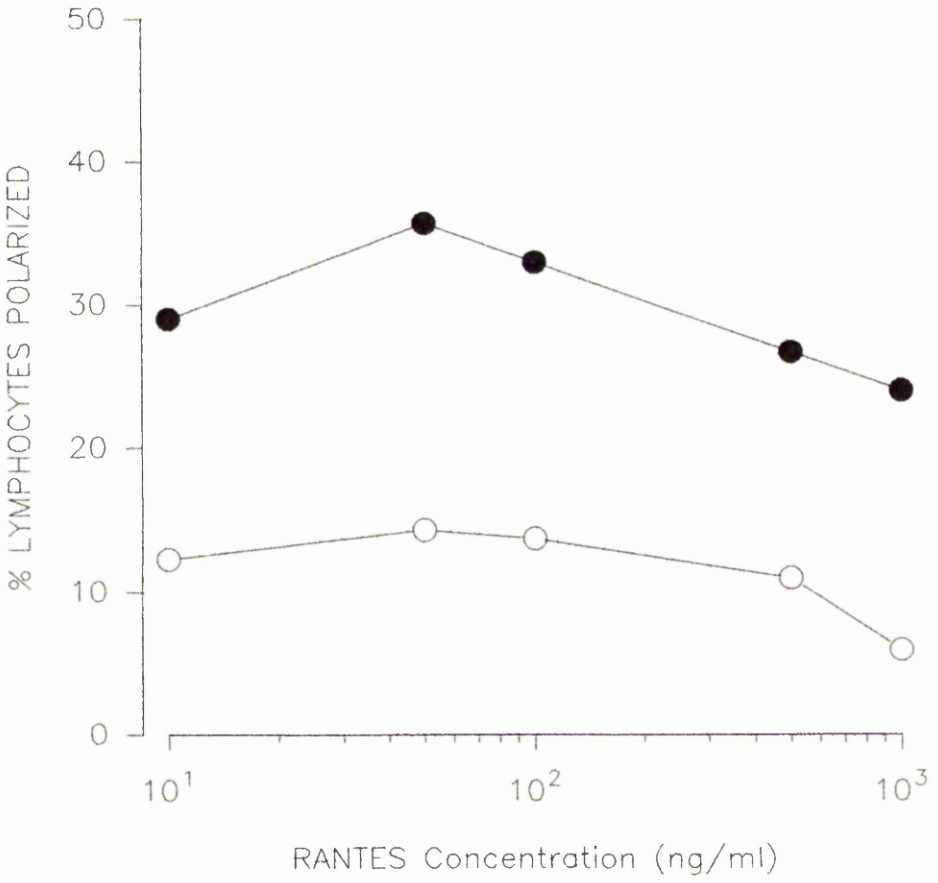


Lymphocytes were tested on day 0 (open symbols), or after overnight culture in FCS (solid symbols). After washing cells, IL-8 from Peprotech (circles) or British Biotechnology (squares) was added at 10, 50, 100, 500 or 1000 ng/ml for 30 minutes, and proportions polarized were determined.

CONTROLS: Day 0 FCS (+) 15.3; HBSS/HSA (-) 7.0
Day 1 FCS (+) 44.7; HBSS/HSA (-) 20.0

FIGURE 4.4

Lymphocyte polarization in response to various doses of RANTES.



Lymphocytes were tested on day 0 (open symbols), or after overnight culture in FCS (solid symbols). After washing cells, RANTES was added at 10, 50, 100, 500 or 1000 ng/ml for 30 minutes, and proportions polarized were determined.
CONTROLS: Day 0 FCS (+) 15.3; HBSS/HSA (−) 7.0
Day 1 FCS (+) 44.7; HBSS/HSA (−) 15.3

undefined cofactor required for response to RANTES. Schall *et al.* (1990) detected cells transmigrating polycarbonate filters and this may measure a different set of responses from those required for morphological polarization. Repeating that strategy, or investigating RANTES as a stimulator of lymphocyte locomotion into collagen gels, could be attempted in conjunction with polarization assays, to see whether other assays give variable responses.

Recently peak activity of RANTES on lymphocytes was reported at 10 ng/ml (Taub *et al.*, 1993b), and receptors for RANTES have been identified (Gao *et al.*, 1993). These were detected on B cells, but not on PHA-activated T cells. Other reports indicate that there may be several receptors for RANTES (Bischoff *et al.*, 1993), and that the affinity of binding of ligands to RANTES receptors does not predict intracellular signalling (Neote *et al.*, 1993).

4.1.5 Other stimulators of lymphocyte locomotion.

Two other well-established stimulators of lymphocyte locomotion are FCS and colchicine (Wilkinson, 1986), although their modes of action are not established. Preliminary experiments confirmed that both cause lymphocyte polarization.

Culture of lymphocytes for a period of greater than 24 hours greatly increases the proportion of polarized, locomotor cells, compared to the proportion found when cells fresh from blood are examined (Wilkinson, 1986). A number of stimulators have been described for such cultures, including α-CD3, PPD,

PHA, and allo-MLR (Wilkinson, 1986), and Con A (Wilkinson *et al.*, 1976). In preliminary experiments, all of these stimulators, as well as PWM, were found to increase the proportion of polarized lymphocytes compared to cells tested on day 0, and also compared to that of cells cultured in medium (HBSS/HSA) alone.

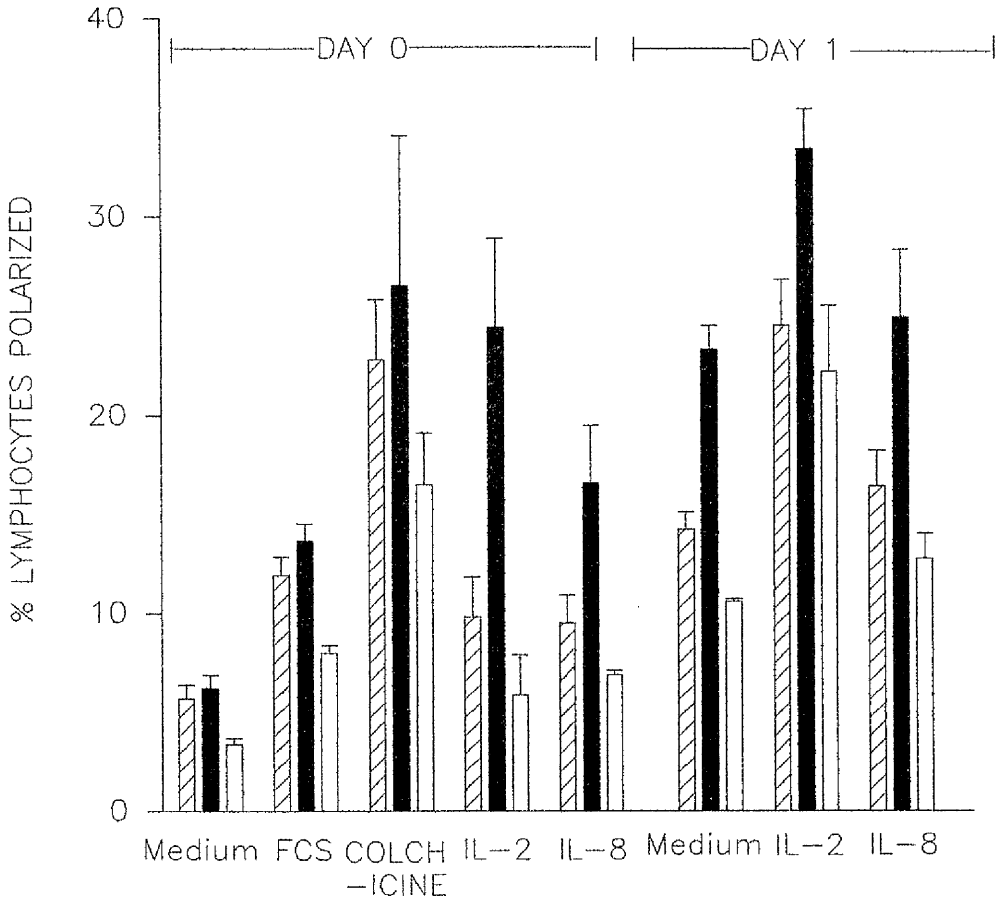
4.2 Polarization of CD45R subsets after short-term stimulation.

A series of preparations of lymphocytes stimulated for 1 hour with the factors described above was stained by the modified APAAP method for CD45RO or CD45RA. Lymphocytes were tested on day 0 with medium (HBSS), colchicine, FCS, IL-2 or IL-8 (from Peprotech), or were cultured overnight in 10 % FCS, washed, and tested with HBSS/MOPS, IL-2 or IL-8. Each factor was tested 3 times, but on various batches of cells. For each preparation, cells which either stained positive or negative were classified as polarized or round.

Results are summarised in figures 4.5 and 4.6. With all the stimulators used a greater proportion of CD45RO+ than of CD45RA+ lymphocytes became polarized. This was the pattern in all individual experiments, except in one experiment in which cells were stimulated with colchicine, and no difference between the subsets was observed. Overall the differences were statistically significant ($p<0.01$, ANOVA test). Similarly a significantly greater proportion of CD45RA- than of CD45RO-lymphocytes became polarized. This was the pattern

FIGURE 4.5

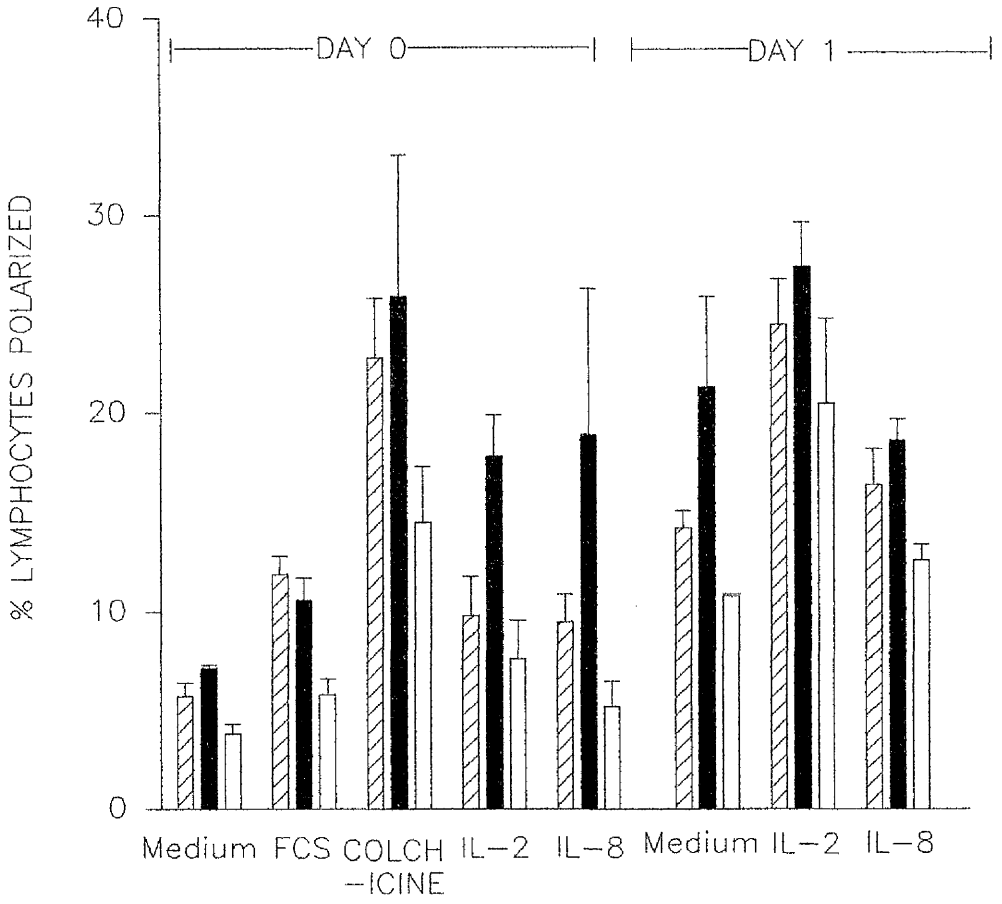
Proportions of CD45RO+ and CD45RA+ lymphocytes polarized after short-term stimulation by various factors.



Lymphocytes were stimulated on day 0, or on day 1 after culture in HBSS/FCS, with medium (HBSS), FCS, colchicine, IL-2 or IL-8 for 1 hour, and stained by the modified APAAP method. Graphs show means and e.s.e. of proportions polarized overall (diagonal bars), among CD45RO+ cells (solid bars), and among CD45RA+ cells (open bars) from 3 experiments for each stimulus.

FIGURE 4.6

Proportions of CD45RA− and CD45RO− lymphocytes polarized after short−term stimulation by various factors.



Lymphocytes were stimulated on day 0, or on day 1 after culture in HBSS/FCS, with medium (HBSS), FCS, colchicine, IL−2, or IL−8, for 1 hour, and stained by the modified APAAP method.

Graphs show means and e.s.e.of proportions polarized overall (diagonal bars), among CD45RA− cells (solid bars), and among CD45RO− cells (open bars), from three experiments for each stimulus.

in every individual experiment.

Some apparently anomalous results were observed after stimulation by FCS. Fewer of both the CD45RA- and CD45RO- populations became polarized than of the overall population. This may result from the fact that among whole lymphocyte populations CD45RO+ and CD45RA-, and CD45RA+ and CD45RO-, populations are not exactly equivalent, and that the two populations are not entirely reciprocal, as was thought to be the case among T cells. Even among T cells, co-expression of CD45RO and CD45RA occurs in a higher proportion of lymphocytes than was originally thought (Richards *et al.*, 1990)

Overall, the proportions of lymphocytes responding to IL-8 were not as high as in the preliminary experiments, nor were they as strong as previously reported (Wilkinson and Watson, 1990; Wilkinson and Newman, 1992). The reason for this is unknown, although other sources of IL-8 were used in many of those experiments.

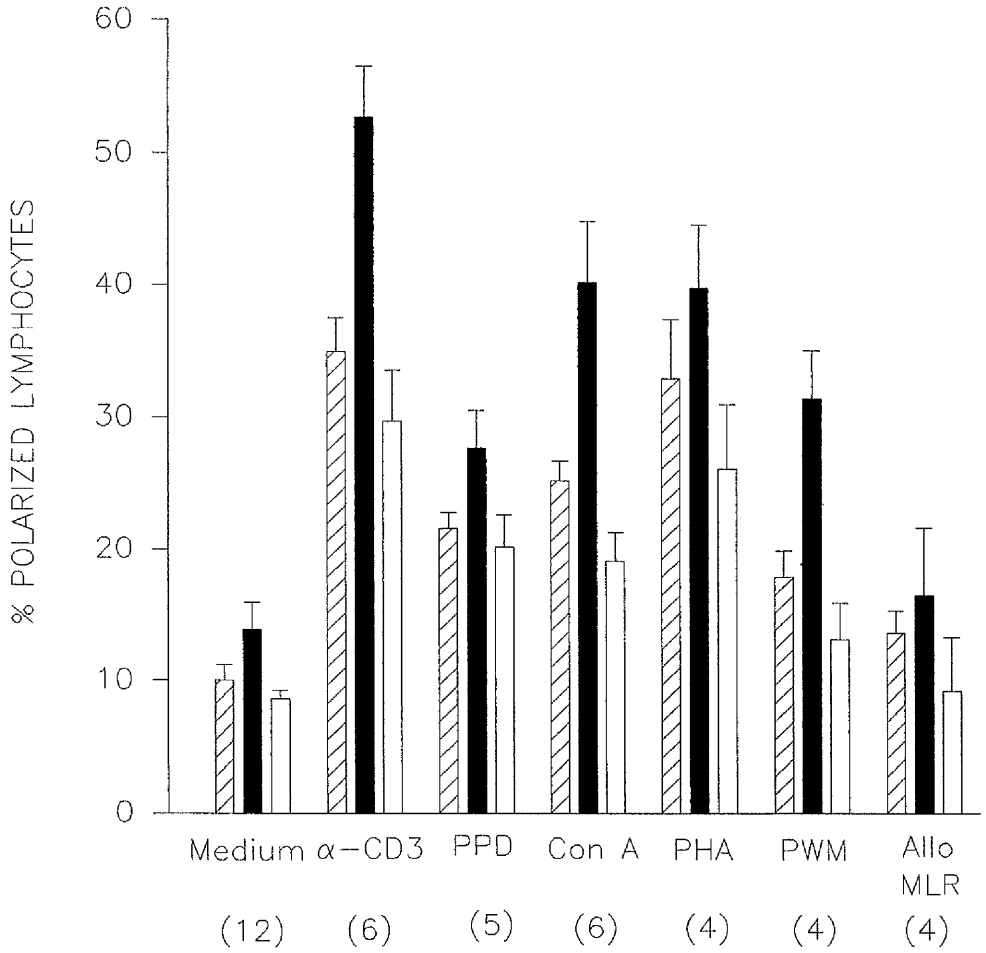
Although few lymphocytes became polarized in response to short-term stimuli, consistent differences were observed between CD45RO+ and CD45RA- cells, and between CD45RO- and CD45RA+ cells, the former containing a greater proportion of polarized cells.

