A T-lymphocyte-derived factor which enhances leukotriene B_4 generation by human neutrophils

A thesis submitted for the degree of Doctor of Philosophy in the University of London (Faculty of Medicine)

by

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I dedicate this thesis

to my parents,

as a token of my

loving devotion

ABSTRACT

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A human blood mononuclear cell (MNC)-derived leukotriene release enhancing factor (LREF) is described. LREF significantly increased IgG-dependent leukotriene B_4 (LTB_4) generation by neutrophils. Supernatants from MNC's incubated with phytohaemagglutinin (PHA) or anti-CD3 monoclonal antibody, as well as PHA-stimulated ER+ lymphocytes, produced a 150-350% increase in LTB_4 generation by IgG-stimulated neutrophils. With PHA, maximal activity was observed 48 hours after culture in serum-free medium. LREF was relatively stable when exposed to low pH (pH 2) or heat (56°C, 60 min). Following progressive purification by gel filtration and chromatofocusing, LREF was associated with proteins of molecular size 35-40 kD and a pI of 5.1-5.5. The molecular weight of LREF by SDS-PAGE was 60 kD. On the basis of molecular weight and biological activities LREF was distinguishable from IL-1, IL-2, IFN-gamma, GM-CSF and TNF. MNC's from atopic individuals produced LREF when incubated with specific allergen. The T cell origin of LREF was established using a long term human CD4+ T cell line specific for the house dust mite. LREF had other biological activities. These included enhanced neutrophil adherence to nylon wool, increased cytotoxicity of opsonized schistosomula of S. mansoni and upregulation of complement receptors (CR1 and CR3). LREF also had a weak neutrophil chemotactic activity. Both corticosteroids and LTB_4, but not disodium cromoglycate or IFN-gamma, inhibited LREF release from PHA-stimulated MNC's in a concentration-dependent fashion. Thus LREF appears to be a hitherto undescribed lymphokine with a range of biological activities and establishes a further link between T cell and neutrophil activation, an observation which may be of importance in our understanding of allergic inflammation.

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ABBREVIATIONS

AA	Arachidonic acid
A 23187	Calcium ionophore
AC	Accessory cell
ADCC	Antibody dependent cell-mediated cytotoxicity
BSA	Bovine Serum Albumin
CBH	Cutaneous Basophil Hypersensitivity
CD	Cluster of differentiation
CF	Chemotactic Factor
Con A	Concanavalin A
CR	Complement Receptor
c.p.m.	count per minute
DEX	Dexamethasone
DSCG	Disodium cromoglycate
ER+	Erythrocyte rosette positive
FACS	fluorescence-activated cell sorter
FITC-RAM	fluorescein isothiocyanate conjugated $F(ab')_2$ fragments of rabbit antibody to mouse immunoglobulins
fMLP	formyl-methionyl-leucyl-phenylalanine
FPLC	Fast Protein Liquid Chromatography
GM-CSF	Granulocyte Macrophage-Colony Stimulating Factor
IEF	Isoelectric focusing
IFN-gamma	Interferon-gamma
IgG	Immunoglobulin G
15 HETE	15 hydroxyeicosatetraenoic acid
LIF	Leukocyte inhibitory factor
LREA	Leukotriene release enhancing activity
LREF	Leukotriene release enhancing factor
LTB_4	5(S), 12(R)-dihydroxy-6, 14 cis-8, 10 trans-eicosatetraenoic acid, leukotriene B_4
$LTC_4/D_4/E_4$	Leukotriene $C_4/D_4/E_4$
MNC	Mononuclear cell
MoAb	Monoclonal antibody
MWCO	Molecular weight cut-off
PBMC	Peripheral blood Mononuclear cell
PBS	Phosphate buffer saline
PG	Preparation grade
PHA	Phytohaemagglutinin
pI	Isoelectric Point
PMA	Phorbol myristic acetate
PMN	Polymorphonuclear cell
PMSF	Phenyl methyl sulfonyl fluoride
RIA	Radioimmunoassay
RP-HPLC	Reverse Phase-High Performance Liquid Chromatography
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TCR	T cell receptor
TNF	Tumor necrosis factor

CHAPTER 1.

GENERAL INTRODUCTION

1.1 Cell-cell interactions in hypersensitivity reactions

The role of leukocytes in host defense mechanisms was first established by Metchnikoff over 100 years ago (Metchnikoff 1884). The term cell-mediated immunity is generally used to describe non-antibody mediated specific immunologic defence mechanisms, and classically it was exemplified by the delayed type hypersensitivity response to tuberculin proteins, in which sensitised T lymphocytes induce the local inflammation associated with lymphocyte and macrophage infiltration. Other variant forms of cell-mediated immunity have been characterised in recent years including cutaneous basophil hypersensitivity (Askenase 1976; Stashenko et al 1977) and contact sensitivity (Asherson & Zambala 1970), although it is now recognised that it is probably incorrect to restrict the term cell-mediated immunity to the classical delayed-type hypersensitivity reaction.

Many of the manifestations of this form of immunologic reactivity do not involve direct cell-cell interactions but rather the actions of soluble effector (mediator) substances called cytokines. Recent in vivo observations indicate that lymphocytes (through the actions of lymphokines) play an important role in the regulation of leukocyte responses and that

these are not necessarily confined to immunologic events (Dinarello & Mier 1987). Though lymphocytes and neutrophils are present together in the histopathology of acute rejection after transplantation and allergic inflammation after allergen skin test, it remains uncertain as to how these cells accumulate and become activated (Robbins & Angell 1976; Zweiman et al 1976; Fleekop et al 1987).

The histological features of delayed-type hypersensitivity reactions in guinea pig are frequently used as the reference point for investigating comparable reactions in other species. Thus, a mononuclear cell infiltrate is regarded as the hallmark of a delayed type reaction. However, in the mouse, neutrophil infiltration is also a common feature of cellular hypersensitivity and precedes the mononuclear cell response (Crowle 1975). Furthermore, when guinea pigs are immunized by procedures that avoid the use of mycobacterial adjuvants, a delayed skin reaction is produced which is relatively nonindurated and is characterized by extensive infiltration of basophils. This is referred to as cutaneous basophil hypersensitivity ("CBH"). CBH reactions are seen in response to a variety of immunogens including contact allergens, vaccinia virus, allogeneic tumour cells, insect bites, and skin

allografts (Colvin & Dvorak 1979). In certain circumstances, the eosinophil may also play a major role. Accelerated reactivity on rejection of antigen at skin sites of healed delayed hypersensitivity reactions has been observed. This second reaction is more intensive and is characterized by the presence of large numbers of eosinophils (Arnason & Waksman 1963).

These various histologic patterns indicate that the typical mononuclear cell infiltrate is only one manifestation of delayed-type hypersensitivity reactions. Common to all of these reactions is the presence of a small number of specifically sensitized lymphocytes that interact with local antigen and release a variety of lymphokines responsible for the effects observed.

Recently, neutrophil activation and LTB_4 generation have been found to come under the influence of cytokines (Roubin et al 1987; Dessein et al 1986).

In acute inflammation, neutrophils are the most important inflammatory cells, and they secrete and elaborate a wide range of inflammatory mediators. Amongst these is leukotriene B_4 (LTB_4), a major 5-lipoxygenase product of arachidonic acid metabolism. LTB_4 is generated by neutrophil after activation (Williams et al 1985) and which is a potent pro-inflammatory mediator, promotes pronounced

chemotaxis of neutrophils (Ford-Hutchinson et al 1980), increases adherence and migration of neutrophils through vascular endothelium as well as increasing vascular permeability (Bjork et al 1982a).

In order to have a better understanding of the relationship between cell mediated immunity and inflammatory cells, I have studied the effect of lymphokines on the capacity of neutrophils to generate LTB_4 *in vitro*. I have used a physiologically relevant stimulus (IgG-coated Sepharose 4B beads) and have also investigated the feedback regulatory effect of LTB_4 on lymphokine generation by lymphocytes.

1.2 Background

1.2.1 Lymphocyte activation and lymphokine generation

The specificity of the immune response depends upon the specific recognition of a very wide variety of antigens by T and B lymphocytes. The T lymphocyte antigen receptor (TCR) consists of a 90 kD disulphide linked heterodimer, composed of one 39-46 kD alpha and one 40-44 kD beta chain (Samelson et al 1983; Kaye & Janeway 1984). This protein complex is termed Ti (in which i=idiotype, reflecting the fact that each TCR has a unique structure capable of interacting with one, and only one, antigenic determinant). TCR is non-covalently associated with CD3 (T3) molecules. The CD3 polypeptide structure is composed of at least three chains: a 20 kD glycoprotein delta-chain, a

25 kD glycoprotein gamma-chain, and a 20 kD nonglycosylated epsilon-chain. It is possible that other subunits may exist (Oettgen et al 1984; Borst et al 1984; Borst et al 1983; Borst et al 1982). The CD3 complex has a transmembrane portion which is phosphorylated during T cell activation and it is therefore thought to act as a transducer for the TCR. TCR only recognises surface bound antigens which are associated with molecules encoded by the major histocompatibility complex (Allison et al 1982; Haskins et al 1983; Meuer et al 1983).

T cells may be activated not only by antigen on appropriate antigen presenting cells, but also by other ligands interacting with the T3/Ti complex. Thus monoclonal antibodies directed against either T3 or Ti can activate T cells under appropriate circumstances, induce proliferative responses and the secretion of lymphokines <u>in vitro</u>. In addition, lectins such as PHA or Con A can be used to activate T cells (Chang et al 1982; Tax et al 1983; Von Wussow et al 1981; Weiss et al 1984; Kanellopoulos et al 1985).

Following cell-surface signals of activation, a series of unknown events occur to induce lymphokine gene transcription. Kinetic studies of the stimulation of the Jurkat T cell line with PHA and phorbol myristic acetate (PMA) revealed that IL-2 and IFN-gamma mRNA transcription peaks at 4-6 hr after

stimulation, and lymphokine generation reaches maximal amounts 24 hr after stimulation (Efrat & Kaempfer 1984; Kronke et al 1984).

Lymphocytes also bear specific hormone receptors which interact with hormones, lymphokines or autacoids (substances which are formed locally and act locally, such as histamine) (Khan & Melmon 1985). Almost all lymphocyte hormone receptors are located on the cell surface except for glucocorticoid receptor.

The prevailing view is that glucocorticoids interact with cytoplasmic receptors, and the glucocorticoid-receptor complexes are translocated to the nucleus where they stimulate synthesis of new messenger RNA and thus of proteins including lipocortin (Hirata et al 1982; Schleimer et al 1984). Lipocortin is a potent inhibitor of phospholipases including phospholipase A2, and its inhibitory action prevents production of both cyclooxygenase and lipoxygenase products of arachidonic acid metabolism.

One study has demonstrated that the inhibitory effect of glucocorticoids on proliferation and IL-2 production is entirely reversed by $5x10^{-9}$ M LTB_4. This action of LTB_4 is specific, in that LTC_4 and LTD_4 cannot reverse the effect of glucocorticoids and LTB_4 does not reverse the inhibition of proliferation induced by PGE_2, histamine or IFN-gamma. Thus LTB_4 may be an essential intermediate in T cell production of

IL-2 (Goodwin et al 1986).

LTB_4 receptors are present on subsets of both T4+ cells and T8+ cells (Payan et al 1984). Although there are no specific receptor antagonists available, the specificity of LTB_4 biologic effects has been evaluated by comparison with other lipoxygenase products. LTB_4 inhibits proliferation of CD4+ cells while it enhances proliferation of CD8+ cells (Payan et al 1984; Gualde et al 1985). One unconfirmed report suggests that LTB_4 can induce the differentiation of T4+ cells to T8+ cells (Atluru & Goodwin 1984). LTB_4 may provide an essential signal for lymphocyte activation. However the relationship between receptor numbers and biologic effect is still unknown.

Numerous observations indicate that cellular co-operation in the immune response is orchestrated by a number of soluble mediators. The term lymphokine was introduced in 1969 to describe "cell-free soluble factors generated by the interaction of sensitised lymphocytes with specific antigen and expressed without reference to the immunological specificity" (Dumonde et al 1969; Maini et al 1969). The term cytokine was introduced later to devote the fact that other cell-types could be the source of soluble mediators. Cytokines are non-immunoglobulin protein mediators secreted by immune cells that amplify and

mediate immune responses. Thus cytokines produced by lymphocytes are termed lymphokines while those produced by mononuclear phagocytes are called monokines. These mediators of immune responses have recently been the focus of a great deal of interest and the subjects of numerous investigations. The application of modern techniques of immunobiology including monoclonal antibody production, long term growth of lymphoid cells in vitro, and the use of recombinant DNA technology has allowed structural analysis and the preparation of large quantities of these molecules. The first group of cytokines to be characterised was the family of proteins with antiviral properties known as interferons (Wheelock 1965). Historically most lymphokines were named according to the assay in which their activity was first described. Ten years ago, the term interleukin was coined to convey the fact that individual cytokines could have multiple activities. The six cytokines defined as IL-1 to 6 have all been sequenced and cloned, have unique spectra of biological activities and all may have significant clinical applications (Hamblin 1988). To date few studies of lymphokine effects on neutrophil activation have been reported.

1.2.2 LTB_4 and its generation by neutrophils

During 1979, Borgeat & Samuelsson described the

generation of several dihydroxy fatty acids by isolated rabbit polymorphonuclear leucocytes (PMN) following incubation with arachidonic acid (AA) (Borgeat & Samuelsson 1979a). They also demonstrated that human PMN generated similar products when stimulated by the calcium ionophore A23187, in the presence of free AA, (Borgeat & Samuelsson 1979). Purification and structural analysis revealed that the products were 5, 12- and 5, 6-dihydroxy compounds with four double bonds at carbons 6, 8, 10 and 14. One of these products was named leukotriene B_4 (LTB_4) (Samuelsson et al 1978) and structural analysis and total chemical synthesis of the molecule, has demonstrated that LTB_4 is 5(S), 12(R)-dihydroxy-6, 14-cis-8, 10-trans-eicosa-tetraenoic acid (Corey et al 1980).

Arachidonic acid metabolites are not stored preformed in neutrophils, but are generated by de novo synthesis following cell stimulation. Most cellular arachidonate is esterified into phospholipids and triglycerides. Stimuli that cause the synthesis of arachidonate metabolites act by first activating cytoplasmic phospholipase A2, stimulating the release of arachidonate from membrane phospholipids. This is a rate limiting step for both cyclooxygenase and lipoxygenase pathways. The most widely studied non-specific stimulus is the calcium ionophore A23187.

Other, more physiological stimuli such as phagocytic stimuli, using zymosan are also capable of inducing arachidonate release from neutrophils.

There have been surprisingly few studies of arachidonate release from neutrophils via receptor activation such as aggregated IgG. How closely the mechanism of ionophore-stimulated arachidonate release resembles receptor mediated arachidonate release is unknown. Some arachidonic acid is oxidatively metabolised by 5-lipoxygenase to 5-hydroperoxyeicosatetraenoic acid (5-HPETE). This unstable intermediate is converted either to its alcohol, 5-hydroeicosatetraenoic acid (5-HETE) (Borgeat & Samuelsson 1979c) or enzymatically to leukotriene A4 (LTA_4) which has an epoxide ring structure incorporating carbons 5&6. (Borgeat & Samuelsson 1979b; Hammarstrom and Samuelsson 1980; Radmark et al 1980). LTA_4 interacts with an epoxide hydrolase to generate LTB_4 (Borgeat & Samuelsson 1979b), nonenzymatic hydrolysis converts a proportion of the LTA_4 to the biologically inactive 6-trans-LTB_4 diasteroisomers (Borgeat & Samuelsson 1979c). Human neutrophils rapidly metabolize LTB_4 by omega-oxidation leading to the formation of 20-OH-LTB_4 and then to 20-COOH-LTB_4 (Powell 1984; Shak & Goldstein 1984; Jubiz et al 1982). After calcium ionophore stimulation, more than 90% of LTB_4 is released

extracellularly, which reaches a maximum at 8 min of incubation (Williams et al 1985). Stimulation of human neutrophils with opsonized zymosan has been reported to result in the generation and release of omega-oxidation products of LTB_4, with relatively small amounts of native LTB_4 (Walsh et al 1981; Claesson et al 1981; Palmer & Salmon 1983). Polymorphonuclear leukocytes (PMNs) incubated with unopsonized zymosan generated substantial quantities of LTB_4 but retained a large proportion of LTB_4 intracellularly (Williams et al 1985). PMN stimulation with IgG-coated beads results in 60% LTB_4 release (Fitzharris et al 1987). The factors which determine the distribution of LTB_4 intracellularly and extracellularly are still unknown.

Properties of LTB_4 which support a pro-inflammatory role for this lipid autacoid include its ability to promote chemotaxis of PMNs (Ford-Hutchinson et al 1980, 1983; Goetzl & Pickett 1980, 1981; Palmer et al 1980; Smith et al 1980), increased adherence to endothelium both in vitro (Gimbrone & Brock 1984; Hoover et al 1984) and in vivo (Dahlen et al 1981; Lindbom et al 1982), migration of PMNs through vascular endothelium in vitro (Cramer et al 1984; Hopkins et al 1984; Migliorisi et al 1984) and in vivo (Dahlen et al 1981; Lindbom et al 1982; Bjork et al 1982a,b; Lundberg et al 1983; Thureson-Klein et al

1984a; Bray et al 1981a), and increased vascular permeability to macromolecules (Bjork et al 1982a; Lundberg et al 1983; Bray et al 1981a; Durham et al 1984; Thureson-Klein et al 1984b, 1986; Wedmore & Williams 1981). Additional biological consequences of LTB_4 include PMN Ca++ influx and release of Ca++ from cytoplasmic pools (Sha'afi et al 1981; Molski et al 1981), transient elevation of PMN cyclic AMP (Claesson 1982; Feinmark et al 1981), stimulation of phospholipase C-mediated formation of inositol triphosphate in PMN (Bradford & Rubin 1985), as well as PMN release of lysosomal enzymes in the presence or absence of cytochalasin B (Goetzl et al 1980; Hafstrom et al 1981; Rae & Smith 1981; Rollins et al 1983). LTB_4 is also capable of stimulating PMN production of superoxide radicals (Serhan et al 1982), and enhancing PMN cytotoxic capacity (Moqbel et al 1983) and C3b receptor expression on PMNs and eosinophils (Goetzl et al 1980; Nagy et al 1982). In recent years, LTB_4 has been found to exert significant effects on various immunological phenomenona (Rola-Pleszczynski 1985) in that it inhibits proliferation of human peripheral blood mononuclear cells (PBMC) (Rola-Pleszczynski 1985a; Gualde et al 1985), inhibits proliferation of T4+ cells and enhances proliferation of T8+ cells (Payan et al 1984; Gualde et al 1985), stimulates T4+ cells to produce IL-2 (Rola-Pleszczynski 1985) and

IFN-gamma (Rola-Pleszczynski et al 1986), inhibits T cells to produce LIF (Payan & Goetzl 1983), inhibits B cells to synthesize immunoglobulin (Atluru & Goodwin 1984), enhances monocytes to produce IL-1 (Rola-Pleszczynski & Lemaire 1985), induces a radiosensitive OKT8(+) (Atluru & Goodwin 1984), increases the subpopulation of T8+ cells (Rola-Pleszczyski 1985a) and reverses glucocorticoid effects on T cell proliferation and IL-2 production (Goodwin et al 1986).

1.2.3 Interactions between neutrophils and lymphocytes

1.2.3a Effects of cytokines on neutrophils

Activated lymphocytes secrete a number of lymphokines which serve as molecular mediators for the recruitment of other leukocytes in order to amplify immunological and inflammatory responses and these lymphokines may be involved with modulation of neutrophil behaviour. PBMC culture supernatants are chemotactic for PMN (Maestrelli et al 1988), powerfully stimulate antibody-dependent killing of tumour cells by PMN (Vadas et al 1983) and inhibit PMN migration (Rocklin 1974). The known effects of purified cytokines on neutrophil functions are follows (summarized in Table 1.1):-

a. Interleukin 1 (IL-1) is a factor produced by a number of cell types. Cells of the macrophage lineage seem to be the major source of IL-1 (Dinarello 1986). Recent studies suggest that endogenous pyrogen (EP) and IL-1 may be identical or similar molecules, highly purified human leucocyte pyrogen (LP) can induce release of specific granule contents from neutrophils (Klempner et al 1978) and can increase oxygen dependent metabolism of neutrophils (Klempner & Dinarello 1979). Exposure of human neutrophils to IL-1 resulted in an immediate rise in intracellular free calcium, also a trivial amount of LTB_4 was detected in the presence of cytochalasin B and arachidonic acid (Smith et al 1987a).

b. Interleukin-2 (IL-2) was decribed as a T cell growth factor secreted by T lymphocytes (Smith 1984). IL-2 can suppress human neutrophil adherence which was enhanced either by recombinant tumour necrosis factor-beta (TNF-beta) or chemotactic peptide formyl-methionyl-leucyl-phenylalanine (fMLP) (Seow et al 1988).

c. Tumour necrosis factor (TNF). TNF alpha is produced by cells of macrophage-monocyte lineage. It is toxic to certain tumour lines *in vitro* and causes haemorragic necrosis of some neoplasms *in vivo* (Old

1985; Beutler & Cerami 1986). TNF has been shown to augment the phagocytosis and antibody-dependent cytotoxic (ADCC) effector function of PMN (Shalaby et al 1985), augments the migration of PMN across the filter (Ming et al 1987), enhances human PMN adherence to nylon wool (Seow et al 1988), increases surface receptor expression (C3bi), degranulation and respiratory burst activity (Klebanoff et al 1986) and enhances leukotriene biosynthesis in neutrophils stimulated with Ca^{2+} ionophore A23187 (Roubin et al 1987). A molecule with a similar spectrum of biological activity is produced by lymphocytes. It was originally named lymphotoxin but has some homology with TNF-alpha and has therefore been renamed as TNF-beta.

d. Granulocyte/macrophage-colony stimulation Factor (GM-CSF). GM-CSF are a group of T cell-derived products responsible for the proliferation of progenitor cells. GM-CSF is also a stimulator of mature human PMN, which enhances cytotoxic activity against antibody-coated targets (Vadas et al 1983), enhances fMLP-stimulated degranulation of cytochalasin B pre-treated neutrophils and fMLP-stimulated superoxide production, enhances surface receptor expression, PMN survival (Lopez et al 1986; Weisbart et al 1985) and cell to cell adherence (Arnaout et al

1986).

e. IFN-gamma. IFN-gamma can increase in PMN phagocytic ability, PMN mediated antibody dependent cell-mediated cytotoxicity (ADCC) (Shalaby et al 1985) and induce the expression of the high affinity Fc receptor for monomeric IgG1 (Shen et al 1987).

Several purified or partially purified cytokines also affect neutrophil function. Neutrophil migration inhibition factor (NIF-T) produced by peripheral lymphocytes in response to mitogens can inhibit the migration of human neutrophils. The biologic activity of NIF-T can be removed by human peripheral blood neutrophils (Weisbart et al 1982) and is proved to be the same molecule as GM-CSF (Weisbart et al 1985). Neutrophil activating factor (NAF) is a semi-purified monokine, (the final NAF preparation contained 150, 50 and 40 ng/ml of IL-1 alpha, IL-1 beta and TNF, respectively, as measured in specific radioimmunoassays). Exposure of PMN to NAF resulted in extracellular release of granule constituents and LTB_4 in the presence of arachidonic acid and cytochalasin B (Smith et al 1987). Another NAF has recently been sequenced and identified as a novel peptide of 6 kD molecules, which can induce neutrophil exocytosis and respiratory burst (Peveri et al 1988). Leukocyte migration inhibition factor (LIF)

is a T lymphocyte product which inhibits neutrophil migration (Rocklin 1974). LIF has been purified to a homogeniety and has various effects on neutrophils including increased adherence and cytotoxic capacity and enhanced degranulation (Borish & Rocklin 1987a, b). A human monocyte derived neutrophil chemotactic factor (MDNCF) which possessed potent chemotactic activity for neutrophils has been purified and sequenced from lipopolysaccharide (LPS) stimulated human monocytes (Matsushima et al 1988). Also a human lymphocyte derived neutrophil activating peptide (LYNAP) is produced by PHA- or Con A-stimulated human T-lymphocytes. LYNAP has potent chemotactic and enzyme degranulating activity in human neutrophils, and it has recently been sequenced (Gregory et al 1988).