4.3 Polarization of CD45R subsets after 72 hour culture.

A series of preparations was made of lymphocytes cultured for 72 hours in various activators, and stained by the

FIGURE 4.7

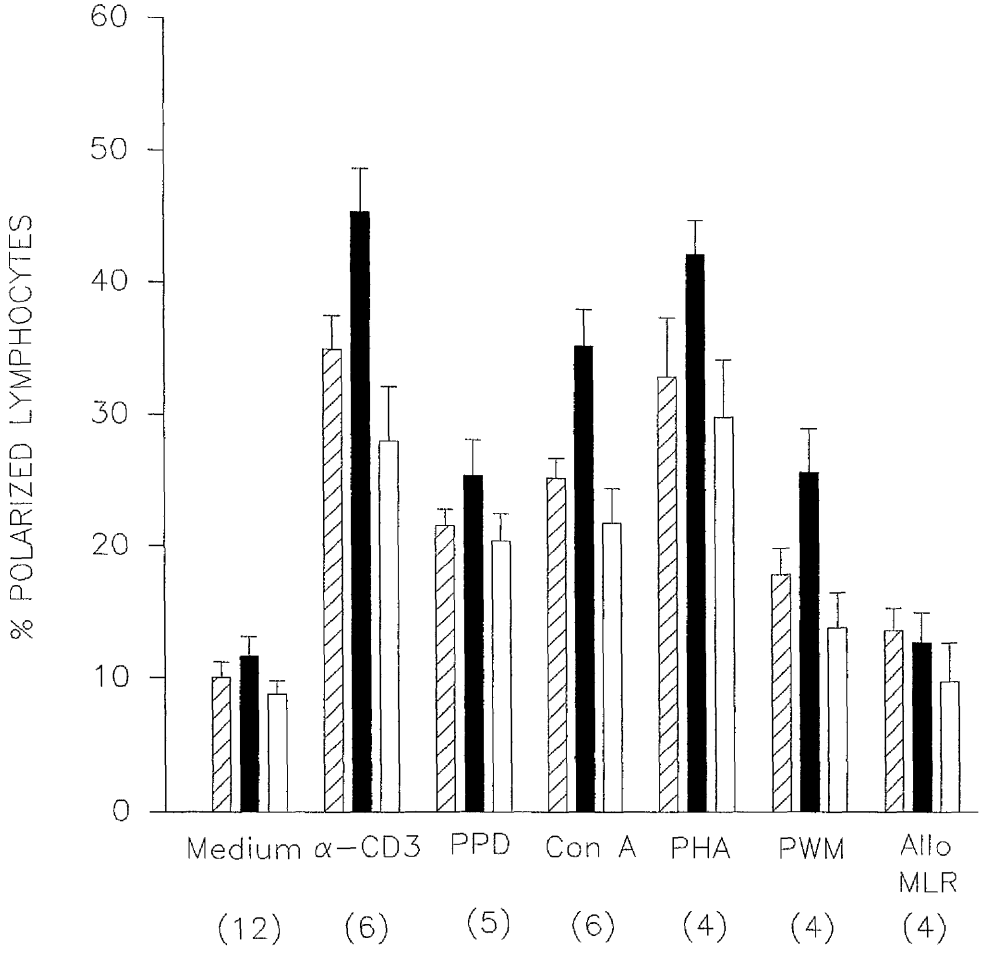
Proportions of CD45RO+ and CD45RA+ lymphocytes polarized after 72 hour culture.



Mononuclear cells were cultured in medium (HBSS/HSA) or various activators, and lymphocytes stained by modified APAAP. Graphs show means and standard errors of the proportions polarized overall (diagonal lines); among CD45RO+ cells (solid bars); and among CD45RA+ cells (open bars). Numbers of experiments are shown in brackets.

FIGURE 4.8

Proportions of CD45RA– and CD45RO– lymphocytes polarized after 72 hour culture.



Mononuclear cells were cultured for 72 hours in medium (HBSS/HSA) or various activators, and lymphocytes stained by the modified APAAP method.

Graphs show means and standard errors of the proportions polarized overall (diagonal lines); among CD45RA– cells (solid bars); and among CD45RO– cells (open bars). Numbers of experiments are shown in brackets.

modified APAAP method for CD45RO or CD45RA.

Results are summarised in figures 4.7 and 4.8. As with the short-term stimuli, a consistent pattern emerged. After culture in all activators, a greater proportion of CD45RO+ than of CD45RA+ lymphocytes became polarized. This was the pattern in all individual experiments. The differences between CD45RO+ and CD45RA+ lymphocytes were statistically significant ($p<0.01$, ANOVA test). Similarly, after culture in all activators, a significantly greater proportion of CD45RA- than of CD45RO- lymphocytes became polarized. This was the pattern in all individual experiments, except in one experiment in which cells were cultured in medium, in which case no difference between the subsets was observed. Again the differences were statistically significant ($p<0.01$, ANOVA test).

An anomaly similar to that found after short-term stimulation of cells with FCS, was observed after 72-hour culture of MNC in allo-MLR. Fewer of both the CD45RA- and CD45RO- populations became polarized than of the overall population after culture in allo-MLR, possibly for reasons similar to those discussed previously with regard to cells stimulated with FCS. The proportions of lymphocytes polarized after culture in allo-MLR were low in these experiments, although they were higher than the proportions of either subject's lymphocytes polarized after culture in medium. Individuals were not selected in any way, and better responses might have resulted if attempts had been made to maximise allorecognition by selecting particular individuals.

4.4 CD25 as a marker of polarized lymphocytes.

As described previously, the proportion of CD25+ cells increases during culture in α-CD3 (see table 4.1). Since expression of CD25 is associated with cell activation, the proportions of CD25+ and CD25- cells which were polarized after 48 hours in α-CD3 was determined by APAAP staining. Results are summarised in table 4.2. It was found that the two populations differed only marginally in their responses. Hence surface expression of CD25 does not correlate with assumption of polarized morphology. It might be interesting to examine whether expression of the IL-2R ß chain correlates with polarized morphology and locomotor activity. However, as discussed previously (section 3.2.2), the antibody against the IL-2R ß chain did not react by APAAP staining, and the receptor may be internalised on binding of IL-2. It has also been reported that, in contrast with freshly-isolated blood lymphocytes, PHA-activated lymphocytes co-express the α and ß chains (Tsudo *et al.*, 1989). Thus on activated lymphocytes, the CD25+ population might be similar to the population expressing the ß chain.

4.5 Receptor redistribution on polarized lymphocytes.

It has been reported that certain molecules become redistributed non-randomly on the surface of lymphocytes, especially on polarized cells. Haston and Wilkinson (1988b) showed anterior localisation of CD45 on polarized lymphocytes,

TABLE 4.2

Proportions of CD25+, and of CD25-, lymphocytes polarized after culture in α-CD3.

CELL POPULATION	% POLARIZED (MEANS ± e.s.e.)
OVERALL	36.6 ± 1.1
CD25 + CELLS	39.9 ± 2.9
CD25 - CELLS	36.3 ± 1.6

Mononuclear cells were cultured with α-CD3 for 48 hours, and lymphocytes were fixed *in situ* and stained for CD25 using the modified APAAP method. Overall, 41.6 ± 6.7 % of lymphocytes were CD25 +.

The table summarises results from 3 experiments.

while Haston and Maggs (1990) similarly demonstrated an anterior distribution of CD45 (and HLA-DR) on polarized human lymphocytes. A similar finding was also reported for Thy 1-2 on polarized mouse lymphocytes (Haston and Shields, 1984), although Unanue *et al.* (1974) demonstrated that asymmetric distribution of surface immunoglobulin was not essential for lymphocyte polarization in response to anti-immunoglobulin.

Preparations of lymphocytes after short-term stimulation, or after culture in a range of activators, were stained by APAAP for CD45RO or CD45RA and examined for evidence of CD45 redistribution. However, in virtually no cells was asymmetric distribution of antigen was evident, although on approximately 5 % of polarized lymphocytes the uropod at the rear of the cell showed reduced staining for CD45 antigen. Thus this method did not demonstrate the receptor redistribution described in other studies.

4.6 Conclusions from polarization assays of CD45R subsets.

The general pattern of results found after phenotypic analysis of polarized lymphocytes using α-CD45RO and α-CD45RA is consistent. With all stimuli, whether after short term response or in longer-term culture, a greater proportion of CD45RO+ and CD45RA- lymphocytes became polarized than of CD45RA+ and CD45RO- cells. Hence the CD45RO+ and CD45RA- populations have a greater proportion of cells with locomotor capacity. This was observed in cells in medium (containing no known stimulus) and the differences were maintained as the

overall proportion of polarized cells increased. A proportion of the CD45RO+ cells always remained round, and a proportion of CD45RA+ cells always became polarized.

The polarization assay is perhaps the simplest assessment of locomotor capacity, and lacks many of the complicating interactions involved when active locomotion is required. On the other hand, this also means that it is not by itself sufficient when locomotion is to be assessed. However the consistent nature of the results obtained suggests that blood CD45RO+RA- lymphocytes have a greater locomotor capacity than do CD45RA+RO- cells.

Because the modified APAAP method failed to detect CD4 (section 3.2.2), the responses of CD4+ lymphocytes, or of CD45R subsets within CD4+ cells could not be determined in polarization assays, and compared to those of CD8+ lymphocytes. Double labelling using alkaline phosphatase and peroxidase is possible on conventionally-fixed cells (Mason and Sammons, 1978), but the method would need to be adapted for polarization assays, and the problems found using α-CD4 after glutaraldehyde fixation might limit those methods. Another approach could involve isolation of CD4+ or CD8+ cells prior to culture, but this might affect their responses, compared to whole mononuclear cell culture.

CHAPTER 5: THE RESPONSE OF CD45RO+ AND CD45RA+ LYMPHOCYTES IN COLLAGEN GEL INVASION ASSAYS.

5.1 Preliminary experiments.

The strategy adopted for investigation of the locomotion of CD45R subsets into collagen gels required that sufficient lymphocytes were recovered from gels for subsequent FACS analysis. In order to separate enough motile lymphocytes for such phenotyping, MNC were cultured for 48-72 hours in various activators, as described previously, to increase the proportion of locomotor cells. Generally culture supernatants were incorporated into gels to act as locomotor attractants.

To confirm that activation of lymphocytes increased the proportions of motile lymphocytes, the proportions of lymphocytes which had invaded gels was determined by counting the cells *in situ* in the gel matrix. Lymphocytes cultured in PPD, allo-MLR, α-CD3 or Con A consistently invaded gels containing culture supernatants in greater proportions than control cells cultured in medium (HBSS/HSA) (table 5.1). Extensive washing of cells cultured in Con A was not found to be necessary to enable lymphocytes to invade gels, although this has been reported (Ratner, Jasti and Heppner, 1988). As shown previously (table 3.5), the proportions of lymphocytes, cultured in α-CD3, which had invaded gels overnight were greater than the proportions polarized in the original cultures, while the proportions invading after 3.5 hours were similar to the proportions polarized in culture. This pattern

TABLE 5.1

Proportions of lymphocytes which invaded collagen gels after culture in various activators, compared to those cultured in medium, and to the proportions polarized after culture.

	% INVASIVE IN ACTIVATOR	% POLARIZED IN ACTIVATOR	% INVASIVE IN MEDIUM	% POLARIZED IN MEDIUM
α-CD3,INVASION OVERNIGHT (n=14)	52.7 ±4.7	37.2 ±2.8	19.6 ±2.5	14.1 ±1.9
PPD, INVASION OVERNIGHT (n=7)	38.9 ±4.7	33.9 ±4.2	28.1 ±5.7	19.6 ±3.4
Allo-MLR, INVASION OVERNIGHT (n=5)	46.5 ±10.4	27.6 ±3.8	30.5 ±9.1	18.4 ±2.2
Con A, INVASION OVERNIGHT (n=4)	45.3 ±13.2	27.5 ±3.5	19.5 ±6.7	15.8 ±1.5
α-CD3, INVASION 3.5 Hr. (n=3)	41.9 ±12.3	37.0 ±4.2	8.6 ±1.5	7.8 ±0.6

Mononuclear cells were cultured in medium (HBSS/HSA), or with activators, for 72 hours, and the proportions that were polarized were determined. Lymphocytes were then allowed to invade collagen gels incorporating culture supernatants, for 3.5 hours, or overnight. The proportions invading were then determined by examination of the gels *in situ*.

The table shows means ± e.s.e. of n experiments.

was also seen after culture in PPD, allo-MLR and Con A. Lymphocytes cultured in PHA did not invade gels containing their culture supernatants, even if cells were extensively washed beforehand. They also failed to invade in response to culture supernatants from α-CD3-stimulated cells. The reasons for the failure of PHA-stimulated cells to invade gels were not furthur investigated. It is possible that cells adhered too strongly to the gel surface, or to each other, to allow migration.

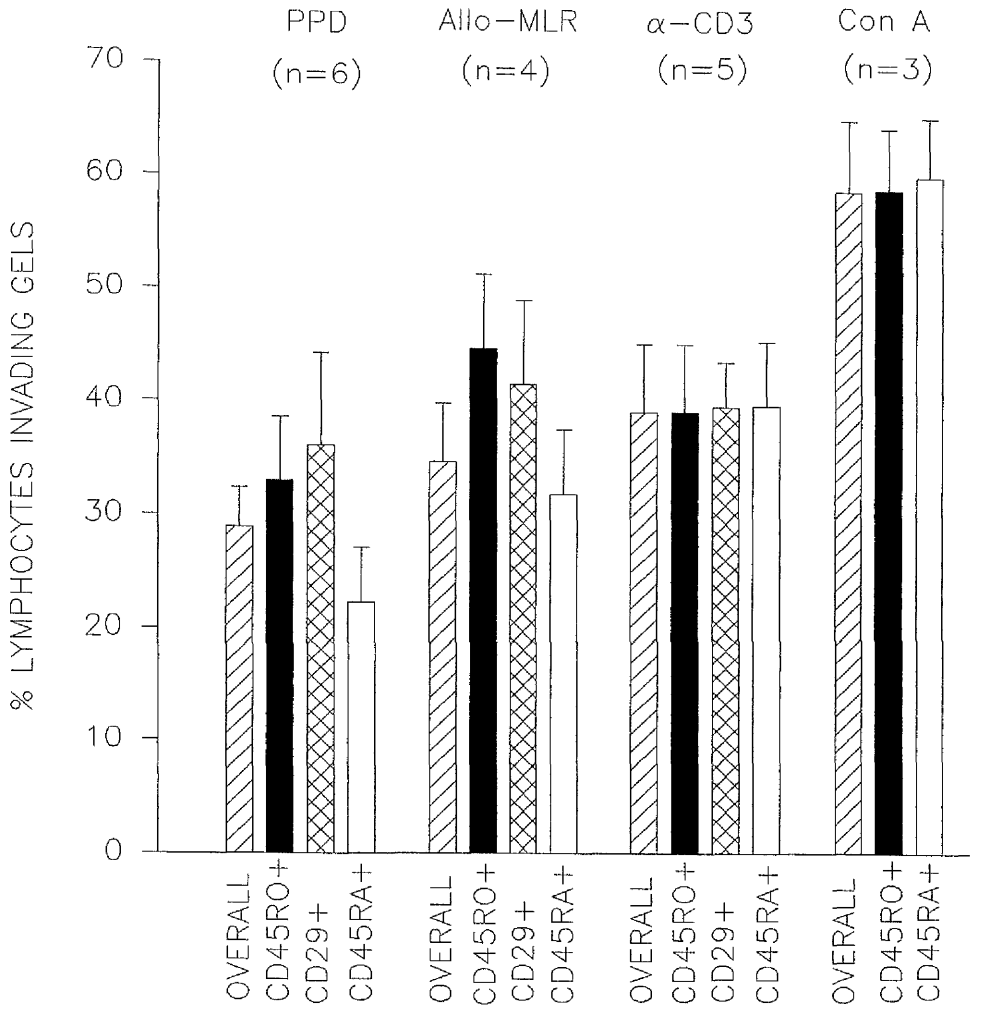
5.2 Overnight invasion of collagen gels by CD45R subsets.

This set of experiments involved staining of cell populations for CD45RO, CD45RA and, when sufficient cells were available, for CD29, after lymphocytes had invaded collagen gels overnight. Non-motile cells washed from the gel surface, and cells retained by gels after washing, and subsequently released after digestion of the collagen, were counted, and stained. The population of cells retained by gels after washing (here termed the invasive population) would, as discussed earlier (section 3.5.4), comprise those which had actively moved into the gel matrix, plus some which may have remained adherent to the gel surface after washing.

Figure 5.1 summarises the results of phenotypic analysis of cultured cells after overnight invasion of collagen gels. Unlike the polarization assays, the pattern of results depended on the activator used to stimulate cells in culture. Generally, responses of cells cultured in PPD resembled those

FIGURE 5.1

Proportions of CD45RO+, CD29+, and CD45RA+ lymphocytes invading collagen gels overnight.



Mononuclear cells were cultured in various stimuli for 72 hours. Culture supernatants were incorporated into gels. The proportions of defined populations which invaded overnight were determined using immunofluorescence and FACS analysis. Graphs show means and standard errors of n experiments.

in allo-MLR, and cells cultured in α-CD3 resembled those in Con A.

In every individual experiment after culture in PPD or allo-MLR, there was a greater proportion of invasive CD45RO+, and CD29+, lymphocytes than of CD45RA+ cells. However results were not statistically significant.

After culture in α-CD3 or Con A, there were generally no consistent differences between the proportions of invasive CD45RO+ and CD45RA+ lymphocytes. In the case of cells cultured in α-CD3, staining for CD29 was also performed, and the proportion of invasive CD29+ lymphocytes was similar to that of the other subsets.