1.2.3b Effects of neutrophil mediators on lymphocytes

Natural killer (NK) cells represent a subpopulation of lymphocytes capable of spontaneous lysis of certain tumour cells, virus-infected cells and possibly normal cells. There is growing evidence that NK cells play an important role in immune surveillance against tumours and certain infections (Herberman & Ortaldo 1981; Talmadge et al 1980). When neutrophils and monocytes are exposed to the tumour-promoting phorbol ester <u>in vitro</u> both are capable of suppressing NK function (Seaman et al

1981). In vivo, the accumulation of phagocytes is temporally associated with decreasing NK activity in tumour tissues and removal of phagocytes restores NK function. Stimulus-dependent secretion of reactive oxygen derivatives by phagocytes appears to be responsible for NK suppression (Niwa et al 1983; Seaman et al 1982).

The effects of cell-free model oxidant systems, MPO-H_2O_2-halide system and H_2O_2 independent of MPO, were also demonstrated to suppress the NK activity and other lymphocyte effector functions (El-Hag & Clark 1987).

LTB_4, which is the major arachidonic acid metabolites released by neutrophils after activation, has also been shown to have immunoregulatory effects. T4 cell proliferation can be inhibited and T8 cell proliferation can be enhanced, also monocyte IL-1 generation can be augmented (Rola-Plezczynski & Lemaire 1985).

1.2.4 The dysfunctions of lymphocytes and neutrophils in allergic diseases and bronchial asthma

1.2.4a Immunoregulatory dysfunctions of lymphocytes (summarized in Table 1.2).

Since the discovery of human IgE in 1966 by Ishizaka et al (Ishizaka et al 1966), it has been generally recognised that common forms of hypersensitivity of immediate type, such as extrinsic

asthma, hay fever, rhinitis, urticaria and occupational allergies (Butcher et al 1976; Maccia et al 1976) are mediated by IgE antibodies. Atopic individuals produce specific IgE antibodies against a wide spectrum of allergens. Specific IgE absorbed by IgE receptors on basophils or mast cells can be crosslinked by allergens (Ishizaka & Ishizaka 1968; Ishizaka et al 1978), leading to degranulation of these cells. Basophils and mast cells release mediators of anaphylaxis, such as histamine, platelet activating factor and leukotrienes. In recent years, it has been increasingly realised that the release of mediators are under the influence of several non-specific factors, among which lymphocyte products such as lymphokines must be counted. Firstly, both basophil promoting activity and interleukin 3 can be shown to regulate the growth and differentiation of basophils and mast cells (Tadokono et al 1983; Razin et al 1981). Secondly, lymphokines are involved in the regulation of IgE synthesis. Several investigations have pointed out that the hyperproduction of IgE is in fact the consequence of a selective lack of suppression (Saxan et al 1980). IL-4 has also been reported to be involved in the regulation and synthesis of IgE (Snapper et al 1988). Thirdly, lymphokines are involved in immediate type hypersensitivity reactions. Supernatants from

cultured human mononuclear cells contain an activity (Histamine releasing factor) that induces within minutes the release of histamine from basophils (Thueson et al 1979a,b) and interferon also can induce an enhancement of IgE-mediated histamine release from human basophils (Hernandez-Asensio et al 1979).

Functional abnormalities of lymphocytes such as a defect in concanavalin A-induced T-suppressor cell function have been described in asthma (Harper et al 1980; Rola-Pleszczynski & Blanchard 1981; Rivlin et al 1981; Hwang et al 1985; Ilfeld et al 1985) and successful immunotherapy has been associated with both an increase in the relative number of T-suppressor cells (Rocklin et al 1980) and an abrogation of the allergen-induced late-phase response (Warner 1976). This suggests that allergic reactions may be associated, at least in part, with abnormal regulation of cell-mediated immunity.

There have been several reports describing allergen-induced proliferation of peripheral blood mononuclear cells (PBMC) from allergic patients (Girard et al 1967; Brostoff et al 1969; Maini et al 1971; Romagnani et al 1973; Gatien et al 1975; Buckley et al 1977; Black & Marsh 1980), and also lymphokine generation by antigen-stimulated PBMC from sensitized donors (Ilonen & Salmi 1982; Rawle et al 1984; Maini et al 1971). However the precise role of

T-lymphocytes and various lymphokines in the pathogenesis of allergic reactions remains undetermined.

1.2.4b Dysfunctions of neutrophils (summarized in Table 1.3).

Neutrophils are a feature of the pathology of asthma (Dunnill 1960) and allergic reactions (Zweiman et al 1976) and in animal models antigen-induced bronchial hyperresponsiveness was associated with neutrophil infiltration (Chung et al 1985). In the rabbit supernatants derived from phagocytosing neutrophils can enhance airway reactivity (Irvin et al 1985), and in the dog model, granulocyte depletion prevented airway hyperresponsiveness induced by exposure to ozone (O'Byrne et al 1984). LTB_4 is the main arachidonic acid metabolite generated by neutrophils which has been found in the sputum of bronchial asthmatic patients (O'Driscoll et al 1984) and in the nasal lavage fluid after nasal challenge with specific allergen (Shaw et al 1985a). Peripheral blood neutrophils from asthmatic subjects have been found to have an increased capacity to generate sulphido-peptide leukotrienes, SRS-A, under calcium ionophore stimulation (Wang et al 1986) and LTB_4 after exercise challenge (Arm et al 1987). They also possess higher 5-lipoxygenase activity (Mita et al 1985). Thus increased LTB_4 generation by neutrophils

might play a role in the augmentation of asthma and allergic inflammation. The recent review about the pathogenesis of asthma suggesting that airway inflammation may be related to bronchial hyperresponsiveness, however the precise stimulus which calls in neutrophils and eosinophils with allergen provocaton is at present not known (Holgate & Finnerty 1988).

1.3 Unresolved issues of the interrelationship between neutrophils and lymphocytes

There are several unresolved questions regarding the relationship between cell mediated immunity and inflammatory cells, although there is circumstantial evidence that lymphocytes and neutrophils might interact with each other as they are often both present in the pathology of chronic inflammation.

1. How do lymphocytes and neutrophils interact, what is the signal, where does the signal come from?
2. Do lymphokines prime neutrophils to generate more LTB_4, is it a general phenomenon or a lymphokine specific reaction?
3. Are leukotriene release enhancing lymphokines generated from lymphocytes after specific allergen stimulation?
4. LTB_4 is a potent pro-inflammatory mediator able to recruit and activate neutrophils, yet also acts as a immunoregulator to stimulate lymphocytes. Is this a

conflict or a synergistic phenomenon?

1.4 Aims of this study

Several lymphokines have been reported to have effects on neutrophils, and LTB_4, one of the principal mediators released by stimulated neutrophils may contribute to the recruitment of neutrophils and cell activation in inflammatory reactions.

The LTB_4 generation by neutrophils in response to a physiologically relevant stimulus *in vitro*, (stimulation using IgG beads), and the effects of lymphokines on the response should provide some insight into the mechanisms by which lymphocytes might modulate the inflammatory response.

Thus the aims of this study were:

1. To attempt to establish whether T-lymphocyte products activate neutrophils in terms of increased LTB_4 production, especially under physiological stimulation.
2. To characterise the lymphokine-leukotriene release enhancing factor by comparing and contrasting it with various human recombinant cytokines.
3. To attempt to identify and generate this lymphokine by allergen-stimulated peripheral mononuclear cell cultures.
4. To ascertain the lymphocyte (or monocyte) origin of leukotriene release enhancing factor.

5. To examine its range of biological activities on neutrophils.

6. To study the effects of mediators and drugs on the release of leukotriene release enhancing factor from mononuclear cells.

Table 1.1

Cytokine effects on neutrophil functions

Designation	Physicochemical characterization	Effects on neutrophils
LIF	MW: 58 kD pI 5-5.5 cell sources: T lymphocytes	Inhibit random migration (Rocklin 1974) Potentiate phagocytosis of opsonized and unopsonized particles (Borish & Rocklin 1987a) Enhance adherence to target cells and potentiate ADCC against erythroleukemic line K562 (Borish & Rocklin 1987b) Induce degranulation and potentiate neutrophil response to fMLP (Borish et al 1986) Increase intracellular calcium (Borish et al 1987)
GM-CSF	MW: 22 kD pI 4.5-4.7 cell source: T lymphocytes	Inhibit migration (Gasson et al 1984) Enhance growth of granulocyte from human bone marrow (Souza et al 1986) Enhance superoxide anion production in response to fMLP and modulate fMLP receptor (Weisbart et al 1985) Enhance ADCC against tumour cell line (Vadas et al 1983) Enhance granulocyte survival and cell surface marker expression (GFA-1, GFA-2, Mo1) (Lopez et al 1986) Enhance granulocyte-granulocyte adhesion and expression of leukocyte adhesion molecules (Mo1, P150.95) (Arnaout et al 1986)
IL-1	MW: 12-18 kD pI 6.5-7.5 cell source: monocytes	Induce release of specific granule contents (Klempner et al 1978) Stimulate oxygen-dependent metabolism (Klempner & Dinarello 1979) Increase cytosolic free calcium and induce production of LTB_4 which was enhanced in the presence of arachidonic acid (Smith et al 1987)
TNF	MW: 17 kD pI 4.8-5.0 cell source: monocytes	Chemotactic for neutrophils (Ming et al 1987) A weak stimulus of respiratory burst and degranulation (Klebanoff et al 1986) Enhance adherence to decron fibre (Seow et al 1988) Increase phagocytic ability against latex beads (Shalaby et al 1985) Enhances LTB_4 biosynthesis stimulated with Ca^{+2} ionophore A23187 (Roubin et al 1987)

Cytokine effects on neutrophil functions (cont'd)

Designation	Physicochemical characterization	Effects on neutrophils
IFN-gamma	MW: 35-70 kD cell source: T lymphocytes	Enhance ADCC against erythrocytes and tumour targets (Hokland & Berg 1981) Enhance ADCC through the high affinity Fc receptor for monomeric IgG (Shen et al 1987) Enhance phagocytosis against latex beads (Shalaby et al 1985)
MDNCF	MW: 10 kD pI 8-8.5 cell source: monocytes	Chemotactic for neutrophil (Yoshimura et al 1987) Induction of MDNCF mRNA by IL-1 and TNF (Matsushima et al 1988)
Monokines	MW: 150, 50, 20, <5 kD pI 4.2-4.3 4.5-4.6 4.9 cell source: monocytes	Enhances LTB_4 generation stimulated with Ca^{+2} ionophore A23187 (Dessein et al 1986)
LYNAP	MW: 10 kD cell source: T lymphocytes	Chemotactic and enzyme degranulating activity in human neutrophils (Gregory et al 1988)
NAF	MW: 6 kD cell source: monocytes	Induce exocytosis and respiratory burst (Peveri et al 1988)

Table 1.2

Mononuclear cell dysfunctions in patients with respiratory allergy

Abnormality	References
Increased proliferation to specific allergen	Buckley et al 1977
Release MIF and IL-2 after allergen stimulation	Ilonen & Salmi 1982 Maini et al 1971
IL-2 production reduced after immunotherapy	Hsieh 1985
T lymphocytes	
T helper cell predominantly proliferated in response to p1 antigen	Rawle et al 1984
T_4 cell proliferation response to mite was decreased after immunotherapy (T_8 cell was increased after immunotherapy)	Hsieh 1984
Decreased T gamma cells and reached normal after immunotherapy	Canonica et al 1979
Activated T cells in circulating during acute asthma	Corrigan et al 1988
Activated T cells increased in circulation after antigen challenge	Gerblich et al 1984
Increase T_8 cells into the lung after allergen induced single early reaction	Gonzalez et al 1987
Suppressor T cell	
Decreased generation of histamine-induced T suppressor cells	Rocklin 1976
Decreased suppression of spontaneous IgE production	Saxan et al 1980
Increase antigen specific suppressor T cell after immunotherapy	Nagaya 1985 Tamir et al 1987

Table 1.3

Neutrophil dysfunctions in patients with respiratory allergy

Dysfunction	References
Decreased intracellular enzyme activity (NBT reduction)	Faulkner et al 1982
Responded more vigorously to casein	Gin et al 1985
Increased 5-lipoxygenase activity	Mita et al 1985
Increased LTB_4 generation	Arm et al 1987
Increased SRS-A generation	Wang et al 1986
Increased complement receptor expression after allergen challenge	Carroll et al 1985
Increase cytotoxicity against complement coated targets after allergen challenge	Moqbel et al 1986
Increased infiltration into airway after allergen-induced late phase reaction	Collins et al 1986

CHAPTER 2.

GENERAL MATERIALS AND METHODS

2.1 General Materials:

2.1.1 Cell preparation media and biochemicals

RPMI-1640 with 20 mM HEPES and 4 mM L-glutamine and Hank's balanced salt solution (Gibco Ltd., Paisley, Scotland)

Penicillin and streptomycin (Flow Lab., Irvine, U.K.)

Phytohaemoagglutin, phenyl methyl sulphonyl fluoride (PMSF), Sodium dodecyl sulphate (SDS). Immunoglobulin IgG, N-formyl-methionyl-leucyl-phenylalanine (fMLP) and Calcium ionophore A23187 (Sigma Chemical Co., Poole, Dorset, U.K.)

OKT-3 (Ortho Pharmaceutical Co., Raritan, N.J., U.S.A.)

Anti-Leu-3a, Anti-Leu-1, Anti-Leu-2a, Anti-CR1 and Anti-CR3 (Becton Dickinson, Sunnyvale Inc., CA., U.S.A.)

LTB_4, LTC_4, LTD_4 and LTE_4 (ICN ImmunoBiologicals, Lisle, U.K.)

14,15-^{3}H-LTB_4 and 14,15-^{3}H-LTC_4, Tritiated thymidine (^{3}H-TdR) (New England, Nuclear/Du Pont, Stevenage, U.K.)

Lymphocult T (Biotex Folex, Frankfurt, FRG)

Ficoll-Paque (Pharmacia, Uppsala, Sweden)

Dextran, Dextran 110 (Fisons, Loughborough, U.K.)

Metrizamide (Nygaard, U.K. Ltd, Birmingham, U.K.)

Bolton and Hunter reagent (Amersham International Plc, Amersham, Bucks).

2.1.2 Buffer solutions

All the chemicals used for buffer solutions were purchased from BDH, Chemical Ltd., Poole, England; except for the following:-

bis-tris from Sigma Chemical Ltd., St. Louis, MO, U.S.A.

Methanol (HPLC grade), Acetic acid (Sequencer grade), and Water (HPLC grade) from Rathburn Chemical Co., Walkerburn, Scotland.

Optiphase 'RIA' from LKB, Fison plc, Loughborough, England.

Protein determination Kit and Silver stain reagents from Bio-Rad, Watford, Herts, U.K.

- Buffer for lysis of red cells (lysis buffer)

  NH_4Cl 8.2 gm and $KHCO_3$ 1.0 gm dissolved in 1 litre distilled water and adjusted to pH 7.2-7.4.

- Buffer for IgG beads preparation

  a) Coupling buffer (0.2M $NaHCO_3$ containing 0.5M NaCl pH 8.7): $NaHCO_3$ 16.8 gm and NaCl 29.2 gm dissolved in 1 litre H_2O, adjusted to pH 8.7.

  b) Blocking buffer (0.1M ethanolamine pH 8.0): 6.1 ml ethanolamine diluted to 50 ml with distilled water, adjusted to pH 8.0 with 6M HCl and made up to 100 ml with coupling buffer.

  c) Acetate buffer (0.1M sodium acetate containing 0.5M NaCl pH 4.0): 13.6 gm sodium acetate

(trihydrate) and 29.2 gm NaCl dissolved in 950 ml distilled water, adjust to pH 4.0 with glacial acetic acid, make up to 1 litre.

- Buffer for gel filtration

  Phosphate buffer saline (PBS)

  PBS was prepared by dissolving 6.8 gm NaCl, 1.16 gm Na_2HPO_4 and 0.43 gm KH_2PO_4 in 1 litre distilled water and adjusting to pH 7.2-7.3.

- Buffer for anion-exchange (Mono-Q column) chromatography

  a) Bis-tris buffer (starting buffer)

     20 mM bis-tris was prepared by dissolving 4.18 gm bis-tris in 1 litre distilled water and adjusted to pH 6.5.

  b) Bis-tris containing 1M NaCl buffer (eluting buffer). Buffer was prepared by dissolving 11.68 gm bis-tris and 0.84 gm NaCl in 200 ml distilled water and adjusted to pH 6.5.

- Buffer for chromatofocusing (Mono-P column)

  a) Bis-tris buffer (starting buffer)

     25 mM bis-tris was prepared by dissolving 5.25 gm bis-tris in distilled water, and adjusted to pH 7.1 with saturated iminodiacetic acid and making up to 1 litre.

  b) Polybuffer 74 (eluting buffer)

     10 ml polybuffer 74 (Pharmacia, Milton Keynes, U.K.) diluted with distilled water and

adjusted to pH 4.0 with saturated iminodiacetic acid then made up to 100 ml.

- Buffer for leukotriene assay

a) PBS buffer

PBS buffer was prepared by mixing up:-

2.8M NaCl	2.5 ml
1.0M Phosphate pH 7.1	1.0 ml
i.e. 1M KH_2PO_4	30 ml
1M Na_2HPO_4	70 ml
2% sodium azide	1 ml
2% sodium azide	0.5 ml
100 mM PMSF (in IMS)	0.1 ml

make up to 50 ml with distilled water.

b) PBS-gel buffer

PBS-gel was prepared by mixing up:-

2.8M NaCl	5 ml
1.0M phosphate pH 7.1	2 ml
2% sodium azide	1 ml
100 mM PMSF (in IMS)	0.1 ml
1.25% gelatin (in water)	20 ml

make up to 100 ml with distilled water.

c) Polyethylene glycol (PEG) buffer

5% PEG buffer was prepared by taking 2.5 gm of PEG 6000, dissolving with 50 ml of PBS-gel buffer.

- Buffer for HPLC

a) Eluting buffer was prepared with HPLC grade methoanol: water: acetic acid: orthophosphoric acid (70:30:0.07:0.03) then degassed and adjusted to pH 5.4 with ammonia.

b) Washing buffer was prepared with methanol: water: acetic acid: orthophosphoric acid (95:4:9:0.07:0.03).

- Buffer for SDS-PAGE

SDS-PAGE buffer was prepared by mixing up:-

50 mM Tris/HCl	(0.651 gm/100 ml)	8 ml
5 mM EDTA	(186 mg/100 ml)	8 ml
10% SDS	(2 gm/20 ml)	10 ml
distilled water		14 ml

5% β-mercaptoethanol was added during reducing conditions

2.1.3 Instruments:

Nucleosil 5u C-18 HPLC column: Macherey-Nagal, Duren, W. Germany.

High performance liquid chromatography (HPLC) pump: Jones Chromatography, Glamorgan, U.K.)

Fast protein liquid chromatography (FPLC) system, chromatofocusing Mono-P column, Anion exchange chromatography Mono-Q column, Superose-12 preparative grade gel for gel filtration column and Phast gel electrophoresis system were from Pharmacia, Milton Keynes, Bucks, U.K.

Speed Vac concentrator: Savant Instruments Inc., Hicksville, N.Y., U.S.A.)

Cell harvester: Titertek cell harvester, Skatron, Lier, Norway.

Orbital shaker, Rock and Roll mixer: Luckhams, Burgess Hill, U.K.

Beta-counter (LKB 1217) and Gamma-counter (LKB 80,000)(LKB, Wallac., Finland).

Amicon ultrafiltration cell, Model 8050 (Amicon Corp. Danver, Mass, U.S.A.)

Laminar flow hood (Class II) (MAT, Canto House, Manchester, U.K.)

Carbon dioxide incubator (Gallenkamp, Loughborough, Leicestershire, U.K.)

Coolspin (MSE Coolspin 2 Centrifuge, Sussex, England)

Microcentrifuge (Microfuge 12, Beckman, High Wycombe, U.K.)

2.2 General Methods:

2.2.1 Peripheral blood mononuclear cell (PBMC) preparation

Human peripheral venous blood from normal healthy donors was mixed with 10 unit/ml of heparin, and diluted 1:1 with RPMI-1640 containing 25 mM HEPES and 4 mM L-glutamine. Thirty millilitres of diluted blood were layered over 15 ml of Ficoll-Paque in 50 ml polypropylene tubes (Falcon 2070), and centrifuged at 1000 g for 25 min at 20°C. The interface cells were

collected, pooled and washed three times with RPMI-1640 at 250 g for 10 min at 20°C. The final PBMC pellet was resuspended in RPMI-1640 tissue culture medium buffered with 25 mM HEPES containing 4 mM L-glutamine, 100 units/ml penicillin and 100 ug/ml streptomycin. Cells were used only when the viability was greater than 95% by trypan blue dye exclusion.

2.2.2 Neutrophil preparation

Heparinized (10 U/ml) peripheral venous blood from normal healthy donors was mixed with one part dextran to five parts blood and sedimented at 37°C for 30 min. Leucocyte rich supernatant was layered over Ficoll-Paque and centrifuged at 1000 g for 20 min. After removal of the cells from the interface, the pellet cells were lysed with lysis buffer at 4°C. Neutrophils were washed twice in RPMI-1640 at 4°C, and counted in a modified haemocytometer using Kimura stain (Kimura et al 1973). Only cell suspensions containing >95% neutrophils were used for experiments.

2.2.3 Cell proliferation assay

Proliferation was assessed by incubating 100 microlitre of $2x10^5$ PBMC in triplicate 96-well round-bottomed microtitre plates (Flow Laboratories, Rickmansworth, Herts, U.K.). Tritiated thymidine (^{3}H-TdR) (0.66 uCi/well) was added during the final 6 hr of incubation. The cells were collected on GF/C, glass fibre paper (Ilacon Ltd., Tonbridge, U.K.) using

a multi-harvester apparatus. Radioactivity was determined in a liquid scintillation beta-counter.

2.2.4 Preparation of IgG-coated beads

These were prepared as described by Shaw et al (1985). Briefly, cyanogen bromide-activated Sepharose 4B beads were swollen in 1 mM HCl, mixed with IgG, usually 7.5 mg per 1 ml of gel in bicarbonate coupling buffer, and incubated overnight on a rock and roll mixer (4°C; pH 8.7). Beads were then incubated with 1M ethanolamine (pH 8) for a further 2 hr (20°C) to block any remaining reactive groups on the Sepharose. The beads were washed with acetate buffer pH 4.0 and bicarbonate buffer pH 8.7 alternately, to remove any non-covalently bound protein. This procedure resulted in preparations containing approximately 1.5 mg IgG/10^6 beads, as determined spectrophotometrically after alkaline (1M NaOH) digestion of the coated beads.

2.2.5 Radioimmunoassay of LTB_4 and LTC_4

Samples were assayed in duplicate with a double antibody radioimmunoassay using an antiserum, raised in rabbits, kindly supplied by Dr A.W. Ford-Hutchinson (Merck, Sharp & Dohme, Rahway, N.J.). The assay was performed as described by Cromwell et al (1985). Briefly, all dilutions were made in phosphate buffered saline pH 7.2 containing 0.25% gelatin and the protease inhibitor phenylmethyl sulphonyl fluoride

(PMSF) $1x10^{-4}$M. Dilutions of standard were made in the range 0.1-7.0 nM for LTC_4 and 0.4-20.0nM for LTB_4. Supernatants were diluted 1:5 for LTB_4 assay and 1:5 and 1:25 for LTC_4 assay. Samples, standards in buffer (100 ul) were mixed with 50 ul of a dilution of antiserum and then 50 ul of tracers [tracers were made up by mixing 2 ul rabbit immunoglobulin (20 mg/ml) together with 0.4 ul 14, 15-^{3}H-LTB_4 or 14,15-^{3}H-LTC_4 (approximately 0.9 and 0.7 p moles respectively) diluted to 50 ul]. Tubes were incubated at 4°C for 16 hr on an orbital shaker prior to the addition of 400 ul of a 1 in 4 dilution of goat anti-rabbit immunoglobulin in buffer containing 5% polyethylene glycol 6000. Tubes were incubated on ice for 24 hr, centrifuged at 2000 g for 15 min at 4°C, and 300 ul of the supernatant was removed and transferred to scintillation vials together with 3 ml of Optiphase scintillation fluid (LKB-Pharmacia, Milton Keynes, Bucks, U.K.). Samples were "counted" on a beta-counter, and a standard curve was constructed with a computerized fit using a smoothed spine method. LTB_4 cross-reactivity in this assay were as follows:- 5(S), 12(R) LTB_4 (100%); 5(S), 12(R) 6-trans LTB_4 (12%); 20-hydroxy LTB_4 (2%); 5(S), 12(S) 6-trans LTB_4 (1.5%) and other arachidonic acid metabolites showed <0.4% cross-reactivity. LTC_4 cross reactivity were as follows:- LTD_4 (70%); LTE_4 (8%); 5(S), 6(R) di-HETE

(0.6%) and other arachidonic acid metabolites showed <0.1% cross reactivity.