5.3 Shorter-term invasion of collagen gels by CD45R subsets.

Unlike the polarization assays described in chapter 4, the response of CD45RO+ and CD45RA+ lymphocytes in assays of where collagen gel invasion proceded overnight apparently depended on which stimuli were used. Experiments were therefore performed to investigate whether the time allowed for invasion of gels affected the response of CD45R subsets.

An increased proportion of lymphocytes invade collagen gels overnight compared to shorter-term (2-6 hour) invasion (Shields, Haston and Wilkinson, 1984). The proportion of lymphocytes invading gels over a 2-6 hour period has been shown to be similar to the proportion which had become polarized in culture (Wilkinson and Higgins, 1987a; Wilkinson and Watson, 1990), suggesting that the population of cells

defined as motile by morphological polarization is the same as that which will subsequently invade collagen gels. Since the response of lymphocytes during such shorter-term invasion more closely resembles those in polarization assays, cultured lymphocytes were only allowed to invade gels for 3.5 hours. MNC were cultured in PPD, or in α-CD3. In this series of experiments, double immunofluorescent labelling was also used, to examine the responses among CD4+ and CD8+ lymphocytes expressing different CD45R isoforms.

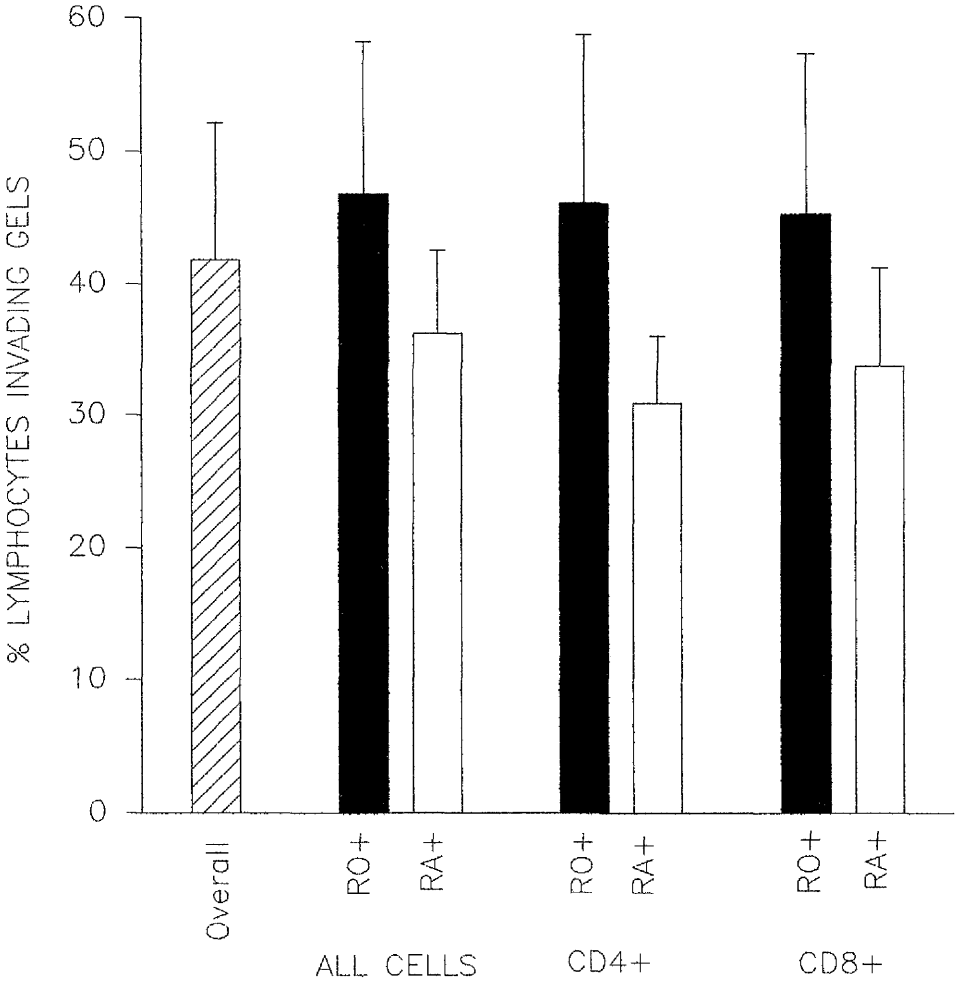
Figure 5.2 summarises the results obtained after culture of MNC in PPD. The response of the total CD45RO+ and CD45RA+ populations was similar to that found after overnight invasion, *i.e.* in all 3 experiments, a larger proportion of CD45RO+ than of CD45RA+ lymphocytes were invasive. This pattern was found within both the CD4+ and CD8+ lymphocyte populations-in each subset a consistently greater proportion of the CD45RO+ than of CD45RA+ cells were invasive.

Figure 5.3 summarises the results after culture of cells in α-CD3. The response of the total CD45RO+, CD29+ and CD45RA+ populations was again similar to that found after overnight invasion, *i.e.* similar proportions of the CD45RO+, CD29+ and CD45RA+ cells were invasive.

Within the CD4+ or CD8+ lymphocyte populations a complicated pattern emerged. In all 4 experiments, proportionately more of the CD4+CD45RA+ cells than of CD4+CD45RO+ cells were invasive. It was found that the proportion of the CD4+CD45RA+ lymphocytes which were invasive in these experiments was similar to that of the CD4+CD29+

FIGURE 5.2

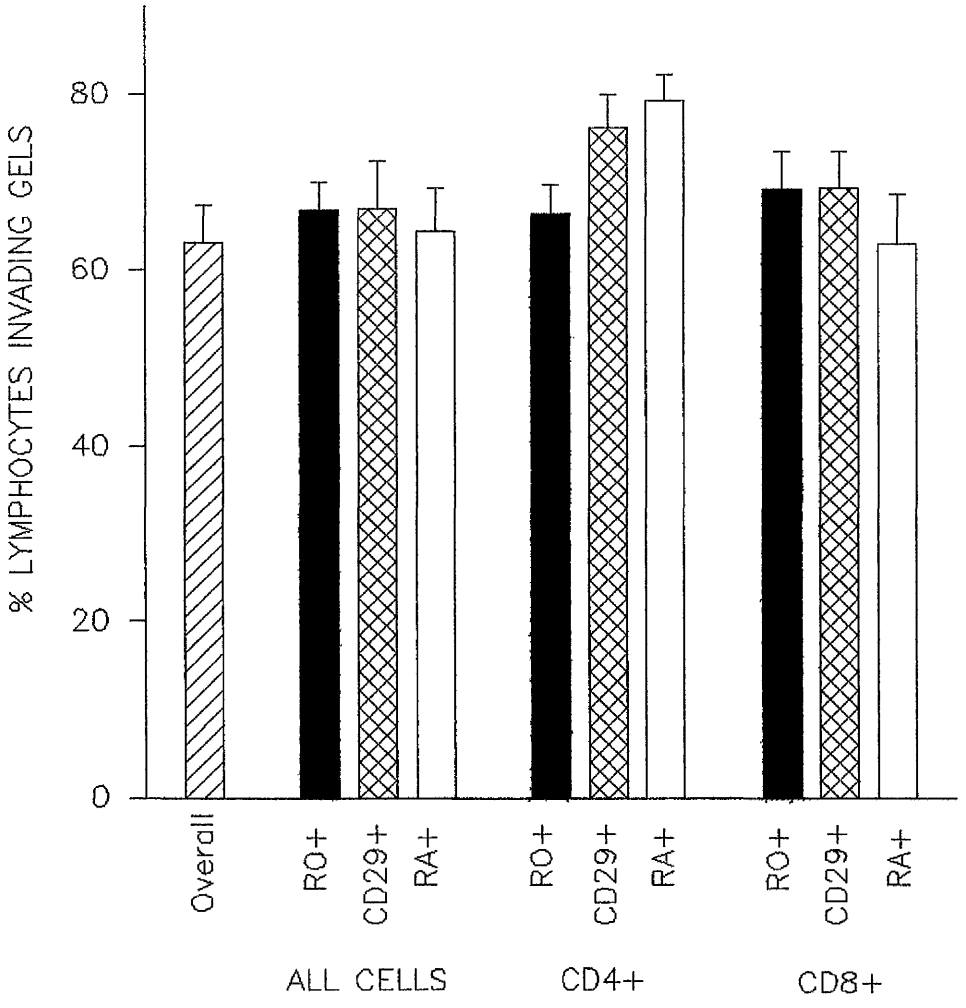
Proportions of CD45RO+ and CD45RA+ cells among CD4+ or CD8+ lymphocytes, cultured in PPD, which invaded collagen gels after 3.5 hours.



Mononuclear cells were cultured for 72 hours in PPD. Culture supernatants were incorporated into collagen gels, and proportions of defined populations which had invaded after 3.5 hours were determined using immunofluorescence and FACS analysis. Graphs show the means and standard errors of 3 experiments.

FIGURE 5.3.

Proportions of CD45RO+, CD29+, and CD45RA+ cells among CD4+ or CD8+ lymphocytes, cultured in α−CD3, which invaded collagen gels after 3.5 hours.



Mononuclear cells were cultured for 72 hours in α−CD3. Culture supernatants were incorporated into collagen gels, and proportions of defined populations which had invaded after 3.5 hours were determined using immunofluorescence and FACS analysis.

Graphs show the means and standard errors of 3 experiments.

cells. This was unexpected, since responses of the CD45RO+ population of lymphocytes are similar to the CD29+ population in many assay systems. However in some responses CD45RO+ and CD29+ cells have been shown to differ, and they may represent overlapping, rather than identical populations (Beverley, 1987). Among CD8+ lymphocytes, in 2 of the experiments proportionately more of the CD45RO+, and more of the CD29+, than of the CD45RA+ cells were invasive. In the other 2 experiments there was little difference between the proportions of invasive CD8+CD45RO+, CD8+CD29+ or CD8+CD45RA+ lymphocytes.

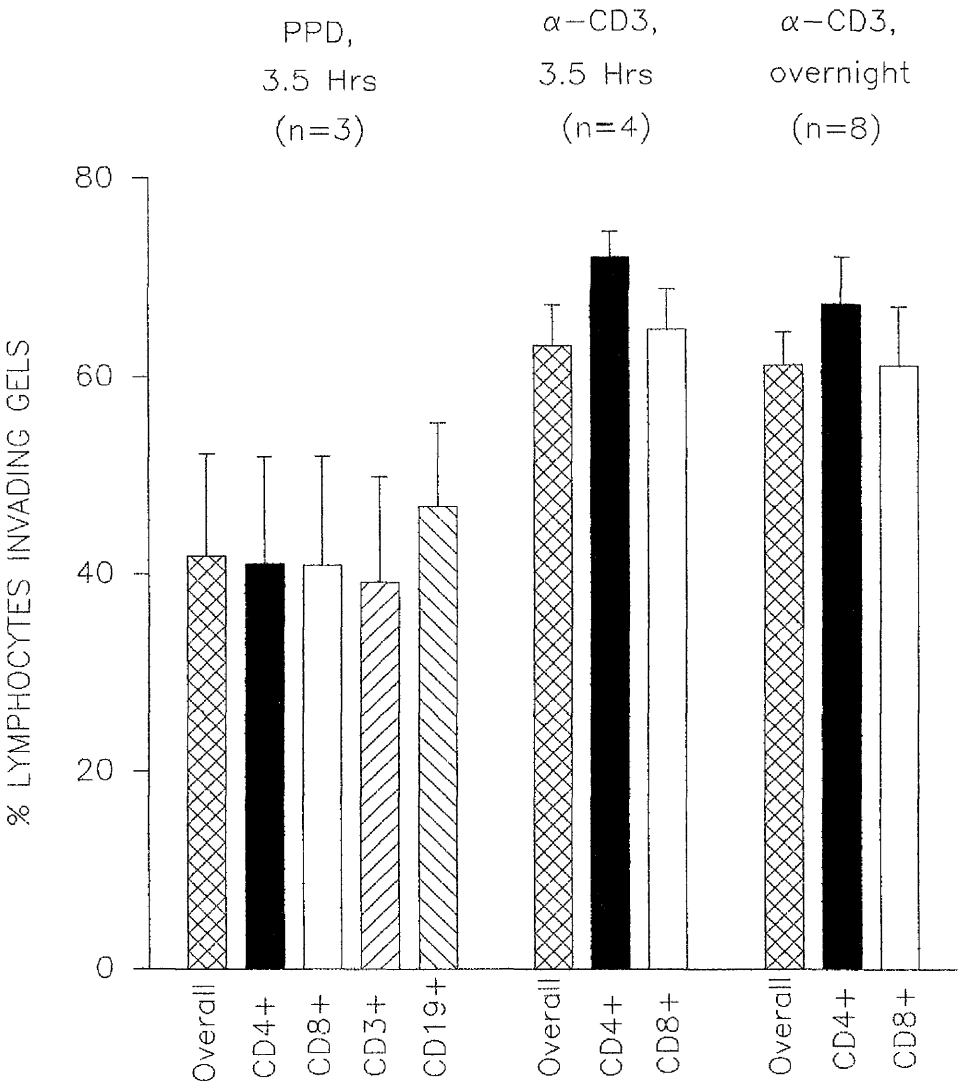
5.4 Invasion of collagen gels by T, B, CD4+ and CD8+ cells.

In view of the apparent differences seen in responses in invasiveness of CD45R subsets after culture in α-CD3 compared to PPD, and the complicated pattern found when comparing CD45R subsets within CD4+ and CD8+ populations, attempts were made to compare the overall proportions of invasive CD4+ and CD8+ lymphocytes, as well as B and T, cells. If overall responses by these subsets differed after culture in α-CD3 or PPD, this may have affected the overall proportions of CD45R subsets which responded, since expression of CD45R isoforms differs among T, B, N.K., CD4+ and CD8+ cells (Morimoto *et al.*, 1985a; Morimoto *et al.*, 1985b; Smith *et al.*, 1986; Jensen *et al.*, 1989; Warren and Skipsey, 1991).

Figure 5.4 summarises the results found after examining the overall CD4+ and CD8+ populations after culture of MNC in

FIGURE 5.4

Proportions of CD4+, CD8+, CD3+ and CD19+ lymphocytes, cultured in PPD or α−CD3, which invaded collagen gels after 3.5 hours, or overnight.



Mononuclear cells were cultured for 72 hours in PPD or α−CD3. Culture supernatants were incorporated into gels, and proportions of defined populations which had invaded after 3.5 hours, or overnight, were determined using immunofluorescence and FACS analysis.

Graphs show means and standard errors of n experiments.

PPD or α-CD3, and invasion of gels for 3.5 hours, plus overnight invasion in the case of cells cultured in α-CD3.

After culture in PPD, there were similar proportions of invasive cells among CD4+ and CD8+ lymphocytes in all 3 experiments. After culture in α-CD3, it was consistently observed that a marginally higher proportion of the CD4+ than of the CD8+ lymphocytes were invasive. This was the case whether cells invaded gels for 3.5 hours, or overnight.

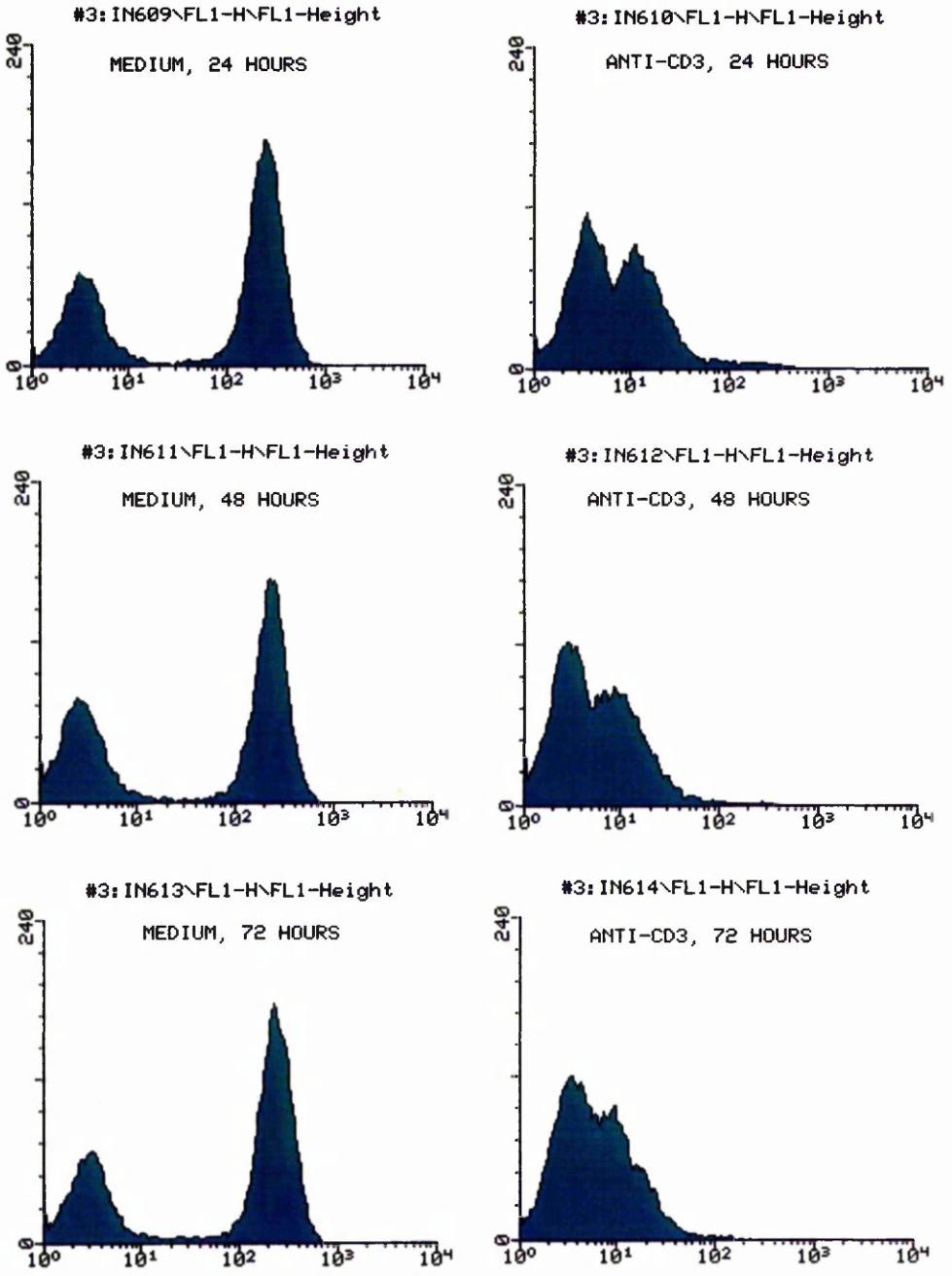
After culture in PPD, it was observed that in all 3 experiments, slightly more of the CD19+ than of CD3+ cells were invasive.

When attempts were made to compare the proportions of T or B cells which had invaded gels after culture in α-CD3, it was observed that such culture modulated CD3 expression on the cell surface. This is illustrated by figure 5.5, which shows that in culture in α-CD3, but not in medium alone, lymphocyte surface CD3 expression is reduced, and it remains reduced throughout a 72 hour period. This is presumably due to receptor internalisation in the continuous presence of the antibody during culture. Preliminary results suggested that culture of cells in PPD had no effect on CD3 expression. The reduction in CD3 expression on culture with α-CD3 meant that direct comparison of CD3+ and B cells after culture in α-CD3 was not possible. Examination of other T cell markers, or culture of cells in other mitogens, might allow T and B cell responses to be compared.

Attempts to examine the responses of CD3+ or CD22+ lymphocytes in polarization assays, after staining by the

FIGURE 5.5

The effect of culture of mononuclear cells in α-CD3 on lymphocyte expression of CD3.



Samples from a batch of mononuclear cells were cultured for various times in medium (graphs on the left of the page), or in α-CD3 (graphs on the right of the page). Cells were washed and stained by immunofluorescence after addition of fresh α-CD3.

Graphs show relative cell numbers on the vertical axis and intensity of green fluorescence on the horizontal axis.

modified APAAP method, encountered similar problems if cells were cultured in α-CD3. As described previously (section 3.2.2), anti-CD4 antibodies did not work by the modified APAAP method, so that comparison of the response of CD4+ and CD8+ cells in polarization assays after culture in PPD or α-CD3 was not possible.

5.5 The role of IL-8 as a locomotor attractant in collagen gels.

Although culture supernatants have been widely used to stimulate lymphocyte locomotion, the active molecules have not been characterised. This has made it difficult to reproduce experiments, and has hampered extrapolation to the situation *in vivo* . Although it had been observed that α-IL-8, but not α-IL-2, reduced the proportion of lymphocytes responding to culture supernatants in polarization assays (P.C. Wilkinson, personal communication), the activity of IL-8 as a stimulator of lymphocyte locomotion into collagen gels had not been addressed. Experiments were therefore performed to examine whether IL-8 could attract lymphocytes into collagen gels; whether α-IL-8 inhibited the stimulation of lymphocyte locomotion by culture supernatants; and how CD45R subsets responded to IL-8 in collagen gel invasion assays.

Tables 5.2 and 5.3 show the results when various agents were added to collagen gels. The proportions of lymphocytes entering the gels after 3.5 hours were counted by examination of the gels *in situ*. Lymphocytes had been cultured for 48 hours in α-CD3, or medium (HBSS/HSA), then washed prior to

TABLE 5.2

The effect of anti-IL-8 antibody on invasion of collagen gels by lymphocytes cultured in α-CD3, in response to culture supernatants.

	AGENT IN GEL	EXPT. # 1	EXPT. # 2	EXPT. # 3	EXPT. # 4	EXPT. # 5
CELLS FROM α-CD3 CULTURES	α-CD3 S/N	44.6	53.9	45.3	45.1	34.3
	α-IL-8 Ab	35.1	36.4	31.6	NT	22.2
	α-CD3 S/N+Ab	29.9	38.4	32.3	31.7	24.2
	MEDIUM	28.2	36.4	28.5	NT	21.6
	HSA S/N	31.7	40.0	25.9	33.6	23.0
CELLS FROM HSA CULTURES	HSA S/N	9.7	12.4	9.8	14.2	15.9
	α-CD3 S/N	18.2	13.9	9.0	13.5	12.5

Ab = Anti-IL-8 antibody.
S/N = Supernatant.
NT = Not Tested.

Mononuclear cells were cultured in α-CD3 or medium (HBSS/HSA) for 48 hours, washed, and allowed to invade gels for 3.5 hours. Gels contained culture supernatants from α-CD3 or HSA cultures; supernatants from α-CD3 cultures plus α-IL8; α-IL-8 diluted in medium; or medium alone. The proportions invading were determined by examination of the gels *in situ*. Other cells were used for experiments shown in table 5.3.

The source of the anti-IL-8 antibody was goat (expts. 1 & 2) or rabbit (expts. 3, 4 & 5), diluted 1/1000 in HBSS/HSA.

TABLE 5.3

The effect of IL-8 on invasion of collagen gels by lymphocytes cultured in α-CD3.

AGENT IN GEL	EXPT. # 1	EXPT. # 2	EXPT. # 3	EXPT. # 4	EXPT. # 5
HBSS	24.8	23.5	21.4	22.2	13.9
IL-8	44.1	40.9	51.8	NT	NT
α-IL-8/HBSS	47.2	46.2	53.7	44.3	23.5
IL-8/α-IL-8	NT	31.0	36.7	NT	NT

Mononuclear cells were cultured in α-CD3 for 48 hours, then washed and allowed to invade gels containing various agents, for 3.5 hours. The proportions invading were determined by examination of the gels *in situ*. The same batches of cells were used for experiments shown in table 5.2.

The source of the α-IL-8 Ab. was goat (expts. 1 & 2) or rabbit (expts. 3, 4 & 5), diluted to 1/1000 in HBSS. The IL-8 (from Dr.A.O.Anderson) was used at 100 ng/ml.

NT = Not Tested.

testing.

From table 5.2 it can be seen that lymphocytes cultured in α-CD3 invaded gels incorporating their culture supernatants in greater proportions than gels containing medium, or supernatants from cells cultured in medium (HBSS/HSA). Cells cultured in medium alone showed lower proportions of invasive cells than those cultured in α-CD3, into gels incorporating supernatants from their own, or α-CD3, cultures. When α-IL-8 was added to α-CD3 culture supernatants prior to incorporation into gels, it significantly reduced the proportions of α-CD3-cultured lymphocytes invading those gels ($p<0.05$, paired *t*-test). α-IL-8 (in HBSS/HSA) had no effect in stimulating lymphocyte locomotion on its own.

Table 5.3 shows results using cells from the same batches as in table 5.2, but in these experiments HBSS, rather than HBSS/HSA, was used as medium. In all experiments in which it was tested, IL-8 caused an increased proportion of lymphocytes, which had been cultured in α-CD3, to invade gels, compared to gels containing medium alone. The responses were reduced, although not completely, by mixing α-IL-8 with the IL-8. One very surprising, yet consistent finding, was that α-IL-8 alone, in HBSS, caused as large a proportion of lymphocytes to invade as did IL-8 itself. This was not the case if HBSS/HSA was used as medium (see table 5.2).

The findings of the experiments described above were thus generally consistent. Addition of α-IL-8 reduced the response of lymphocytes cultured in α-CD3 to their own culture supernatants. The response was reduced to the same level as

that of the same cells to HBSS/HSA culture supernatants, or to HBSS/HSA alone. This suggests that IL-8 is a crucial factor in the increased lymphocyte locomotor activity seen in such α-CD3 culture supernatants, although other factors may also be important. IL-2 is not produced in sufficient quantities to be an active agent by itself in supernatants from MNC cultured in α-CD3 or PPD (Wilkinson and Newman, 1992). It is interesting that the anti-IL-8 antibodies were active at a dilution of 1/1000, sufficient to neutralise 10 ng/ml of IL-8 (Wilkinson and Newman, 1992). Levels of IL-8 in α-CD3 culture supernatants have been measured at approximately 60 ng/ml (Wilkinson and Newman, 1992); thus it would appear that complete neutralisation of the IL-8 may not be necessary. Recombinant IL-8 (at 100 ng/ml) could itself act as a locomotor attractant in this assay, and this could be partially blocked by addition of α-IL-8 at 1/1000.

In all cases, a large proportion of cells cultured in α-CD3 responded to HBSS/HSA, or to HBSS alone. Substances released into culture supernatants may nevertheless be required for directional responses, or for maximal numbers to respond, but this was not tested. Cells cultured in HBSS/HSA alone showed very little locomotor activity, even if α-CD3 culture supernatants were incorporated into the gels, suggesting that the capacity of lymphocytes to respond requires activation of lymphocytes, as previously suggested (Wilkinson, 1986).

The finding that the α-IL-8 antibodies alone caused lymphocytes to move into gels, if they were diluted in HBSS,

but not apparently if they were diluted in HBSS/HSA, or mixed with IL-8, was unexpected, and the reasons for these findings are not clear. Anti-immunoglobulin has been reported to cause chemotaxis of lymphocytes (Unanue, Ault and Karnovsky, 1974), but this was presumably via the $F(ab')_2$ fragment of the antibody. Blood lymphocytes express Ig Fc receptors constitutively and expression can be increased by activation of T cells, especially via the T cell receptor complex (reviewed by Sandor and Lynch, 1993). Human Fc receptors bind mouse immunoglobulins (reviewed by Van de Winkel and Capel, 1993), and so may also bind immunoglobulins from other species, such as the goat and rabbit, from which the antibodies used in these experiments were derived. Experiments with isotype-matched goat and rabbit immunoglobulins might show if the effect was specific for the α-IL-8 antibodies. The mouse α-CD3 used to activate lymphocytes did not cause freshly-isolated blood lymphocytes to polarize after short-term exposure (Wilkinson and Higgins, 1987a), and the α-CD3-containing culture supernatants did not consistently stimulate cells cultured in HBSS/HSA alone in the current experiments (table 5.2). It might be useful to examine whether lymphocytes cultured in α-CD3, then washed, would respond to mouse immunoglobulins. In the current experiments, the α-IL-8 antibodies caused lymphocyte locomotion, but it is not clear why mixture of the antibodies with IL-8 itself, or with HSA, would abolish its effect.

5.6 Invasion of collagen gels by CD45R subsets in response to IL-8.

Some preliminary experiments were carried out on lymphocytes cultured in PPD, allo-MLR or α-CD3, and allowed to invade gels containing recombinant IL-8 at 100 ng/ml, for 3.5 hours.

Results are shown in table 5.4. Generally the phenotype of invasive lymphocytes was similar to that of cells responding to their own culture supernatants, as described previously. After culture in PPD or allo-MLR, a larger proportion of CD45RO+, and of CD29+, lymphocytes were invasive than of CD45RA+ cells. Proportions of invasive CD4+ and CD8+ cells were similar. After culture in α-CD3, similar proportions of CD45RO+, CD29+ and CD45RA+ lymphocytes were invasive. The only difference between cells responding to IL-8, as compared to cells responding to their culture supernatants, was observed in the single experiment with α-CD3-cultured cells. More CD8+ than CD4+ cells were invasive, whereas the converse was found in response to culture supernatants. As with the other experiments outlined above, it would be interesting to see whether the results were consistent. If responses to IL-8 were similar to responses to culture supernatants, this might suggest that IL-8 alone would be sufficient to act as the selective locomotor stimulus in culture supernatants.

TABLE 5.4

The phenotype of lymphocytes, cultured in various activators, invading collagen gels containing IL-8.

PROPORTIONS INVASIVE	CULTURE CONDITIONS		
	PPD	Allo-MLR	α-CD3
OVERALL	24.7	20.5	52.8
CD45RO+	26.4	21.5	55.7
CD29+	27.5	26.7	53.2
CD45RA+	19.1	16.9	53.4
CD4+	22.3	19.1	54.0
CD8+	22.3	17.8	63.6

Mononuclear cells were cultured for 48 hours in PPD, allo-MLR or α-CD3, washed, and allowed to invade collagen gels containing 100 ng/ml IL-8 (from Dr.A.O.Anderson) for 3.5 hours. Cells were washed from the surface or digested from the gels. The phenotypes of non-motile and invasive populations were determined after immunofluorescence and FACS analysis.

5.7 The activation status of lymphocytes invading collagen gels.

As discussed previously, acquisition of locomotor capacity is a feature of activated lymphocytes, and is associated with cell growth (Wilkinson, 1986). Since FACS data from numerous gel invasions was available, the mean forward scatters of invasive, and non-invasive lymphocytes in each of 25 separate experiments were derived and compared. In these gel invasions, in which lymphocytes cultured in various activators had invaded gels for 3.5 hours or overnight, no consistent differences in mean forward scatter values were evident. The overall mean forward scatter value for non-invasive cells was 105.0 (e.s.e. 2.6), while the overall mean forward scatter value for invasive cells was 107.1 (e.s.e. 2.2). Thus the invasive and non-invasive populations appear to be of similar size. However measurement of forward scatter is an indirect measure of cell volume. Possibly use of a more direct measurement of cell size would be more definitive in comparing the sizes of the two populations.

Another marker of the activation status of lymphocytes is CD25 (Uchiyama *et al.*, 1981). Experiments were therefore performed in which lymphocytes cultured in α-CD3 were allowed to invade collagen gels, and the non-motile and invasive populations were stained for CD25.

Table 5.5 summarizes the results. In all experiments, more of the CD25+ than of the CD25- lymphocytes were invasive. However the results suggest that expression of CD25 is not a

TABLE 5.5

Proportions of CD25 +, and of CD25 -, lymphocytes which invaded collagen gels after culture in α-CD3.

CELL POPULATION	% INVASIVE (MEANS ± e.s.e.)
OVERALL	59.8 ± 3.5
CD25 + CELLS	65.7 ± 4.4
CD25 - CELLS	55.9 ± 3.0

Mononuclear cells were cultured with α-CD3 for 48 hours. Lymphocytes were then allowed to invade collagen gels incorporating their culture supernatants for 3.5 hours. Non-motile and invasive cells were stained for CD25 by immunofluorescence and analysed on a FACS.

Overall, 40.3 ± 1.0 % of lymphocytes were CD25 +.

The table summarises results from 3 experiments.

marker of cells with locomotor capacity; an observation similar to that found after similar experiments using polarization assays (see table 4.2), although differences in the collagen gel invasion assay were more marked.

5.8 Conclusions from collagen gel invasion assays.

The results found after phenotypic analysis of lymphocytes invading collagen gels were not as clear-cut as those from polarization assays. It was found that after culture in PPD or allo-MLR, the differences observed between CD45RO+ and CD45RA+ cells in collagen gel invasion assays were similar to those in polarization assays-proportionately more of the CD45RO+ cells (and here of the CD29+ cells) than of the CD45RA+ cells were invasive. This was found to be the case among both CD4+ and CD8+ cells. After culture in α-CD3 or Con A, no such differences were seen, unlike the pattern found in polarization assays. This may reflect a difference in lymphocytes after culture in antigens (PPD or allo-MLR) as opposed to polyclonal mitogens (α-CD3 or Con A). It is possible that the gel invasion assay detected a difference in cell adhesiveness or invasive capacity that morphological polarization in suspension does not reveal. The pattern of results was similar whether invasion proceeded for 3.5 hours, or overnight.

Attempts to use the collagen gel invasion assay to assess locomotor capacity of other subsets such as T cells and B cells, or CD4+ and CD8+ cells, as overall populations, were

limited by the finding that surface CD3 is reduced in culture in α-CD3. It was found that after culture in α-CD3, but not PPD, slightly more of the CD4+ than CD8+ lymphocytes were invasive. Culture in PPD resulted in a slightly higher proportion of CD19+ than of CD3+ cells being invasive. Since the APPAP technique was unsuitable for staining CD4 or CD19 (see section 3.2.2), it could not be used to compare the findings from gel invasions to those in polarization assays.

Attempts to examine the activation status of cells retained by collagen gels did not greatly supplement the established association between lymphocyte activation and acquisition of locomotor capacity. Slightly more of the CD25+ than of the CD25- lymphocytes, cultured in α-CD3, invaded collagen gels in response to their culture supernatants. No increase in forward scatter was seen among invasive cells, compared to non-invasive cells. Cell sizing by visual methods, or use of other activation markers, may reveal more marked differences. Culture of lymphocytes in other activators (for example antigens such as PPD), might also reveal such differences, especially since no differences in the proportions of CD45R subsets were found among invasive lymphocytes (themselves activation markers), after culture in α-CD3.

The results found using IL-8 and α-IL8 supplement other work which implicates IL-8 as a major, possibly pivotal, cytokine in the locomotor responses of lymphocytes to culture supernatants. IL-8 can itself cause polarization, invasion of collagen gels and chemotactic invasion of polycarbonate

filters. Anti-IL-8 appears to abolish much of the activity of culture supernatants in polarization and collagen gel invasion assays. Preliminary phenotypic analysis of invasive lymphocytes responding to IL-8 shows a similar pattern to that of cells responding to culture supernatants, which implies that IL-8 might by itself be capable of preferential stimulation of locomotion of antigen-stimulated CD45RO+ lymphocytes.