2.2.6 Extraction of LTB_4 with C_{18} Sep-Pak

Sep-paks (Waters Associates, Northwich, Cheshire) were pretreated by sequential washing with tetrahydrofuran (10 ml), methanol (10 ml) and 10% methanol pH 4.0 (10 ml). Samples diluted ten fold with 11.1% methanol and adjusted to pH 4.0 with acetic acid were loaded, and the Sep-pak washed with 10% methanol (20 ml) prior to elution with methanol (3 ml). After elution, samples were dried by Speed-Vac concentrator 200 H and kept at -80°C until assayed.

2.2.7 Reverse phase HPLC

Samples from C_{18} Sep-pak columns were resuspended in HPLC running buffer and separared on a 5 ug Nucleosil C_{18} column (Macherey-Nagal, Duren, W. Germany) eluted at 1.0 ml/min with running buffer [methanol:water:acetic acid:orthophosphoric acid (70:29:0.07:0.03), pH 5.4] for 30 min followed by 20 min gradient to washing buffer [methanol water: orthophosphoric acid: acetic acid (95:4.9:0.07:0.03)]. Each HPLC fraction was dried on the Speed-Vac concentrator and reconstituted for assay of LTB_4 immuno-reactivity.

2.2.8 Statistics

A paired-t test was used to compare LTB_4

production with control values. Logarithmic values were employed where appropriate to normalise the data.

The data is expressed as the percentage enhancement of LTB_4 generation by neutrophils following IgG-dependent stimulation and was calculated by the following formula:

$$\frac{LTB_4 \text{ experimental} - LTB_4 \text{ control}}{LTB_4 \text{ control}} \times 100\%$$

where LTB_4 experimental is the concentration of LTB_4 contained in the supernatants from IgG-stimulated neutrophils pretreated with PBMC supernatants. LTB_4 control refers to the concentration of LTB_4 contained in the supernatant from IgG-coated beads activated neutrophils pretreated with medium alone.

CHAPTER 3.

LEUKOTRIENE RELEASE ENHANCING FACTOR (LREF) PRODUCTION AND DIFFERENTIATION FROM OTHER CYTOKINES

3.1 Introduction

The effect of cytokines on neutrophil functional activity has been extensively studied (for a review see chapter 1). Attention has been focused mainly on the biological activity of neutrophils, whereas very little is known about the effect of cytokines on neutrophil LTB_4 generation. Among the lymphokines that act on neutrophils, the best studies are leukocyte migration inhibitory factor (LIF) and chemotactic factor (CF). Both are actively secreted by lymphocytes in response to antigen or non-specific mitogen (Rocklin 1974; Yoshida et al 1976). Neither LIF nor CF have been investigated for their effect upon LTB_4 generation from neutrophils, but GM-CSF and TNF have been shown to enhance leukotriene generation from granulocytes in response to the calcium ionophore A23187 (Roubin et al 1987; Owen et al 1987).

Generation of LTB_4 under calcium ionophore stimulation suggests a theoretical means by which neutrophils could contribute to the amplification of inflammatory reactions (Palmer & Salmon 1983), however the physiological relevance of this remains uncertain, because of the artificial nature of the stimulus. Other more physiological agents, e.g. opsonized and unopsonized zymosan, have been used to activate neutrophils to generate LTB_4 (Walsh et al 1981; Claesson et al 1981; Clancy et al

1983). Another relevant physiological stimulant, unphagocytosable IgG-coated Sepharose beads, has been reported to initiate LTB_4 generation from neutrophils, and in this system the capacity to generate LTB_4 was augmented by a bacterial product fMLP (Fitzharris et al 1987). Since many cytokines have been reported to modulate neutrophil functions, it is of interest to evaluate the effect of cytokine on LTB_4 generation using this IgG-bead stimulation system. Using this model, we could assess whether interactions between lymphocytes and neutrophils in the inflammatory reaction, such as occur when antigen has been recognized by specific IgG antibodies, can contribute to the activation of neutrophils and the generation of inflammatory mediators.

Phytohaemagglutinin (PHA) stimulated human peripheral blood mononuclear cell (PBMC) culture is a widely used method for lymphokine generation (for a review see chapter 1). In this study, I established the mononuclear cell culture conditions, and evaluated the effects of supernatants on the capacity of neutrophils to generate LTB_4 using IgG-coated Sepharose beads as the trigger. I have also compared the activity of PBMC supernatants with some well characterized recombinant cytokines including IL-1-beta, IL-2, IFN-gamma, GM-CSF and TNF.

3.2 MATERIALS AND METHODS

3.2.1 Peripheral blood mononuclear cell (PBMC) cultures

Human peripheral blood mononuclear cell (PBMC) after separation were resuspended at a concentration of $2x10^6$ cells/ml. One millilitre of cell suspension in 12x75 mm tissue culture tubes was incubated with different concentrations of PHA for varying lengths of time in a 5% CO_2 atmosphere at 37°C and 95% humidity. After incubation the cell suspensions were centrifuged at 400 g for 10 min at 4°C, and the sterile cell-free supernatants were aliquoted and stored at -80°C until tested. Cell viability, as assessed by trypan blue dye exclusion was >90%. Controls included a) medium alone, b) cell supernatants incubated in the absence of PHA.

3.2.2 Stimulation of neutrophils

Neutrophils ($1x10^6$) were suspended in 0.25 ml RPMI-1640, supplemented with calcium and magnesium chloride to a final concentration of 0.6mM and were incubated with equal volumes of PBMC supernatants for various periods of time at 37°C. IgG-coated beads ($1x10^6$ in 0.5 ml RPMI-1640) were added and incubated for 15 min, without agitation. The incubation was terminated by centrifugation at 400 g for 10 min at 4°C. The cell free supernatants were removed and stored at -80°C prior to assay. The cell/bead pellet

was extracted with 500 ul of 100% methanol for 16 hr at 4°C. The cell extracts were removed and evaporated to dryness in a Speed Vac concentrator and were resuspended in PBS prior to radioimmunoassay. Five recombinant proteins, including rIL-1 beta (5-40 U/ml), rIL-2 (1-1000 U/ml), rIFN-gamma (1-1000 U/ml), rTNF (1-1000 U/ml) and rGM-CSF (0.2-4 ng/ml) were examined to assess their influence on LTB_4 generation by neutrophils in the same system.

The dose response to IgG beads was also evaluated. After incubation with PBMC supernatants neutrophils were incubated with different numbers of IgG beads for 15 min. The cell free supernatants were assayed for LTB_4.

3.2.3 Preparation and stimulation of eosinophils

Eosinophils were obtained from patients attending the Brompton Hospital's Allergy Clinic and from laboratory staff. These individuals were suffering from allergic rhinitis, and some had extrinsic asthma. All had a peripheral blood eosinophilia of greater than 10% and were not taking any systemic medication. Peripheral blood was collected into tubes containing preservative-free heparin (10 U/ml) and mixed with 0.2 volumes of 6% dextran 110 at room temperature. The leucocyte rich plasma layer was removed after 60 min, the cells were washed twice in RPMI-1640, recovered by centrifugation at 200 g for 8 min, and 1 ml aliquots,

each containing the cells from 10 ml blood, were layered onto discontinuous metrizamide gradients in 15 ml conical tubes. The gradient consisted of 2 ml aliquots of 25, 23, 22, 21, 20 and 18% w/v metrizamide in Tyrodes/gelatin buffer (Vadas et al 1979). Tubes were centrifuged at 1200 g for 45 min at 20°C, and the eosinophils were recovered from the 23/25% interface. The cells were washed twice in RPMI-1640 and reconstituted to $4x10^6$cells/ml. Aliquots of this suspension (250 ul) were pre-treated with an equal volume of LREF (active fraction recovered from gel filtration), or buffer (buffer fraction) then challenged with 500 ul of IgG-beads for 45 min at 37°C without agitation.

The cell free supernatants were collected by the centrifugation (400 g, 10 min, 4°C) and stored at -80°C prior to LTC_4 assay.

3.2.4 Neutralization effect of anti-GM-CSF antibody on the activity of PBMC supernatants

PBMC supernatants or GM-CSF (4 ng/ml) were mixed with different dilutions of anti-GM-CSF antibody (the kind gift of Dr Sue Watt, Institute of Cancer Research, London, UK) for 60 min at 4°C. After neutralization the supernatants or GM-CSF were tested for their effect on LTB_4 generation from neutrophils (as described in 3.2.2).

3.2.5 Stability of LREA in PBMC supernatants

PBMC supernatants were divided into three equal portions to test for acid and heat stability and control treatment. For stability to acid pH, 1M HCl was added to supernatants until pH reached 2. After 5 min, the pH was adjusted back to pH 7.4 by the addition of 1M NaOH. For heat inactivation, supernatants were incubated in a 56°C waterbath for 60 min. The untreated and heat inactivated samples were diluted to the same volume as acid inactivation samples. The samples were stored in -80°C until tested.

3.2.6 Physicochemical characterization

3.2.6a Gel filtration

Gel filtration chromatography was performed on HR 16/50 columns of Superose 12 prep grade in a fast protein liquid chromatography system (FPLC) (Pharmacia). Two millilitres of a ten-fold concentrated PBMC supernatant (Speed Vac Concentrator 200H, Savant Instruments Inc., Hicksville, N.Y., U.S.A.) were applied to the column previously equilibriated with phosphate-buffered saline (PBS). Chromatography was performed at 20°C with a flow rate of 0.5 ml/min and 1 ml fractions were collected. The column was calibrated with molecular weight markers (Blue Dextran 2000 kD, thyroglobulin 669 kD, aldolase 158 kD, bovine serum albumin 67 kD, ovalbumin 43 kD,

chymotrypsinogen 25 kD and ribonuclease A 13.7 kD (Pharmacia). Each FPLC fraction was tested for neutrophil LTB_4 release enhancing activity.

3.2.6b Chromatofocusing

Three millilitres of the peak of neutrophil LTB_4 release enhancing activity obtained from Superose 12 were concentrated to 1 ml and dialysed for 16 hr at 4°C against 0.025M bis-tris-iminodiacetic acid buffer, pH 7.1, and applied to a Mono-P HR 5/20 column (Pharmacia) which had previously been equilibriated with the same buffer. A 10% solution of polybuffer 74-iminodiacetic acid, pH 4, was used for elution at a flow rate of 0.5 ml/min. A linear pH-gradient of 40 ml from pH 7 to 4 was generated and 1 ml fraction were collected. The fractions were dialysed and subsequently tested for neutrophil LTB_4 release enhancing activity. The column was regenerated with 2M iminodiacetic acid disodium salt and reequilibriated with starting buffer before each experiment.

3.2.7 IL-2 bioassay

IL-2 activity in FPLC fractions was assayed in terms of their ability to support the growth of a human IL-2 dependent T-cell line (Gillis et al 1978). For bioassay, $2x10^4$ cells were washed and resuspended to 100 ul RPMI-1640 culture medium, 50 ul of PBS containing 1 mg/ml BSA and 50 ul of test sample.

After 24 hr incubation, cultures were pulsed with 0.66 uCi (24.4 KBq) of ^{3}H-TdR in 10 ul. After a further 16-18 hr incubation the well contents were harvested onto glass fibre paper using a multi-harvester and the incorporated radioactivity determined by liquid scintillation counting.

In order to determine units of IL-2, rIL-2 was tested at serial two-fold dilutions starting at 1 U/ml. The results generated in this bioassay were evaluated by a logarithmic plot of ^{3}H-TdR uptake (counts per minute) against the logarithmic dilution of IL-2. The IL-2 titre in test samples was calculated. Culture medium and 5 ug/ml of PHA were incubated in the assay as controls. Experiments were performed in triplicate.