CHAPTER 6: CELL SIZING.

6.1 Area measurement APAAP-stained preparations.

Lymphocytes were fixed after stimulation by FCS on day 0 or after culture in various activators for 72 hours. Preparations were stained by the modified APAAP method. Positively-staining lymphocytes were classified visually, and the areas of 50 round, and 50 polarized, cells were measured.

In the first experiment, areas were derived using a cell image processor (Edwards, Robson and Campbell, 1987). In the second experiment the outlines of selected cells were traced manually and the areas of the outlines derived using a bitpad (Wilkinson *et al.*, 1988).

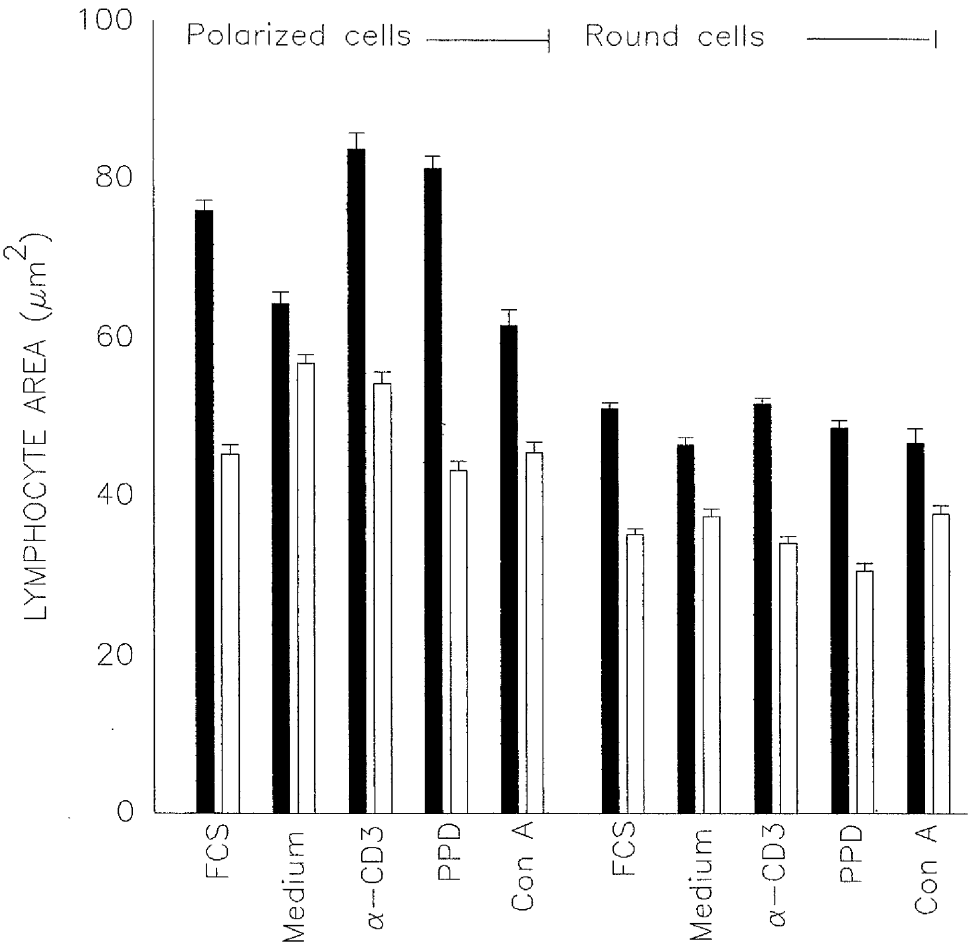
6.1.1 Areas of CD45R subsets from cell image processing.

Measurements of the areas of 50 round and 50 polarized lymphocytes staining positive for CD45RA or CD45RO are summarised in figure 6.1. Cells were tested on day 0 after stimulation by FCS, or after culture with medium (HBSS/HSA), PPD, α-CD3, or Con A.

With all stimuli CD45RO+ lymphocytes were significantly larger in area than CD45RA+ cells ($p<0.01$, Mann-Whitney U test). This was the case for both round and polarized cells.

FIGURE 6.1

Cell areas of polarized or round, CD45RO+ or CD45RA+, lymphocytes, measured using cell image processing.



Lymphocytes were stimulated on day 0 with FCS, or cultured for 72 hours in medium, α–CD3, PPD or Con A, Cell areas of CD45RO+ cells (solid bars), or CD45RA+ cells (open bars) were measured using cell image processing of slides stained by the modified APAAP method. In each pairing CD45RO+ cells were significantly larger than CD45RA+ cells ($p<0.01$, Mann Whitney U test).

6.1.2 Areas of CD45R subsets from bitpad tracing of outlines.

Again areas of 50 round and 50 polarized cells staining positive for CD45RO or CD45RA were measured, but in this experiment a wider range of stimuli was used. Results are summarised in figure 6.2.

After stimulation with FCS (day 0), and after culture in medium (HBSS/HSA), αCD3, or Con A, CD45RO+ lymphocytes were significantly larger in area than CD45RA+ cells ($p<0.01$, Mann-Whitney U test). This was the case among round and polarized cells.

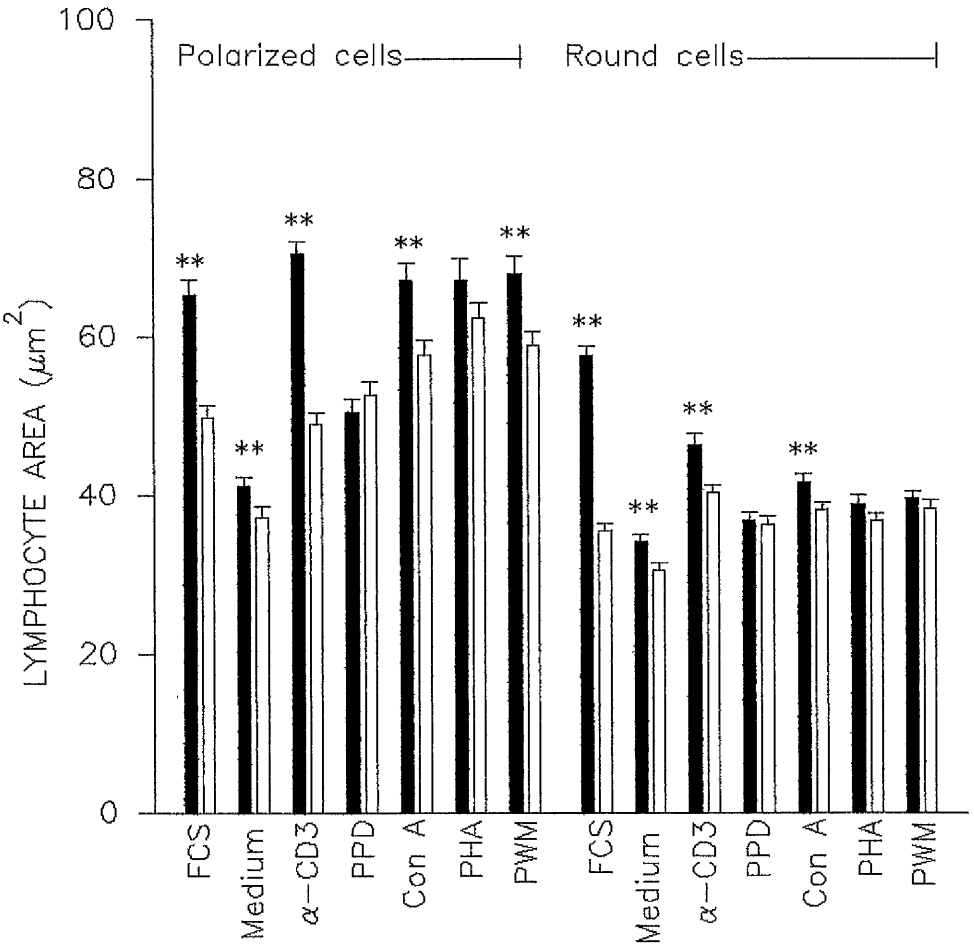
After culture in PWM, polarized CD45RO+ lymphocytes were significantly larger than polarized CD45RA+ cells. However no differences were found between round CD45RO+ and round CD45RA+ cells.

After culture in PHA, both round and polarized CD45RO+ lymphocytes were on average larger in size than the equivalent CD45RA+ cells. However these differences were not statistically significant.

In this experiment, after culture in PPD, no differences in cell areas between CD45RO+ or CD45RA+ were found, either among round or polarized cells. This contrasts with the previous experiment, when CD45RO+ lymphocytes were larger than CD45RA+ cells after culture in PPD. The reasons for this are not clear. The two methods used to derive cell areas give equivalent results (Dr. G.G. Reid, personal communication). Further experiments in which MNC were cultured in PPD, and the two methods were directly compared, might help to determine

FIGURE 6.2

Cell areas of polarized or round, CD45RO+ or CD45RA+, lymphocytes, measured using bitpad tracing of cell outlines.



Lymphocytes were stimulated on day 0 with FCS, or cultured for 72 hours in medium, α−CD3, PPD, Con A, PHA or PWM. Cell areas of CD45RO+ cells (solid bars), or CD45RA+ cells (open bars) were measured using bitpad tracing of outlines of cells stained using the modified APAAP method. Graphs show means and standard errors of 50 cells for each category. ** Indicates where CD45RO+ cells were significantly larger than the corresponding CD45RA+ cells ($p<0.01$, Mann Whitney U test).

some of the reasons for this discrepancy.

Cell area measurement of populations of cells containing polarized and round cells is complicated by the need to consider polarized cells separately from round cells. This is because the relationship between cell area and cell volume in polarized cells is not as exact as that in round cells, and non-spherical objects are larger in area than spherical objects of the same volume (Wilkinson, 1986: Wilkinson and Islam, 1989). This makes comparison of the overall size of populations impossible if different overall proportions of cells become polarized in different stimuli; if different proportions of defined populations become polarized; and if expression of phenotypic markers is not reciprocal. Each of these limitations applies to the CD45R subsets cultured in various activators.

An approach to overcome this complication, *i.e.* cooling cells to 4°C so that all become round, was recently described (Wilkinson and Newman, 1992). This should enable the overall size of defined populations to be determined.

6.2 Measurement of forward scatter of CD45R subsets.

An approach to obtain values for the overall size of a lymphocyte population without the need to consider polarized cells separately from round cells is measurement of forward scatter values on a FACS (Parks, Lanier and Herzenberg, 1986; Buckle and Hogg, 1990). It has been reported that there are no apparent differences in the forward scatter of polarized or

round cells (Eisele *et al.*, 1991). To confirm this, glutaraldehyde-fixed lymphocyte populations containing different proportions of polarized cells (assessed by microscopy) were acquired for FACS analysis (figure 6.3). Lymphocytes cultured in α-CD3 or medium were fixed *in situ* in experiment 1, or were allowed to invade collagen gels incorporating their culture supernatants (experiment 2). Populations were washed from the surface or digested from the gels in experiment 2. Those digested from gels were re-warmed in supernatant, and both populations fixed. Figure 6.3 shows that forward scatter is not affected by cells being in polarized morphology, and that no discrete populations were evident despite a range of proportions of polarized lymphocytes being present.

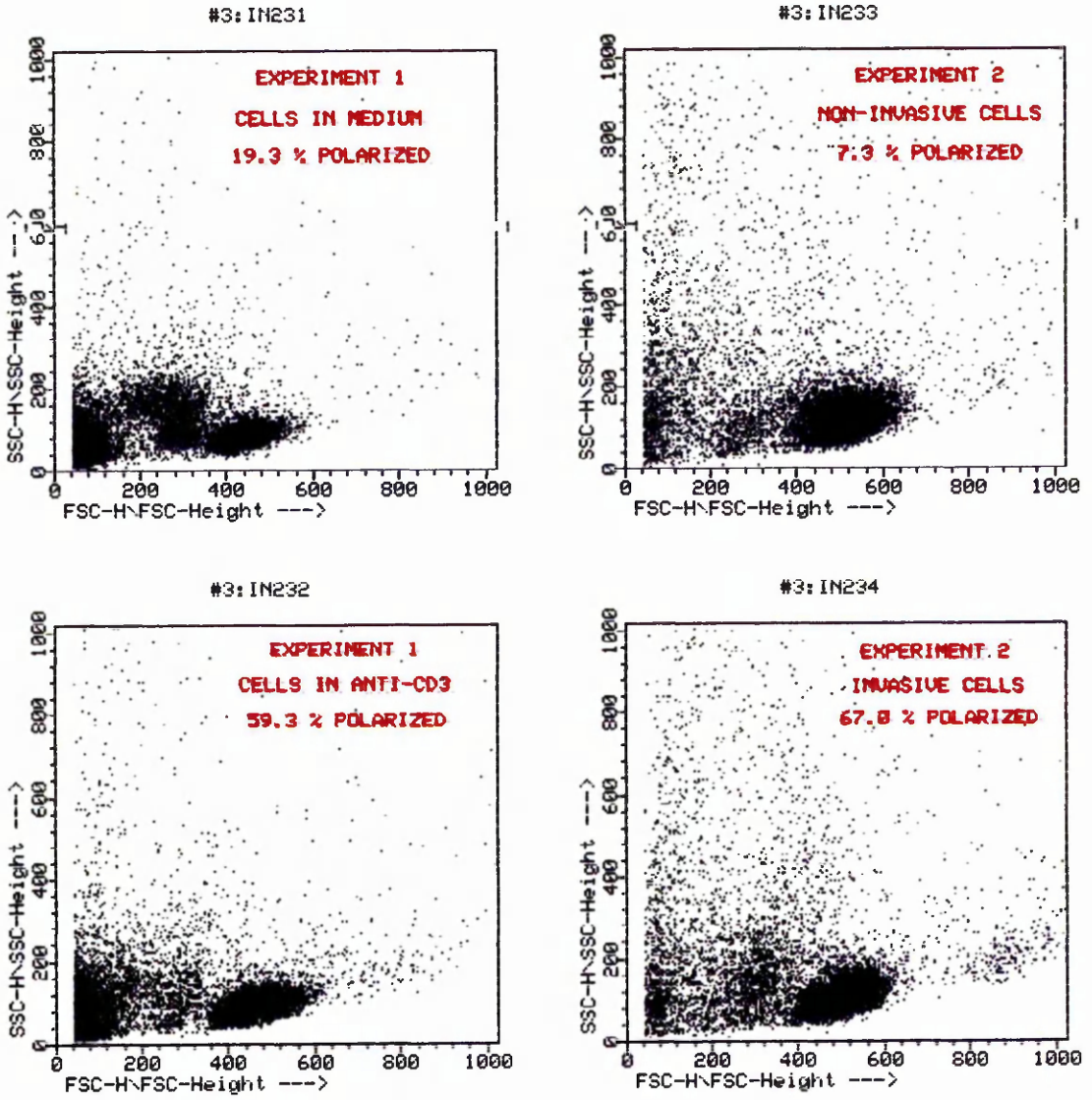
To determine the forward scatter of the CD45RO+ and CD45RA+ populations, lymphocytes were stained by immunofluorescence and acquired by the FACS, on day 0, or after 72 hours of culture in medium, αCD3, PPD, Con A, PHA, PWM, or allo-MLR.

Lymphocytes were selected by gating using leucogate staining and forward and side scatter profiles, then a second gate was used to select cells positive for CD45RO or CD45RA by fluorescence. The mean forward scatter of these cells was derived, although software allowing the values for individual cells was not available. Results are summarised in figure 6.4.

In every individual experiment, and with all activators, the mean forward scatter of CD45RO+ lymphocytes was

FIGURE 6.3

Forward and side scatters of lymphocyte populations containing various proportions of polarized cells.



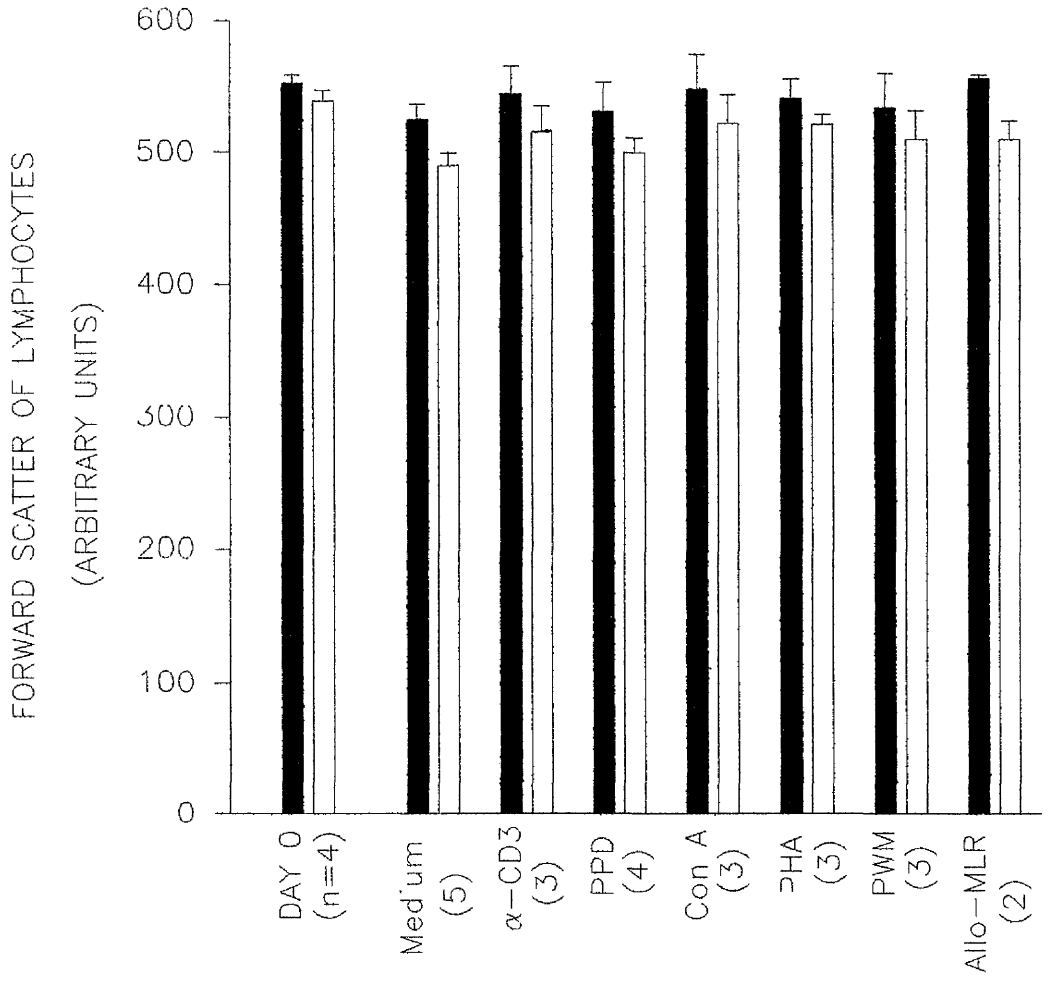
Mononuclear cells were cultured for 48 hours in medium ± anti-CD3. In experiment 1 samples were fixed directly from culture. In experiment 2, invasive and non-invasive populations among α-CD3-stimulated lymphocytes were separated after 3.5 hour invasion of collagen gels. Invasive cells were re-stimulated with culture supernatant, and both populations fixed.

Proportions polarized were determined, and forward and side scatter measured on a FACS.

Graphs show side scatter on the vertical axis, and forward scatter on the horizontal axis.

FIGURE 6.4

Cell volumes of CD45RO+ or CD45RA+ lymphocytes measured using forward scatter values from FACS analysis.



Lymphocytes were stimulated on day 0 with FCS, or cultured for 72 hours in medium, α-CD3, PPD, Con A, PHA, PWM or allo-MLR. Mean forward scatter of CD45RO+ cells (solid bars) or CD45RA+ cells (open bars) was derived from FACS analysis of fluorescently-labelled cells. Graphs show the means and standard errors of n experiments. In all individual experiments CD45RO+ cells were larger than CD45RA+ cells ($p<0.01$, Student's t-test).

significantly greater than that of CD45RA+ cells ($p \leq 0.01$, Student's *t* test). Differences in forward scatter appeared not to be as marked as those found after lymphocyte area measurement. The results of forward scatter of CD45R subsets on day 0 confirm those of Buckle and Hogg (1990), who found that the mean forward scatter of LFA-3+ lymphocytes was higher than that of LFA-3- cells, the LFA-3+ cells being equivalent to CD45RO+ cells.

6.3 Experiments following on from measurement of cell size.

6.3.1 Introduction.

A potential complication when comparing the sizes of CD45R subsets after culture is the well-established conversion of CD45RA+ lymphocytes to expression of CD45RO during culture in various activators. Thus different cell populations could be stimulated to change phenotype and be re-classified varyingly according to the activator, which would affect comparisons of CD45RO+ and CD45RA+ cells after culture in medium, or in different activators.

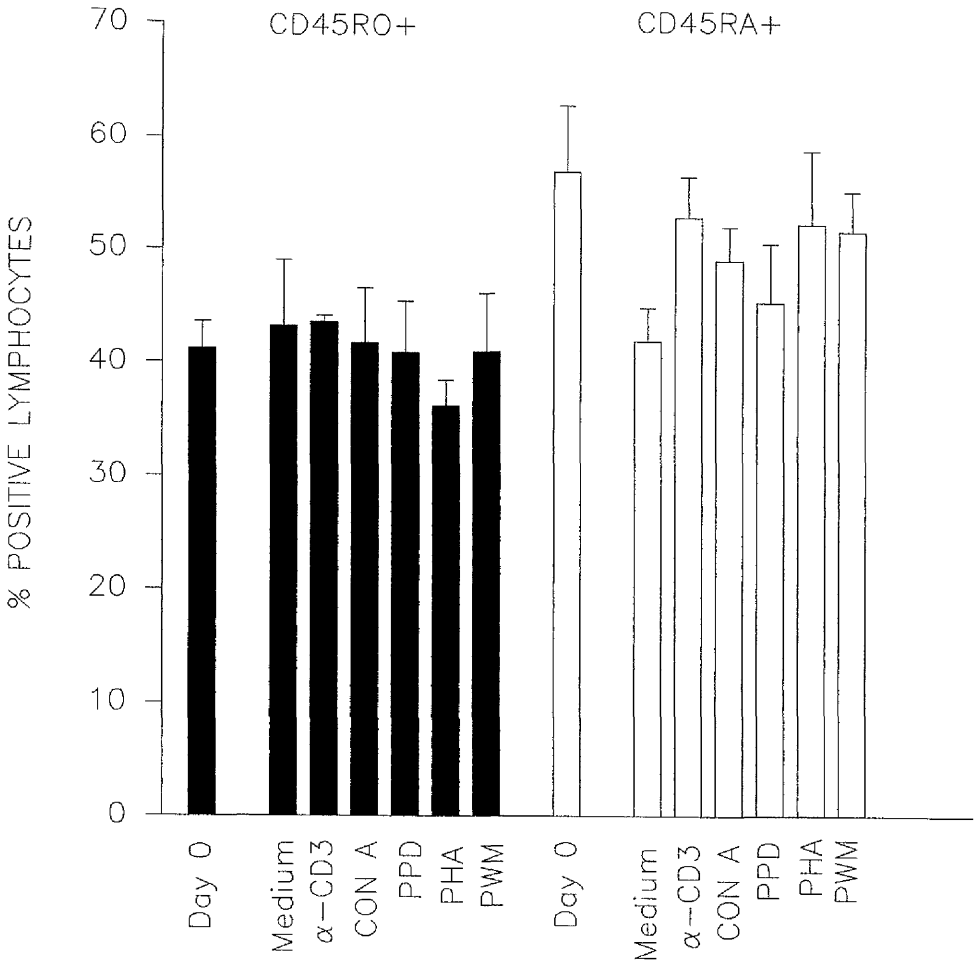
6.3.2 Changes in CD45R phenotype of cultured lymphocytes.

Experiments were undertaken to examine whether CD45RA+ lymphocytes would change phenotype under the culture conditions used for stimulation of locomotion in this project.

Figure 6.5 summarises the findings. Although there were

FIGURE 6.5

Proportions of lymphocytes staining for CD45RO or CD45RA after culture with various activators.



Lymphocytes were stained by immunofluorescence for CD45RO (solid bars) or CD45RA (open bars) on day 0, or after 72 hours in medium, α−CD3, Con A, PPD, PHA or PWM. Graphs show means and standard errors of three experiments.

slight variations in individual experiments, overall there was a slight decrease (of 5-15%) in the proportion of CD45RA+ cells by day 3 (including cells in medium alone). The proportion of CD45RO+ cells remained virtually unchanged after 3 days in culture with all of the stimuli used. Thus populations of lymphocytes examined on day 3 appear to be similar in expression of CD45R isoforms, particularly of CD45RO. However the CD45RA+ population might contain slightly different populations of cells depending on the stimulus used.

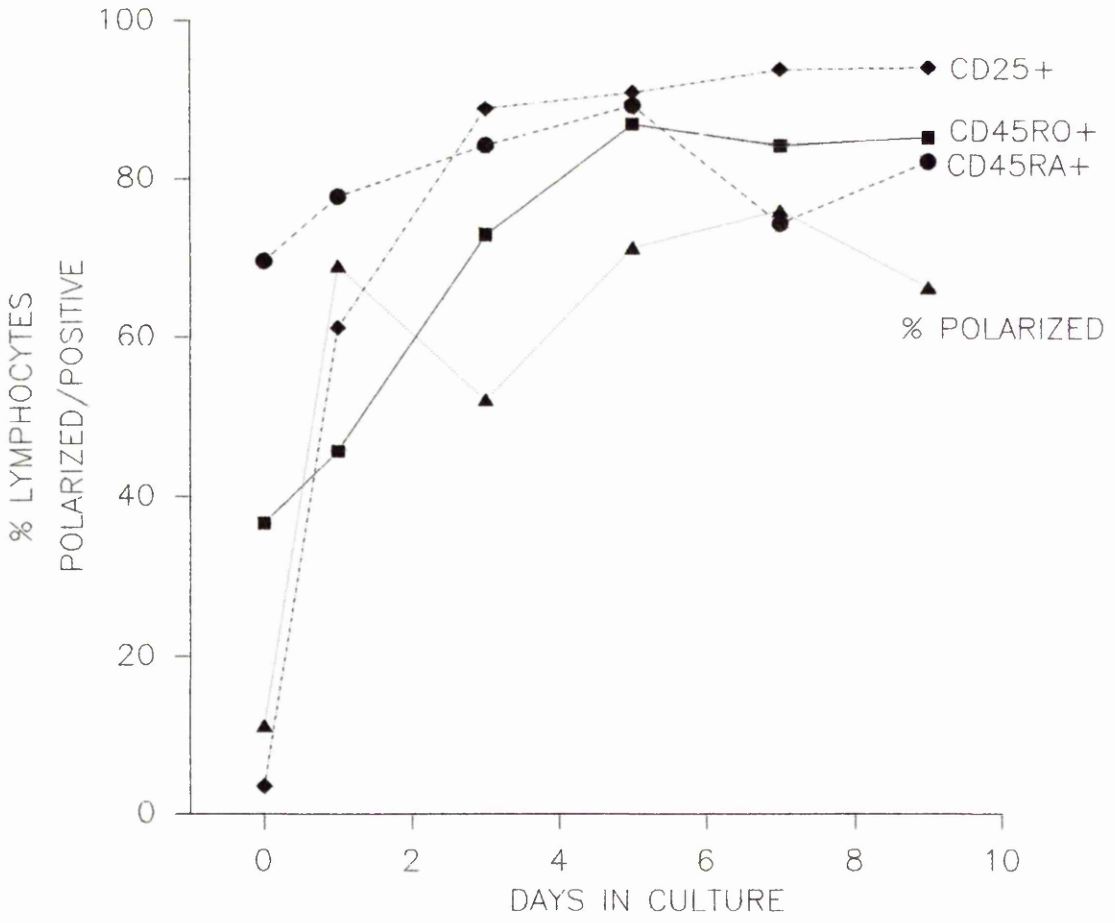
6.3.3 Culture of MNC in RPMI/FCS.

The changes in CD45R phenotype described above are not as marked as those reported previously (Akbar *et al.*, 1988; Byrne, Butler and Cooper, 1988; Deans, Boyd and Pilarski, 1989; Wallace and Beverley, 1990). These studies examined separated cell populations, cultured for at least 72 hours in PHA, using RPMI/FCS as a culture medium. To see whether culture of MNC in a RPMI/FCS could affect the kinetics of subset conversion, plus lymphocyte activation and locomotion, cells were cultured in RPMI/FCS and stimulated by PHA over a 9-day period. Samples were removed at various times for staining for CD45RO, CD45RA and CD25, and for polarization assays.

Results are summarised in figure 6.6. This indicates that expression of CD45R isoforms was different from that seen when the medium used was HBSS/HSA (see figure 6.5). It was

FIGURE 6.6

Changes in proportions of polarized cells, and in expression of CD45RO, CD45RA and CD25 after culture of lymphocytes with PHA in RPMI/FCS.



Samples were taken from mononuclear cell cultures on days 0, 1, 3, 5, 7 and 9. Proportions polarized (triangles on graph) were determined, as were proportions positive after immunofluorescence for CD45RO (squares), CD45RA (circles) or CD25 (diamonds) using FACS analysis.

also observed that virtually all lymphocytes were CD25+ after 72 hours. In addition the proportion of polarized cells was high throughout most of the experiment. Many blast-like lymphocytes were observed, and cell shapes were much more variable, than had been observed previously after culture in HBSS/HSA. Designation of lymphocytes as polarized or round was thus less straightforward, and cells might more accurately have been classified as round or non-round. Assessment of active locomotion of such cells would be informative, since loss of spherical morphology is not necessarily associated with motility (Wilkinson *et al.*, 1988).

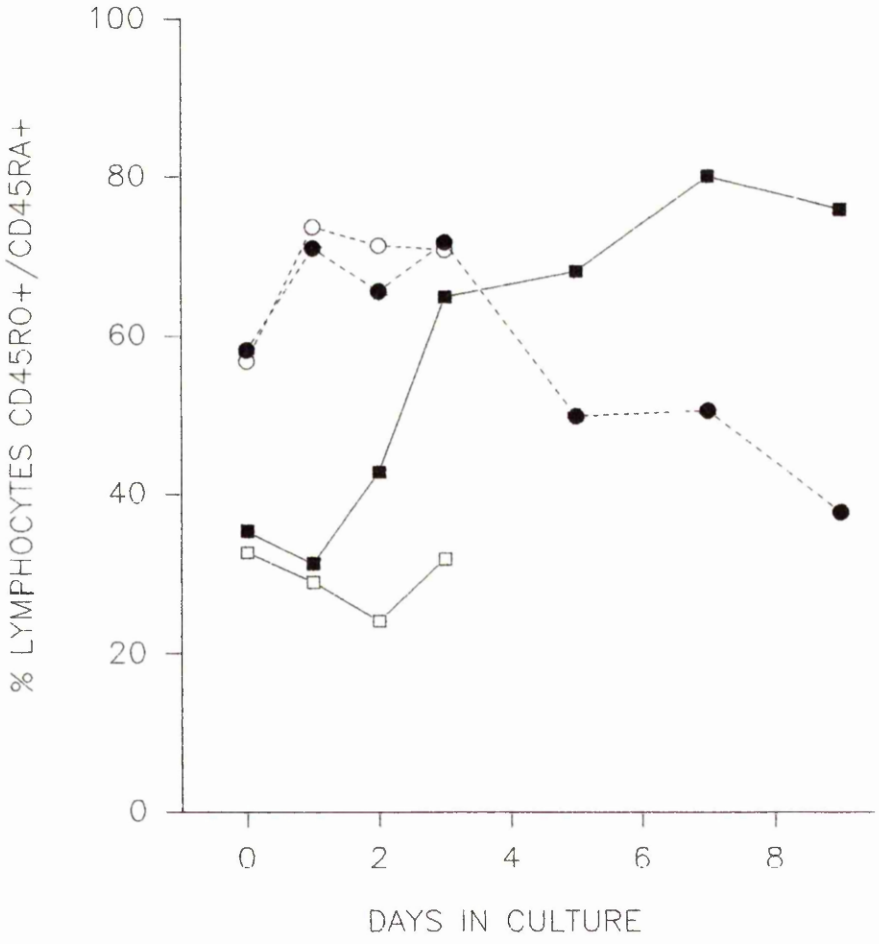
To extend some of the above findings, and also to investigate differences in cell size in different culture media, an experiment was conducted in which MNC were cultured in α-CD3, either in HBSS/HSA (over 3 days) or in RPMI/FCS (over 9 days).

Results are summarised in figures 6.7 and 6.8. These show that the proportions of lymphocytes staining for CD45RO, CD45RA and CD25+ were all different in cells cultured in HBSS/HSA compared to those in RPMI/FCS. In this experiment there was a slight increase in CD45RA expression at 72 hours, unlike the experiment shown in figure 6.5, although the reason for this is not known. The proportion of cells becoming polarized, or at least non-spherical, was also different in the two media.