3.2.8 IFN-gamma radioimmunoassay

IFN-gamma was measured with SUCROSEP(R) IFN-gamma immunoradiometric assay (Boots-Celltech Diagnostics Ltd., Slough, U.K.). Samples and dilutions of IFN-gamma standard, and the appropriate controls were incubated with an ^{125}I-labelled anti-IFN-gamma monoclonal antibody. The monoclonal antibody/ IFN-gamma immune complex was immobilised by incubation with a sheep anti-IFN-gamma antibody coupled to solid phase. The separation of bound from unbound labelled monoclonal antibody was obtained by a sucrose layering system (SUCROSEP(R)). The radioactivity in the assay

tubes was counted using a gamma-scintillation counter. A standard curve was constructed plotting log ^{125}I counts versus log IFN-gamma standard concentrations. IFN-gamma concentrations in known samples were interplotted. The assay was performed in triplicate.

3.3 Results

3.3.1 The production of leukotriene release enhancing activity of neutrophils (LREA).

Supernatants obtained from PHA-stimulated human PBMC cultures contained an activity which, when added to neutrophils (60 min, 37°C), enhanced LTB_4 generation elicited by IgG-coated Sepharose beads (Fig 1). Maximal effect was obtained in cultures stimulated with 5 ug/ml PHA, and maximal enhancement was observed with a 50% dilution of PHA-stimulated PBMC supernatants. The activity decreased in a concentration-dependent fashion when 20% and 10% dilutions of the supernatant were tested. PHA (5 ug/ml) alone did not enhance neutrophil LTB_4 generation.

Time course studies indicated that the activity was present in both 6 hr and 12 hr cultures (Fig 2), but significant levels of activity were only seen in 48 hr and 72 hr cultures ($p<0.01$). Cell proliferation measured by ^{3}H-TdR incorporation, was also significantly increased after 48 hr in PHA-stimulated cultures when compared with 24 hr cultures and controls ($p<0.05$).

3.3.2 Time course of the effect of pre-incubation of neutrophils with PBMC supernatants on LTB_4 generation.

When 1:2 dilutions of PHA-stimulated PBMC supernatants were incubated with neutrophils prior to stimulation with IgG-coated beads, LTB_4-enhancing effect was observed after only 5 min (Fig 3). Maximal effect occurred at 60 min, and decreased thereafter. Unstimulated PBMC supernatant produced slight activity after 10 min pre-incubation. This was sustained until 120 min, but was relatively weak and not significantly different from control. Neutrophils pre-treated for 60 min with stimulated PBMC supernatants showed an average enhancing activity of 250% when compared with supernatants from unstimulated PBMC supernatants (Fig 3.3).

Insignificant enhancement of LTB_4 generation occurred following incubation of neutrophils with buffer or PHA (5 ug/ml) over the same time course.

Ten different PBMC supernatants (obtained from 10 different donors) were tested for their leukotriene release enhancing activity against neutrophils from the same donor. The amount of leukotriene release enhancing activity was variable between PBMC donors ranging from 150%-350% enhancement of LTB_4 generation (Fig 3.4a). Intracellular LTB_4 was also evaluated, and that too was found to be significantly increased

($p<0.05$) (Fig 3.4b).

3.3.3 Generation of LTB_4 by LREA-treated neutrophils in response to stimulation with IgG-coated Sepharose beads

The effects of altering the numbers of IgG-coated beads added to $1x10^6$ pre-treated neutrophils (pre-incubated with PBMC supernatants) are shown in Fig 3.5. There was a dose-dependent increase of LTB_4 released with increasing numbers of beads. LTB_4 concentrations in pre-treated neutrophil supernatants without beads or in PBMC supernatants were negligible (less than 1 nM). Pre-treatment of neutrophils with fMLP ($5x10^{-8}M$) for 60 min also showed an increased LTB_4 release.

The identity of LTB_4 immunoreactivity generated by neutrophils pre-treated with supernatants from cultured PBMC or buffer, and stimulated with IgG-coated beads was confirmed by RP-HPLC (Fig 3.6) and assay of LTB_4 immunoreactivity in the fractions. The major peak of immunoreactivity coeluted with synthetic LTB_4, and a relatively small amount of activity was associated with material having a retention time comparable with that of 5(S), 12(R) 6-trans LTB_4.

3.3.4 Stability of leukotriene release enhancing activity

Leukotriene release enhancing activity was relatively heat and pH stable (Table 3.1). In five experiments, PBMC supernatants heated at 56°C for 60 min lost less than 15% of their activity, when compared with untreated PBMC supernatants. Exposure of PBMC supernatants to low pH (pH 2) for 5 min, reduced the activity by 28% when compared with that of untreated PBMC supernatants (Table 3.1).

3.3.5 Comparison of LREA with recognised cytokines

The LREA from PHA-stimulated PBMC supernatants was compared with several recombinant cytokines, including rIL-1 beta, rIL-2, rIFN-gamma, rTNF and rGM-CSF (Fig 3.7). In five experiments, rIL-1 beta showed a small but statistically insignificant enhancing activity when used at a concentration of 10 U/ml. No activity was observed at higher concentrations (20 and 40 U/ml). rIL-2 and rIFN-gamma did not enhance LTB_4 generation by neutrophils when tested over the dose range of 1 to 1000 U/ml. rGM-CSF enhanced LTB_4 generation when used at concentrations of 1, 2 and 4 ng/ml, but the activity only achieved a statistically significant level at a dose of 4 ng/ml. rTNF also exhibited a similar degree of activity, and the optimal effect was observed using concentrations of 100 and 1000 U/ml. The activities of rGM-CSF and

rTNF were 50% less than that observed with PHA-stimulated PBMC supernatants.

Pre-treatment of GM-CSF with anti-GM-CSF antibody totally abolished the leukotriene release enhancing activity of GM-CSF whilst treatment of the PBMC supernatant with anti GM-CSF antibody had no effect on the leukotriene release enhancing activity (Fig 3.8).

3.3.6 Physicochemical characterisation

PBMC supernatants concentrated 10 times and fractionated by gel filtration using Superose 12 PG in an FPLC system. Each column fraction was tested for LTB_4 enhancing activity. A major peak of activity was consistently observed (ten experiments) which eluted together with material of molecular weight approximately 35-45 kD (Fig 3.9). Small amounts of activity were detected at 60 kD and below 25 kD. The concentration of IL-2 and IFN-gamma were measured in each fraction from two FPLC separations. IL-2 activity was detected within the low molecular weight range of 13.7-25 kD and IFN-gamma activity was confined to fractions of molecular weight over 45 kD.

The material was further purified by chromatofocusing on a Mono-P column. Fractions were focused on a pH gradient between 7.1 and 4.0. The main peak of LREA was located between pH 5.1 and 5.5 and in addition, there were two small peaks of activity at pH 6 and pH 4.5 (Fig 3.10).

3.3.7 The effects of LREF on LTC_4 release by eosinophils

LREF recovered after gel-filtration was tested on LTB_4 generation by neutrophils as well as LTC_4 generation by eosinophils. Stimulation of eosinophils was the same as neutrophils, except the incubation time of IgG beads was extended to 45 min. Results showed that the capacity of neutrophils to generate LTB_4 was significantly enhanced ($p < 0.01$), whilst the capacity of eosinophils to generate LTC_4 eosinophils was not enhanced (Fig 3.11).

3.4 Discussion

Cultured human PBMC released an activity which was able to enhance the capacity of neutrophils to generate LTB_4 after *in vitro* physiological stimulation, with IgG-coated Sepharose beads. It had a molecular weight and isoelectric point range of between 35-40 kD and pI 5.1-5.5, respectively. For these reasons I have designated the material "leukotriene release enhancing factor (LREF)".

Some LREF-like activity was produced after 6 to 12 hr culture without stimulation. This "spontaneous" production was absent in 48 and 72 hr cultures (Fig 1&2). Spontaneous production of factors by cultured PBMC has been reported, including migration inhibition factor (MIF) (Arvilommi & Rasanen 1975) and histamine releasing factor (HRF) (Thueson et al 1979a). The significant and sustained release of LREF by PBMC cultures for 48 and 72 hrs in the presence of PHA correlated with proliferation as assessed by ^{3}H-TdR incorporation (Fig 3.2). The time course of LREF production appeared to be similar to that of many other lymphokines (Rocklin 1974; Kaplan et al 1985; Parker & Metcalf 1974). The large variation in the amounts of LREF generated by PBMC from different donors (Fig 3.4) was comparable with that for IL-2 production by human PBMC (Gearing & Bird 1987).

I have shown that PHA-stimulated PBMC generating

LREF under serum-free culture conditons, conditions also used by Rocklin (Rocklin 1974) for the production of LIF. This medium not only prevents other proteins interfering with the LTB_4 assay, but facilitates the purification of LREF. Although this culture condition might be thought to be unphysiological, the cell viability after 48 hr and 72 hr culture, was still over 90%. The importance of serum proteins in short term cell culture remains uncertain. Some investigators even found serum had inhibitory effects on cell growth in vitro, especially for lectin-activated T cells (Morse et al 1977; Brown et al 1983).

Using IgG-coated beads as a stimulant, LTB_4 and LTC_4 were found to be generated by neutrophils and eosinophils respectively (Fitzharris et al 1987; Shaw et al 1985). My results also showed that LTB_4 released into supernatant and retained intracellularly were both increased by LREF (Fig 3.4).

The dose-dependent increase of LTB_4 generation with increasing numbers of IgG-beads and the extremely low LTB_4 concentrations in PBMC supernatants and unstimulated neutrophil supernatants suggest that LTB_4 was newly generated by neutrophils and depended on the triggering by IgG beads.

LTB_4 is reported to have a direct activating effect on neutrophils, causing aggregation and may

even increase LTB_4 generation (Ford-Hutchinson et al 1980). Since monocytes can also release LTB_4 (Williams et al 1984), it might be thought that the activity of LREF was due to LTB_4 itself. However, LTB_4-induced activation signals and responses in neutrophils are short-lived (within minutes) (Omann et al 1987) while the maximum effect of LREF on neutrophils required 60 min incubation. Furthermore, the concentration of LTB_4 in the PBMC supernatants was negligible (Fig 3.5), suggesting that the LREF activity was not due to LTB_4.

Several different cytokines which can modify neutrophil activity have been described, and it is important to consider whether LREF represents a novel lymphokine or simply an additional activity of previously described lymphokines acting singly or in combination. IFN-gamma and IL-2 are important lymphokines produced by PBMC (Wheelock 1965; Gearing & Bird 1987). Neither of these had enhancing activity on LTB_4 generationfrom neutrophils in my assay system (Fig 3.7) and furthermore IFN-gamma and IL-2 did not coelute with LREF on FPLC (Fig 3.9). LREF, like IL-1 beta and IL-2 (Rosenwasser & Dinarello 1981; Mochizuki et al 1980), was relatively heat and acid (pH 2) stable (Table 3.1) while IFN-gamma was unstable at pH 2 and at 56°C (Wheelock 1965), suggesting that LREF is distinct from IFN-gamma. It has been reported

that GM-CSF specifically enhances LTC_4 generation by eosinophils and can enhance LTB_4 generation by neutrophils in a calcium ionophore stimulation system (Silberstein et al 1986; Roubin et al 1987) and that TNF activity is very low in serum-free PBMC cultures stimulated with PHA (Stone-Wolff et al 1984). TNF and GM-CSF both stimulated the neutrophil respiratory burst and degranulation (Klebanoff et al 1986; Weisbart et al 1985), and we have shown that they have some activity in my system (Fig 3.7). Since their molecular weights are close to 25 kD, they may account for some of the activity in the lower molecular weight fractions from FPLC (Fig 3.9). However, the maximal effect of physiological concentrations of TNF and GM-CSF resulted in only 60% increases of LTB_4 production, whereas the PHA-stimulated PBMC supernatant in the same set of experiments produced 200% enhancement. Anti-GM-CSF antibody also can blocked the effect of GM-CSF but not the effect of PHA-stimulated PBMC supernatant (Fig 3.8) suggesting that the activity is unlikely to be due to TNF or GM-CSF. IL-1 beta, which has a different molecular weight (17 kD) and pI (7.0), has been reported to stimulate arachidonic acid metabolism in smooth muscle cells, and neutrophils, but only trivial amounts (<1 pmoles $LTB_4/10^6$ cells). The effect depended on supplementary arachidonic acid and cytochalasin B

(Smith et al 1987). In my system, IL-1 beta had no significant effect on LTB_4 generation from neutrophils, suggesting that LREF was independent of IL-1 beta.