There were also differences in cell size as assessed by forward scatter, depending on the culture medium. Figure 6.9 shows that cells cultured in HBSS/HSA plus α-CD3 showed little

FIGURE 6.7

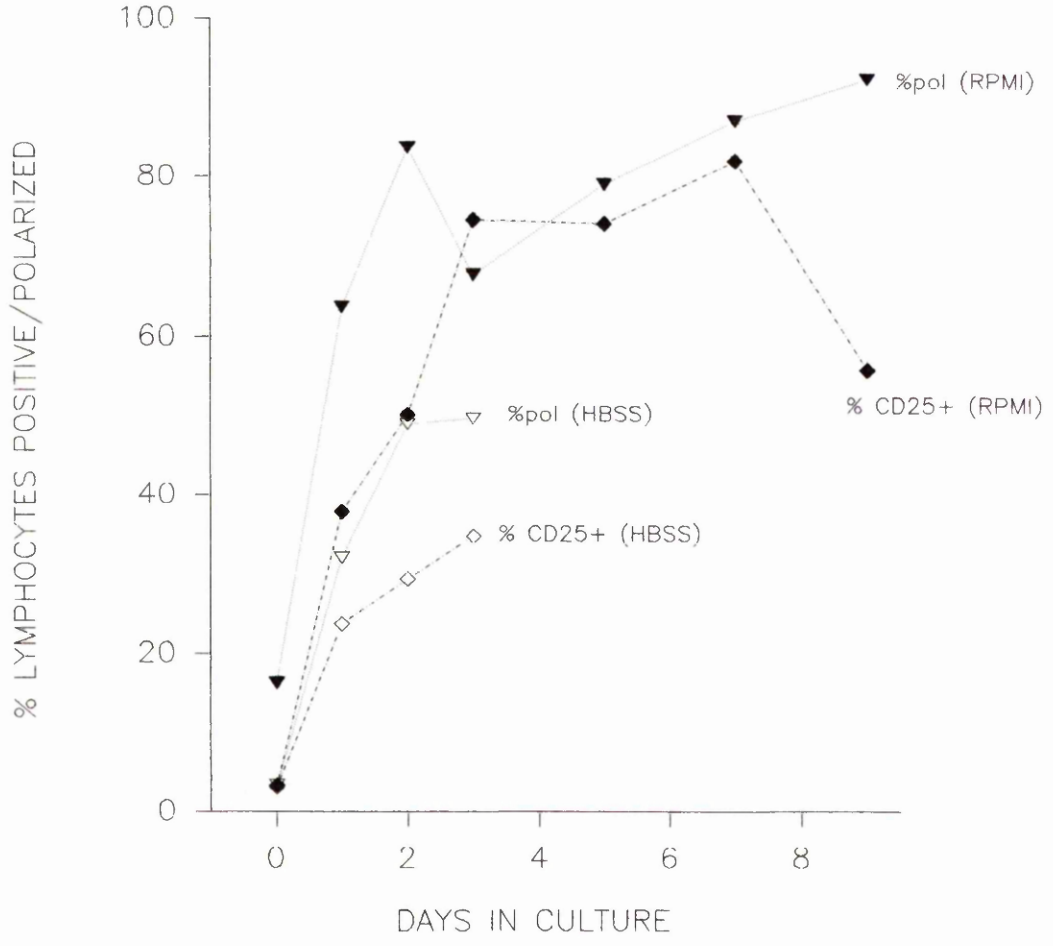
Changes in expression of CD45RO and CD45RA after culture of lymphocytes with α–CD3, in RPMI/FCS or in HBSS/HSA.



Mononuclear cells were cultured with α–CD3, either in RPMI/FCS (solid symbols) or in HBSS/HSA (open symbols). Samples were taken on days 0, 1, 2 and 3 from HBSS/HSA cultures; and on days 0, 1, 2, 3, 5, 7 and 9 from RPMI/FCS cultures. Proportions of lymphocytes positive for CD45RO (squares) or CD45RA (circles) were obtained using immunofluorescence and FACS analysis .

FIGURE 6.8

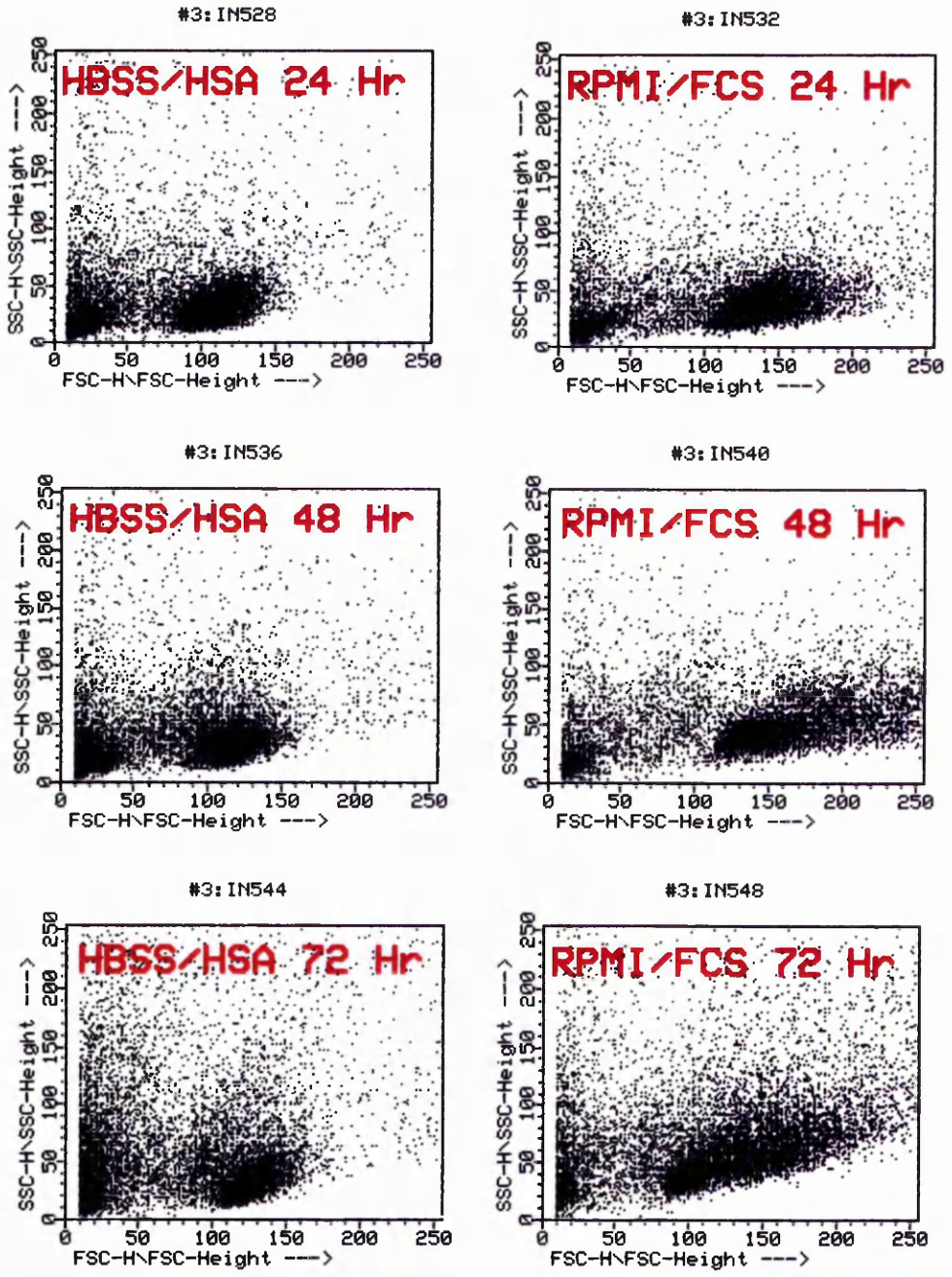
Changes in proportions of cells polarized, and in expression of CD25, after culture of lymphocytes with α–CD3, in RPMI/FCS or in HBSS/HSA.



Mononuclear cells were cultured with α–CD3, either in RPMI/FCS (solid symbols) or in HBSS/HSA (open symbols). Samples were taken on days 0, 1, 2 and 3 from HBSS/HSA cultures; and on days 0, 1, 2, 3, 5, 7 and 9 from RPMI/FCS cultures. Proportions of polarized lymphocytes were determined (triangles). The proportions of lymphocytes positive for CD25 (diamonds) were obtained using immunofluorescence and FACS analysis.

FIGURE 6.9

The effect of culture in HBSS/HSA or RPMI/FCS on forward and side scatter of α-CD3-stimulated lymphocytes.



Mononuclear cells were cultured for various times with α-CD3, in HBSS/HSA or RPMI/FCS, and forward and side scatter measurements of unstained lymphocytes obtained on a FACS.

Graphs show side scatter on the vertical axis, and forward scatter on the horizontal axis.

increase in forward scatter over the 72 hour period, while a progressive increase in forward scatter was evident if cells were cultured in RPMI/FCS plus α-CD3. Figure 6.10 shows the appearance of cells stimulated by α-CD3, in HBSS/HSA or in RPMI/FCS. Large numbers of blast-like cells were present in the RPMI/FCS cultures, but none in the HBSS/HSA cultures.

These experiments show that choice of *in vitro* culture medium markedly affected all of the responses assessed, and this may explain why lymphocytes cultured in HBSS/HSA showed different dynamics of change in expression of CD45R isoforms from those reported in other studies.

6.4 Conclusions to cell size analysis.

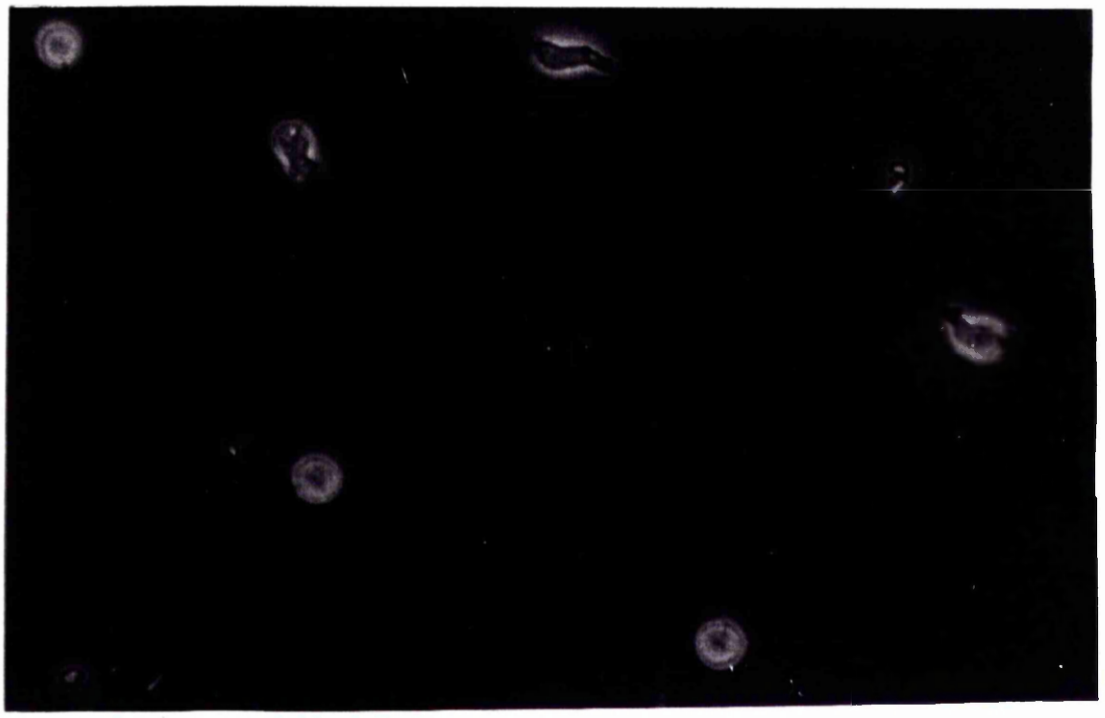
It can be concluded that, in most of the experiments described, CD45RO+ lymphocytes were larger than CD45RA+ cells. Taken together with the increased proportion of CD45RO+ which become polarized, this supports the hypothesis that cell growth is associated with acquisition of locomotor capacity (Wilkinson, 1986). Increased size did not absolutely predict acquisition of polarized morphology, since round CD45RO+ lymphocytes were also larger than round CD45RA+ cells.

When assessed by forward scatter measurement, CD45RO+ cells were consistently larger under all conditions. Most of the preparations used to derive cell areas by cell image processing or cell outline measurement also showed this pattern. Exceptions were found after cell outline measurement

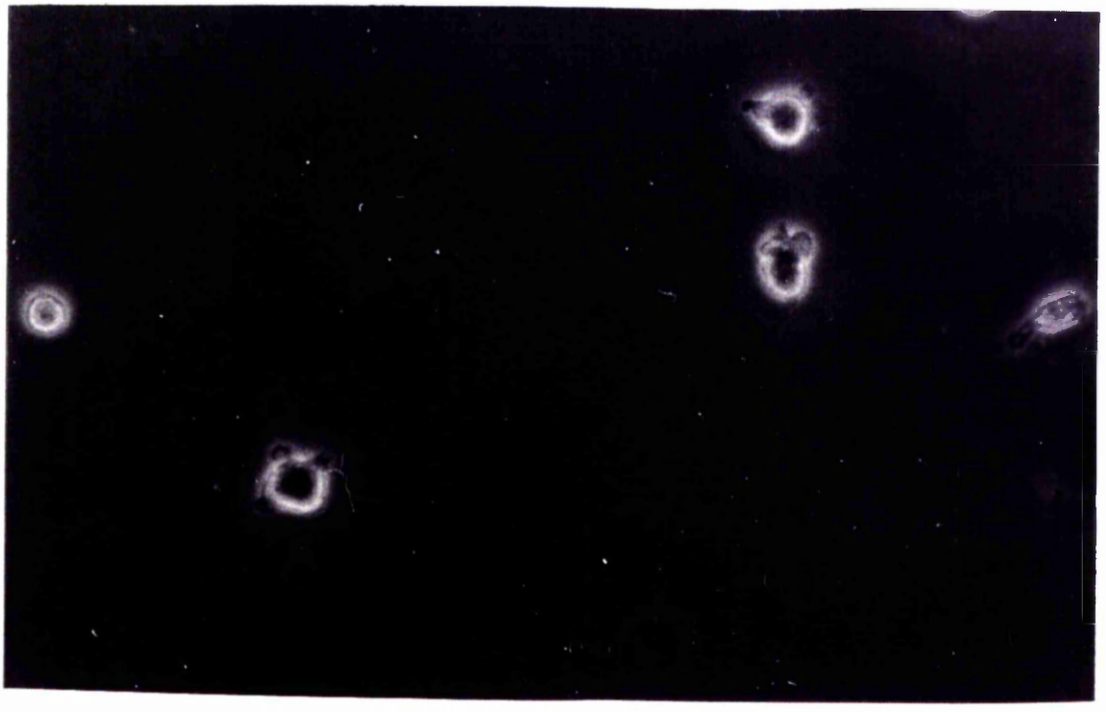
FIGURE 6.10

Appearance under phase contrast of lymphocytes after culture for 72 hours in α-CD3, in HBSS or in RPMI.

A:- Cells cultured in HBSS/HSA/α-CD3.



B:- Cells cultured in RPMI/FCS/α-CD3.



of cells cultured in PPD and PHA, and round cells after PWM stimulation.

Experiments which followed on from these findings firstly demonstrated that cells defined as CD45RO+ and CD45RA+ were probably similar cell populations, since proportions of lymphocytes staining for these CD45R isoforms were similar under various culture conditions; and secondly demonstrated that choice of culture medium markedly affected all of the lymphocyte responses tested. Culture of MNC in RPMI/FCS increased morphological and phenotypic indicators of activation *i.e.* increased lymphocyte size, presence of blast-like cells and a high proportion of CD25+ cells. It would be interesting to relate such indicators to effects on cell locomotion.

CHAPTER 7: GENERAL DISCUSSION.

7.1 Introduction.

This project aimed to examine the *in vitro* locomotion of human blood lymphocytes, in relation to expression of CD45RO and CD45RA, and also to examine some of the defined factors stimulating such locomotion.

As detailed previously, differential expression of CD45R isoforms on lymphocyte subsets has been extensively studied as a potential marker of cell function. It would appear that no stable, distinct function either of the molecules themselves, or of cells expressing them, is entirely or exclusively marked by any one CD45R isoform, although studies have been performed with a restricted number of isoforms in a range of mammalian species, which has to some extent hampered attempts to draw general conclusions. However the consensus based on these studies suggests that CD45RO is a marker of recent activation of lymphocytes. While recent activation is not a stable, irreversible, phase of lymphocyte lifespan or functional capacity, it has obvious relevance to *in vivo* lymphocyte localisation, and functioning of the immune system. It is of course possible that other distinct functions of lymphocytes expressing different CD45R isoforms may emerge in the future, and that results obtained to date can be re-interpreted accordingly.

In this project the locomotion of CD45R subsets was

examined in two major assay systems *i.e.* the polarization assay, which is the simplest assessment of locomotion of leucocytes, requiring no interaction with other surfaces; and invasion of collagen gels, which provide a more complex, but more physiological, substratum for leucocyte locomotion. The size of motile lymphocytes was also examined, since this has previously been related to activation status, *in vitro* locomotion, and recirculation patterns of lymphocytes *in vivo* (Parrott and Wilkinson, 1981).

7.2 Development of methods.

The first task was to adapt methods to allow simultaneous phenotyping of responding lymphocytes.

A modified APAAP method allowed phenotypic analysis of lymphocytes in the polarization assay. This method appears to preserve locomotor morphology, and give specific staining for many lymphocyte surface markers. It could potentially be applicable for other markers on lymphocytes, and other cell types. It should be noted that not all lymphocyte markers were detectable using the modified APAAP method, so that the suitability of the method would have to be determined for each marker.

FACS analysis of the phenotype of lymphocytes recovered after invasion of collagen gels was straightforward, since exposure to collagenase did not affect any of a range of lymphocyte markers examined. The only drawback to this method was that separation of motile lymphocytes from those merely

adherent to the gel surface (and thus resistant to being washed off) was not possible.

Cell sizing of defined populations of lymphocytes was also straightforward- by measurement of forward scatter of lymphocytes which had invaded collagen gels; or by combining cell area measurement by bitpad or cell image processing of images obtained after staining by the modified APAAP method. However the approach used in this project meant that polarized and round lymphocytes were present, and the need to consider these as separate populations made interpretation of results difficult. This drawback can be prevented by cooling cells prior to fixation.

The methods described could all prove useful for other studies of the locomotor behaviour of defined lymphocyte subsets within phenotypically mixed populations, as well as other cell types.

Other observations demonstrated modulation of surface CD3 expression during culture in α-CD3, and such modulations are of importance both for phenotypic analysis of cells, and presumably also for cell signalling in lymphocytes.

7.3 The locomotion of CD45R subsets.

In polarization assays, a significantly higher proportion of CD45RO+CD45RA- lymphocytes than of CD45RA+CD45RO- cells responded. This was the case using short-term stimulation of lymphocyte polarization by a range of substances on day 0 , or after overnight culture in FCS.

The same pattern was observed when examining lymphocytes polarized *in situ* after culture in a range of antigens or mitogens.

After culture of mononuclear cells in PPD or allo-MLR, CD45RO+ (and CD29+) lymphocytes invaded collagen gels containing their culture supernatants in greater proportions than CD45RA+ cells. This was observed within both the CD4+ and CD8+ subsets of lymphocytes. No differential invasion of gels by CD45R subsets was observed if mononuclear cells were cultured in α-CD3 or Con A, although clear differences were evident in polarization assays.

The reasons for the different patterns of response of CD45R subsets in polarization assays and collagen gel invasion assays after culture in α-CD3 or Con A are not easy to explain, since the two assay systems measure different, although related, responses. One possible way to further investigate the responses of CD45R subsets cultured in α-CD3 or Con A, and relate them to differing assay systems, would be to isolate each subset and film them responding to culture supernatants, in suspension or on collagen gels. Hence the relationships between morphology and active locomotion, and any differences evoked by the collagen matrix, could be investigated.

PPD and allo-MLR are antigens, while α-CD3 and Con A are polyclonal mitogens, and this distinction could be important if it affects the response of lymphocytes in locomotion assays, and indeed in other assay systems. Potentially different mechanisms of signalling could occur as a result of

interaction of specific antigens with the T-cell receptor or surface immunoglobulin on B cells, as opposed to signalling via CD3 or other surface receptors for mitogens. It is also possible that the presence of molecules such as Con A or PHA on the cell surface might affect the adhesiveness of lymphocytes for various substrata, which might help to explain the observation that culture of lymphocytes in PHA appeared to prevent lymphocyte invasion of collagen gels, despite the presence of morphologically polarized cells. Further work testing the responses of lymphocytes cultured in antigens or polyclonal mitogens might establish any differences.

It is concluded that blood CD45RO+ lymphocytes contain a greater proportion of cells with locomotor capacity as assessed in this study by morphological polarization, and invasion of collagen gels (at least after culture in PPD and allo-MLR), compared to CD45RA+ lymphocytes. Since CD45RO+ lymphocytes tested on day 0 displayed similar preferential polarization compared to CD45RA+ cells, it appears that this property is maintained during lymphocyte activation, rather than occurring as a result of cell activation. It should also be emphasised that a proportion of CD45RO+ lymphocytes always failed to respond in locomotion assays, and that a proportion of CD45RA+ cells always responded. It would therefore appear that surface expression of CD45R isoforms did not define lymphocytes with *in vitro* locomotor capacity, but rather populations with differing relative responses. Possibly examination of subsets within either population might give a

more definitive marker of locomotor potential.

The results obtained from the analysis of the locomotion of CD45R subsets are in general agreement with related studies elsewhere, which used filter assays to assess lymphocyte locomotion, after various methods of cell preparation, stimulation and determination of phenotype. Preferential migration of CD45RO+ (or CD29+) lymphocytes compared to CD45RA+ cells has been demonstrated in response to RANTES and interferon-inducible protein-10 (Schall *et al.*, 1990; Taub *et al.*, 1993a; Taub *et al.*, 1993b). The preferential migration of CD29+ lymphocytes compared to CD45RA+ cells in response to MIP-1α and MIP-1ß reported by Taub *et al.* (1993a) was not observed if CD45RO+ lymphocytes were compared to CD45RA+ cells (Schall *et al.*, 1993). As has been shown in other assay systems, responses of CD29+ lymphocytes were generally similar to the CD45RO+ population in this project.

Thus CD45RO+ lymphocytes have been shown to contain a greater proportion of cells with locomotor capacity in many *in vitro* studies of lymphocyte locomotion. CD45RO+ lymphocytes have also been shown to have enhanced migration compared to CD45RA+ cells across monolayers of endothelial cells (Masuyama *et al.*, 1992). The significance of the enhanced locomotor capacity of CD45RO+ lymphocytes relies on further knowledge of the relationship between *in vitro* locomotion, and other functions, of lymphocytes. This is necessary since, as discussed previously, results obtained in *in vitro* studies of

lymphocyte locomotion using defined factors appear to depend to a large extent on the assay system and the strategies used to prepare lymphocytes prior to testing. Experiments carried out during this project suggest that choice of culture medium (*i.e.* HBSS/HSA, compared to RPMI/FCS) may influence the activities of lymphocytes, including responses in locomotion assays. Further studies might define such influences, which will clearly affect the interpretation of *in vitro* experiments, and extrapolation to the situation *in vivo*.

The enhanced response of CD45RO+ lymphocytes in locomotion assays, after culture in a wide range of stimuli, is in contrast to the variable differences between CD45R subsets reported using proliferation assays. CD4+CD45RO+ and CD8+CD45RO+ cells, compared to CD4+CD45RO- and CD8+CD45RO- cells respectively, show increased proliferation to IL-2 and PPD (Taga *et al.*, 1991); CD4+CD45RA+ cells, compared to CD4+CD45RA- cells, show increased proliferation to Con A, and slightly increased proliferation to PHA, PWM and allo-MLR (Morimoto *et al.*, 1985a); CD3+CD45RO+ cells, compared to CD3+CD45RO- cells, show similar proliferation to PHA (Smith *et al.*, 1986; Akbar *et al.*, 1988), and to allo-MLR (Merkenschlager *et al.*, 1988). Comparative proliferative responses of CD45R subsets to α-CD3 appear to vary (Beverley, 1990).

All of the mitogens and antigens used in this study increased the proportion of motile lymphocytes, and it would be interesting to examine whether this increase is due to

direct stimulation of these lymphocytes, or if "bystander" lymphocytes become motile as a result of stimulation of other cells. In the HBSS/HSA medium used, very few lymphocytes appear to undergo blast transformation or mitosis, unlike the findings when MNC were cultured in RPMI/FCS. It is possible that in HBSS/HSA, lymphocytes remain activated but unable to undergo cell division, and this may represent an interesting state of cell cycle as far as motility is concerned.

7.4 Sizing of CD45R subsets.

Previously acquisition of lymphocyte locomotor capacity has been related to cell activation (Wilkinson, 1986). Lymphocytes judged to be activated on the basis of size and enhanced uptake of thymidine appear to show distinct recirculation pathways, and localisation patterns, *in vivo*, compared to small, resting lymphocytes. However small lymphocytes also recirculate, and must therefore presumably be capable of locomotion, but this capacity appears not be evident *in vitro*.

Freshly-isolated blood CD45RO+ lymphocytes have been shown to be larger than CD45RA+ cells, and show enhanced levels of surface activation and adhesion molecules (Sanders, Makgoba and Shaw, 1988; Maurer, Felzmann and Knapp, 1990; Buckle and Hogg, 1990; Shimizu *et al.*, 1990a; Shimizu *et al.*, 1990b; Taga *et al.*, 1991; Zola and Flego, 1992; Horgan *et al.*, 1992).

In this study it was shown using mean forward scatter

measurement that on day 0, and after culture in a wide range of activators, CD45RO+ lymphocytes were significantly larger than CD45RA+ cells. Using bitpad measurement it was observed that within both polarized and round populations obtained on day 0 or after culture in a range of activators, CD45RO+ lymphocytes were significantly larger than CD45RA+ cells. Although area measurement by cell image processing gave generally similar results to those obtained using bitpad measurements, there were some inconsistencies compared to results from bitpad measurement. An unexpected observation was that invasive lymphocytes cultured in a range of activators, and recovered from collagen gels, were similar in mean forward scatter to non-invasive cells. Possibly examination of CD45R subsets within the invasive populations, or a more direct measurement of the sizes of the two populations would have confirmed whether lymphocyte size is related to capacity to invade collagen gels.

The general pattern of results from cell sizing does however emphasise the relationship between activation status (here suggested by expression of CD45RO and cell enlargement), and *in vitro* locomotor capacity among lymphocytes. Another commonly-used marker of lymphocyte activation is CD25. During the course of this study it was shown that, although consistently more of the CD25+ than of the CD25- lymphocytes were shown to be polarized, and to invade collagen gels, expression of CD25 did not define cell populations with enhanced locomotor capacity.

The term activation in regard to lymphocytes generally

relies on the test system in use. Thus activation to become motile, to enlarge, and to express other functional capacities will involve different stimuli and *in vitro* conditions, and cells within different subsets at various stages of differentiation. If precise, specific and stable markers of the activation status of lymphocytes were defined, they might enable the exact relationship between lymphocytes motile *in vitro*, and those which localise at sites of inflammation *in vivo*, to be established.

7.5 Relating *in vitro* locomotion to *in vivo* localisation.

Reports of experiments in sheep and humans have suggested that lymphocyte subsets defined using differential expression of CD45 isoforms use different recirculation pathways (Picker *et al.*, 1990; Shimizu *et al.*, 1991a; Shimizu *et al.*, 1991b; McKay, Marston and Dudler, 1990; McKay *et al.*, 1992). There is an enrichment of CD45RO+ lymphocytes compared to CD45RA+ *in situ* in the gut and in sites of inflammation, and also compared to proportions of the two populations among lymphocytes in the blood (James *et al.*, 1986; Emery *et al.*, 1987; Pitzalis *et al.*, 1987b; Kingsley *et al.*, 1988; Modlin *et al.*, 1988; Harvey, Jones and Wright, 1989; Foster *et al.*, 1990; Markey *et al.*, 1990; Pitzalis *et al.*, 1987a; Pitzalis *et al.*, 1987b; Silverman *et al.*, 1993). Although it could not be determined whether the predominance of CD45RO+ lymphocytes arose as a result of cells switching from CD45RA+ phenotype, *in vivo* experiments suggest that CD45RO+ lymphocytes localise

preferentially at sites of injection of PPD too quickly for CD45R isotype switching to occur (Pitzalis *et al.*, 1991; Picker *et al.*, 1993).

Thus there is considerable evidence suggesting that CD45RO+ lymphocytes in humans, and their equivalents in sheep, display characteristics of activated lymphocytes as described previously *i.e.* enhanced *in vitro* locomotor capacity, separate recirculation pathways and localisation in the gut and at sites of inflammation *in vivo*, compared to resting cells. However it should be noted that small lymphocytes also traffic through sites of chronic inflammation, such as the rheumatoid synovium. A more direct strategy to examine the relationship between *in vitro* locomotion and *in vivo* localisation might involve separation of lymphocytes motile *in vitro* (for example using digestion of invasive cells from collagen gels), and their reintroduction into a model of inflammation.

It has become established that vascular endothelium plays a crucial role in the arrest of leucocytes from the circulation prior to traversal of extracellular matrix and localisation within tissue. CD45RO+ lymphocytes show enhanced adhesion, compared to CD45RA+ cells, to endothelial cell monolayers, and to the extracellular matrix components fibronectin and laminin (Cavender *et al.*, 1987; Pitzalis *et al.*, 1988; Damle and Doyle, 1990; Shimizu *et al.*, 1990a; Shimizu *et al.*, 1991a; Ikuta *et al.*, 1991), and this may play a role in selective extravasation of CD45R subsets. It should be noted that enhanced adhesion to endothelium, or to

extracellular matrix, need not necessarily facilitate locomotion or influence localisation.

Despite the fact that there are many unknown factors influencing the movement of lymphocytes both *in vitro* and *in vivo*, it is certainly feasible that preferential adhesion to non-specialised endothelium, and preferential locomotor capacity, could potentially enable blood CD45RO+ lymphocytes to leave the circulation and localise at sites of inflammation. This may relate to nature and function of CD45RO+ lymphocytes activated *in vivo*, and to their recirculation and localisation within the body. Further understanding of such mechanisms might facilitate delineation and manipulation of immune responses.

7.6 The role of IL-2 and IL-8 in lymphocyte locomotion.

The second major objective of the project was to examine defined factors capable of stimulating lymphocyte locomotion, particularly IL-2 and IL-8. If any defined molecules can be established to be active in recruitment of lymphocytes, or of particular lymphocyte subsets, strategies of manipulation by modulation of levels of such molecules should be possible.

One of the earliest cytokines reported as stimulating lymphocyte locomotion was IL-2 (Kornfeld *et al.*, 1985). Since then relatively few reports of this activity have occurred. Experiments described in this project indicate that human

recombinant IL-2 (obtained from Glaxo) stimulated locomotion in a large proportion of lymphocytes freshly-isolated from blood, or cultured overnight in FCS. It is interesting, but puzzling, that human recombinant IL-2 obtained from Genzyme appeared to have much less activity in similar assays. Possibly similar variations in activities have contributed to the uncertainties about whether IL-2 is capable of stimulating lymphocyte locomotion. Whatever the reasons for the variations in activity between IL-2 from different sources, a role for IL-2 in lymphocyte locomotion raises the possibility that activated lymphocytes could recruit other lymphocytes at particular sites. IL-2 also has a crucial role in lymphocyte activation and differentiation, which may relate to the acquisition of locomotor capacity. Results shown in this project confirm that IL-2R α chain expression is low on lymphocytes fresh from blood or cultured overnight in FCS, and as the effects of IL-2 on the locomotion of blood lymphocytes tested on day 0 are abolished by blocking the IL-2R ß chain using a specific antibody (P.C. Wilkinson, personal communication), it appears that on these cells the ß chain is important for signal transduction for lymphocyte locomotion, although on activated cells the α chain may also play a role. Further work might establish the signalling systems by which IL-2 stimulates lymphocyte locomotion, potentially allowing manipulation of such mechanisms.

Culture supernatants have proved to be a useful source of stimulators of leucocyte locomotion. Many of the active

molecules have recently been defined, including the molecule now termed IL-8. Several reports have defined a role for IL-8 as a stimulator of lymphocyte locomotion *in vitro* (Larsen *et al.*, 1989a; Bacon, Westwick and Camp, 1989; Leonard *et al.*, 1990; Wilkinson and Watson, 1990), and a lymphocyte attractant *in vivo* (Larsen *et al.*, 1989a; Larsen *et al.*, 1989b; Endo *et al.*, 1991), although neutrophils were the predominant cell type attracted. Experiments outlined in this project suggest a pivotal role for IL-8 in the lymphocyte attractant activity of supernatants from mononuclear cell cultures in α-CD3. A similar pivotal role for IL-8 in the lymphocyte attractant activity of supernatants from mononuclear cell cultures in both α-CD3 and PPD has been demonstrated in polarization assays (Wilkinson and Newman, 1992). Furthermore levels of IL-8, but not of IL-2, in α-CD3 or PPD culture supernatants were found to be sufficient to stimulate lymphocyte locomotion, and clearly differential production of cytokines will influence the responses elicited by culture supernatants.

The role of IL-8 and other members of the chemokine family as locomotor attractants for leucocytes is currently being investigated extensively (reviewed by Miller and Krangel, 1992). A complicated pattern is emerging, with each chemokine stimulating selective, but in many cases overlapping, leucocyte types and subsets. RANTES has been demonstrated to stimulate lymphocyte locomotion *in vitro* (Schall *et al.*, 1990; Taub *et al.*, 1993a; Taub *et al.*, 1993b), although in this project responses to RANTES in polarization assays were inconsistent. No simple explanation is evident,

but choice of assay system may be important-for example Taub *et al.* (1993a) demonstrated that RANTES, but not IL-8, was active in filter assays.

As discussed previously, chemokines can be synthesised by a variety of different cell types, in response to a range of stimuli. Receptors for chemokines can be specific for one or more members of the family at different affinities, and may be expressed on different cell types, the effects apparently being dependent on the concentration of chemokine used. Thus control of chemokine production and receptor expression appears to be complex. Potentially this may allow a repertoire of regulatory effects on the functions of various leucocyte populations, although such a role *in vivo* is still to be established.

7.7 Expression of CD45 as a marker of lymphocyte function.

Despite a considerable amount of research in a variety of assay systems, many questions remain about the role of lymphocyte surface CD45, but it is clear that it plays a crucial role in many lymphocyte activities. Evidence of different functions by the various isoforms of CD45 remains elusive, but expression of such isoforms as a marker of differing lymphocyte function has stimulated considerable interest, and such differences may become apparent.

Although examination of expression of CD45R isoforms has yet to provide fully-established markers of lymphocyte function, the use of monoclonal antibodies to define surface

markers allows results to be re-interpreted as more information becomes available about the ligand recognised.

Application of phenotypic analysis, such as expression of CD45R isoforms, to studies of lymphocyte locomotion may offer the chance to investigate the recirculation and localisation of defined populations. The observations discussed previously suggesting a link between the preferential localisation of CD45RO+ lymphocytes at sites of inflammation *in vivo*, and the *in vitro* phenomenon of lymphocyte locomotion, might suggest that approaches enabling the mechanisms involved may be delineated and manipulated. This may potentially increase understanding of the immune system in general, and may eventually contribute to strategies resulting in therapeutic benefit.

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LIST OF PUBLICATIONS.

The following publications include work presented as part of this thesis:-

Newman, I. & Wilkinson, P.C. (1992). "Methods for phenotyping polarized and locomotor human lymphocytes". *Journal of Immunological Methods*, 147, 43-50.

Wilkinson, P.C. & Newman, I. (1992). "Identification of IL-8 as a locomotor attractant for activated human lymphocytes in mononuclear cell cultures with anti-CD3 or purified protein derivative of *Mycobacterium tuberculosis*". *The Journal of Immunology*, 149, 2689-2694.

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