There are two lymphokines which have similar physicochemical characteristics to LREF. Phagocytosis--inducing factor (PIF) was thought to be derived from T lymphocytes following culture with Con-A (Margolick et al 1986). Its molecular weight was within the range 35 to 55 kD, with an estimated pI of 5.0 to 6.0. The target cells for PIF were monocytes (U937 cell line) and its effects on neutrophils have not been determined. Leukocyte inhibitory factor (LIF) has also been shown to be produced by PBMC after PHA stimulation (Rocklin et al 1974). The molecular weight of LIF was around 58 kD with a pI ranging from 5.0 to 5.5. LIF has also been shown to increase in the cytotoxic potential of neutrophils (Borish et al 1986; Borish & Rocklin 1987a), but its effect on LTB_4 generation by neutrophils has not been assessed.

Thus LREF is apparently distinct from rIL-1 beta, rIL-2, rGM-CSF, rTNF, and rIFN-gamma, but I can not at present distinguish LREF from LIF or PIF. Complete characterization of the molecules of PIF, LIF and LREF will be necessary before their precise relationship can be determined.

Thus, PBMC is a source of LREF which is able to

increase the immunological release of LTB_4 from human neutrophils. Generation of this activity was enhanced by culturing with PHA. The regulatory effect exerted by LREF on the LTB_4 generation of neutrophils gives further evidence of the close relationship between cell mediated immunity and inflammatory cells.

3.5 Summary

In an attempt to characterise some of the interactions between mononuclear cells and granulocytes, I have examined mononuclear cell culture supernatants for their ability to enhance the immunological release of LTB_4 from human neutrophils.

Supernatants from phytohaemagglutinin (PHA) stimulated human peripheral mononuclear cells (PBMC) produced a 3 to 3.5 fold increase in LTB_4 generation by IgG-stimulated neutrophils. Maximal release was observed after 12 hours' incubation, several hours before ^3H-thymidine uptake was detected. The activity appeared to be distinct from recognized cytokines, including interleukin 1-beta, interleukin-2, gamma-interferon, tumor necrosis factor and granulocyte/macrophage-colony stimulating factor. FPLC gel filtration (Superose 12 PG) and chromatofocusing revealed that the major peak of leukotriene release enhancing activity (LREA) was associated with molecules of molecular weight in the range 35-45 kD and an isoelectric point (pI) of 5.0-5.5. LREA appeared to have no effect on LTC_4 generation by human eosinophils.

Table 3.1

Heat and low pH stability of the LTB_4 release enhancing activity in supernatants of PHA-stimulated PBMC cultures

	LTB_4 (pmoles/10^6cells)	% enhancement
Untreated PBMC SN[a]	12.7 ± 2.2	87 ± 15
56°C, 60 min	11.7 ± 1.8	72 ± 11
pH2, 5 min[b]	10.8 ± 4.6	59 ± 23
Medium alone	6.8 ± 1.7	-

[a]SN (supernatant)

[b]With 1 N HCl and readjusted after 5 min to pH 7.2-7.4 with 1 N NaOH. The results are expressed as mean ± s.e.m. of 5 experiments.

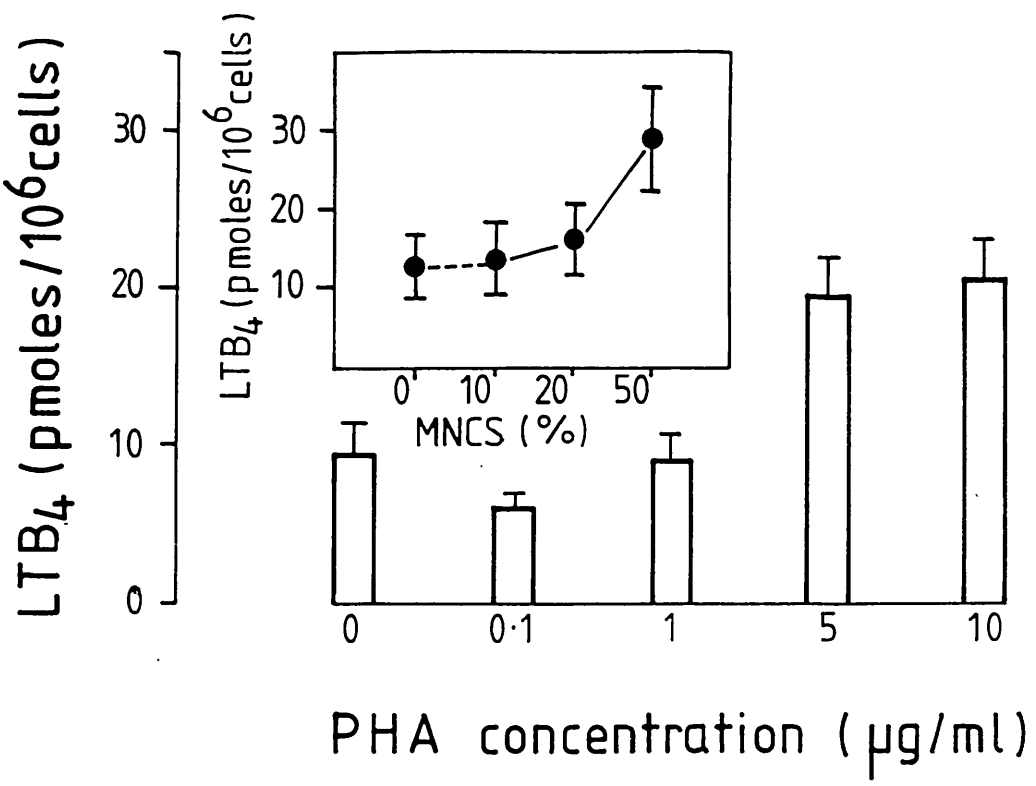

Figure 3.1: Effect of PBMC supernatant, cultured with various concentrations of PHA, on IgG-dependent LTB_4 generation by human neutrophils (n=5). INSET: Effect of various percentage concentrations of PHA-stimulated PBMC supernatants on subsequent generation of LTB_4 by neutrophils (n=4). PHA (5 ug/ml) alone was added to neutrophils, LTB_4 generation was 8.1 ± 1.5 p moles/10^6 cells.

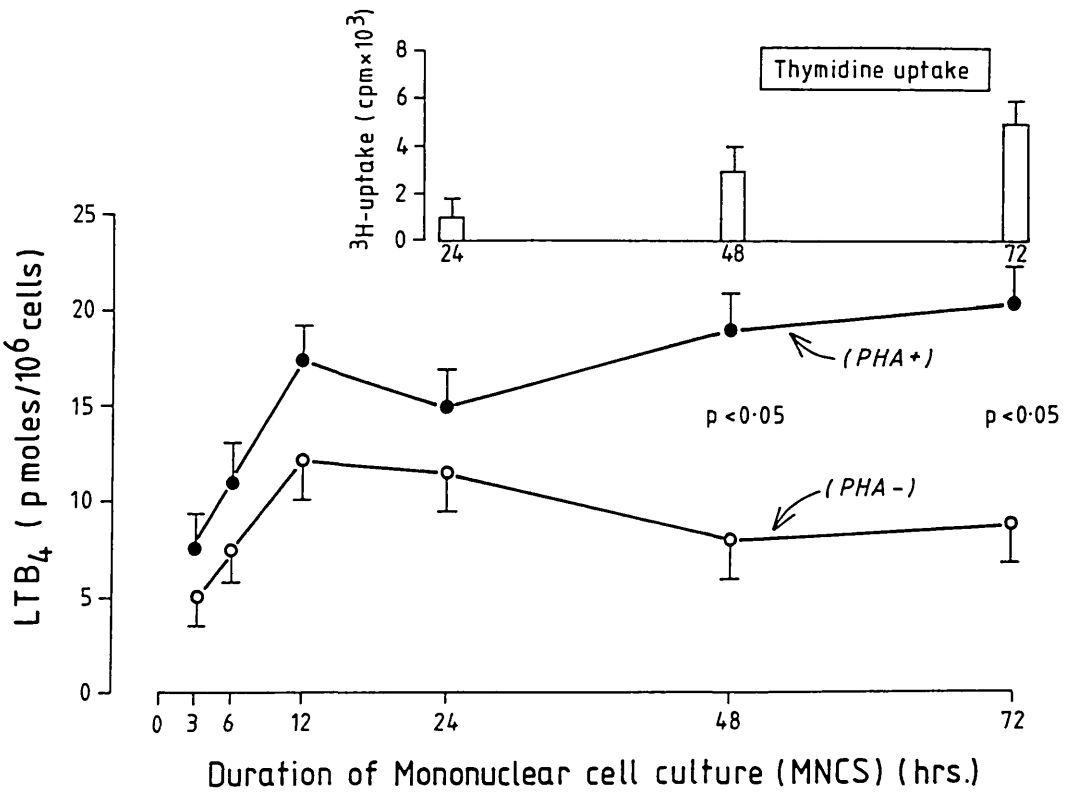

Figure 3.2: Duration of PBMC cultures required to effect optimal enhancement of LTB_4 generation by human neutrophils (n=6). INSET: Cell proliferation (expressed as ^{3}H-TdR incorporation) at 3 time points during culture in the presence of PHA (5 ug/ml). Each column represents mean cpm±s.e.m. of triplicate counts. Neutrophils alone obtained 5.5±2.1 p moles/10^6 cells.

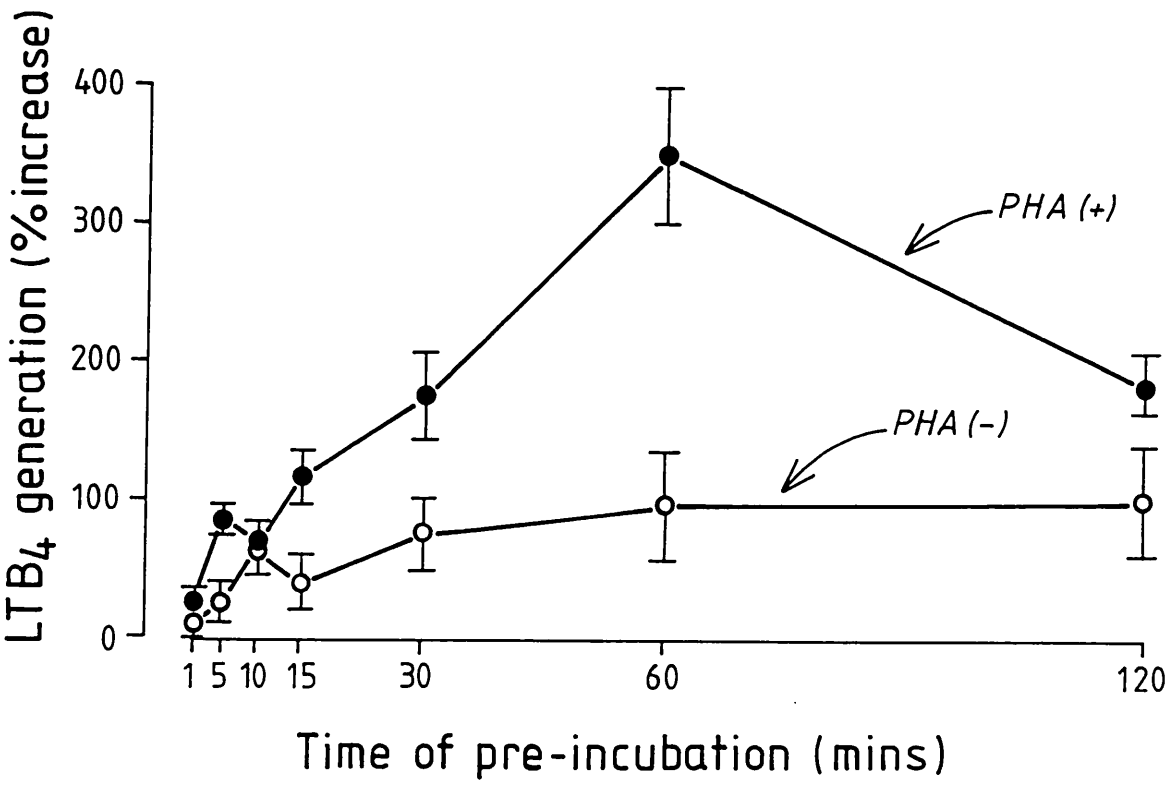

Figure 3.3: Time course of the effect of PBMC supernatants with human neutrophils on the percentage increase in LTB_4 generation. Neutorphils treated with supernatants from stimulated (closed circle) and unstimulated PBMC cultures (open circle) were compared with untreated neutrophils (4.7 ± 1.3 p moles/10^6 cells). Each point represents mean$\pm$s.e.m. (n=6). Neutrophils pre-treated with buffer or PHA over the same time periods showed no significant enhancement of LTB_4 generation.

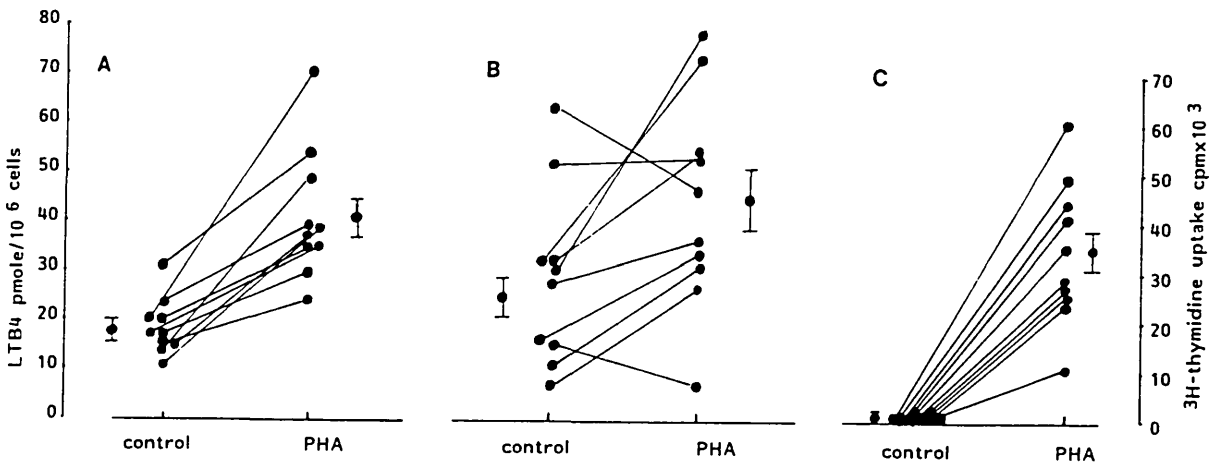

Figure 3.4: Extracellular and intracellular distribution of immunoreactive LTB_4 generated by PBMC supernatant pre-treated neutrophils in the IgG-beads stimulation system. PBMC from 10 different donors were stimulated with or without PHA for 48 hours, and supernatants were tested for neutrophil LTB_4 release enhancing activity (neutrophils obtained from one donor). PBMC supernatants, obtained from either with or without PHA stimulation, were incubated with neutrophils (60 min, 37°C). Cells were then stimulated with IgG beads (15 min, 37°C). Cell free supernatants were assayed for immunoreactive LTB_4 (A) and cell pellets were extracted with methanol then assayed for immunoreactive LTB_4 (B). PBMC proliferation was expressed as ^{3}H-TdR incorporation, each point represents mean$\pm$s.e.m. of triplicate counts (C).

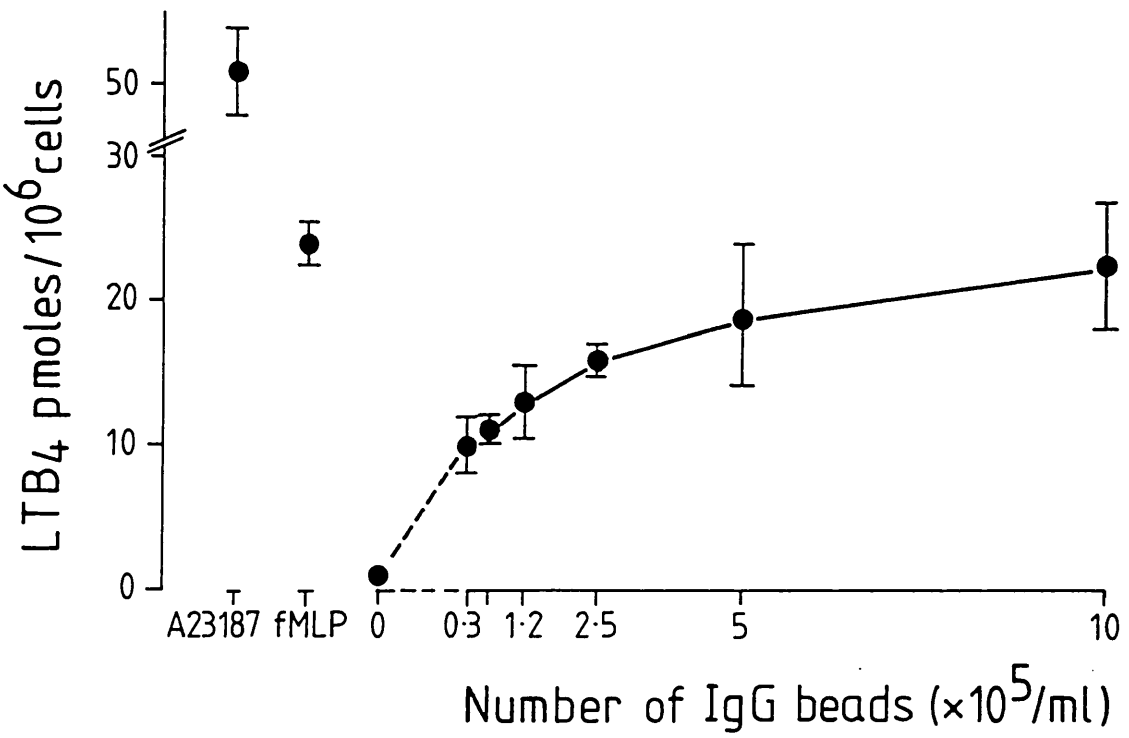

Figure 3.5: Generation of LTB_4 by PBMC supernatant-treated neutrophils in response to the stimulation of IgG-coated Sepharose beads. Neutrophils were pre-incubated (60 min, 37°C) with PBMC supernatants then treated with various concentrations of IgG-beads (15 min, 37°C). Cell free supernatants were then assayed for LTB_4 concentration. fMLP ($5x10^{-8}M$) and A 23187 ($5x10^{-6}M$) were used as positive controls.

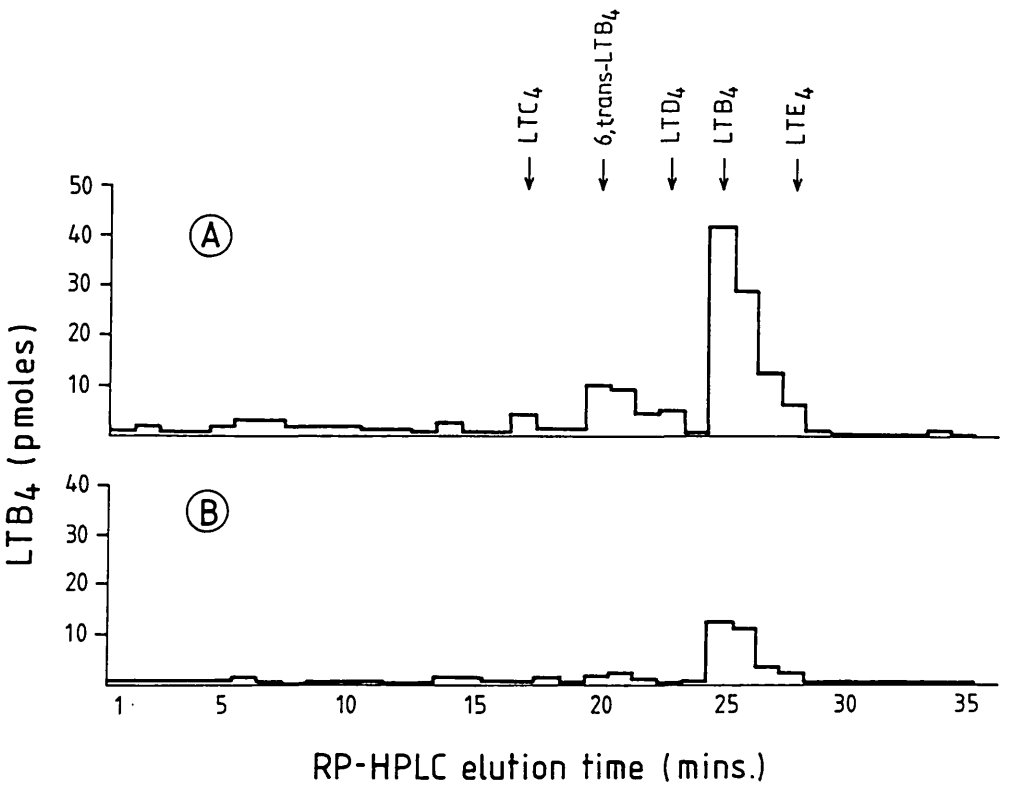

Figure 3.6: Confirmation of the identity of LTB_4 generated by neutrophils, using a combination of RP-HPLC and RIA. Neutrophils were pre-incubated with (A) and without (B) PBMC supernatants (60 min) and stimulated with IgG-coated beads (15 min). Cell free supernatants were extracted using C-18 Sep-Pak columns and the concentrated polar lipid mediators were subsequently fractionated on RP-HPLC (Nucleosil C-18 column). Each HPLC fraction was assayed for immunoreactive LTB_4. Synthetic LTB_4, 6-trans-LTB_4, LTC_4, LTD_4 and LTE_4 were indicated.

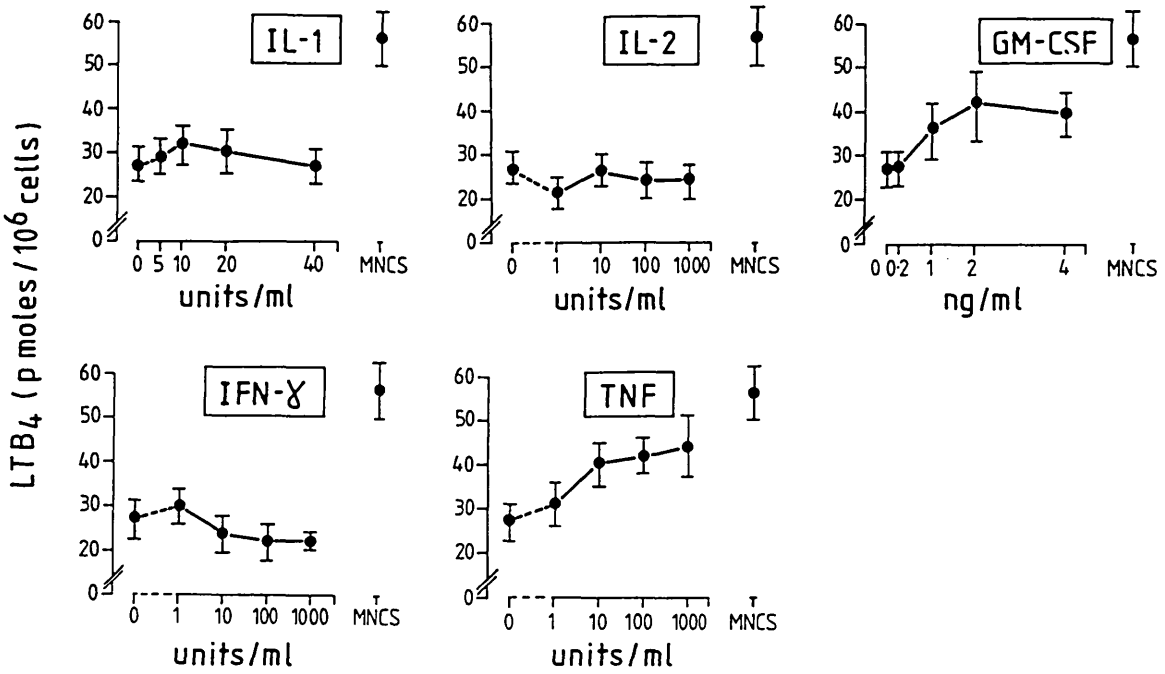

Figure 3.7: Dose response of the effect of various cytokines on LTB_4 production by human neutrophils in an IgG-beads stimulation system. Neutrophils were incubated (60 min, 37°C) with various doses of recombinant cytokines and PBMC supernatants (48 hrs, PHA+ culture). Cells were then stimulated with IgG-coated beads (15 min, 37°C) and the cell free supernatants were subsequently assayed for LTB_4. The results represent mean±s.e.m. values of 5 experiments.

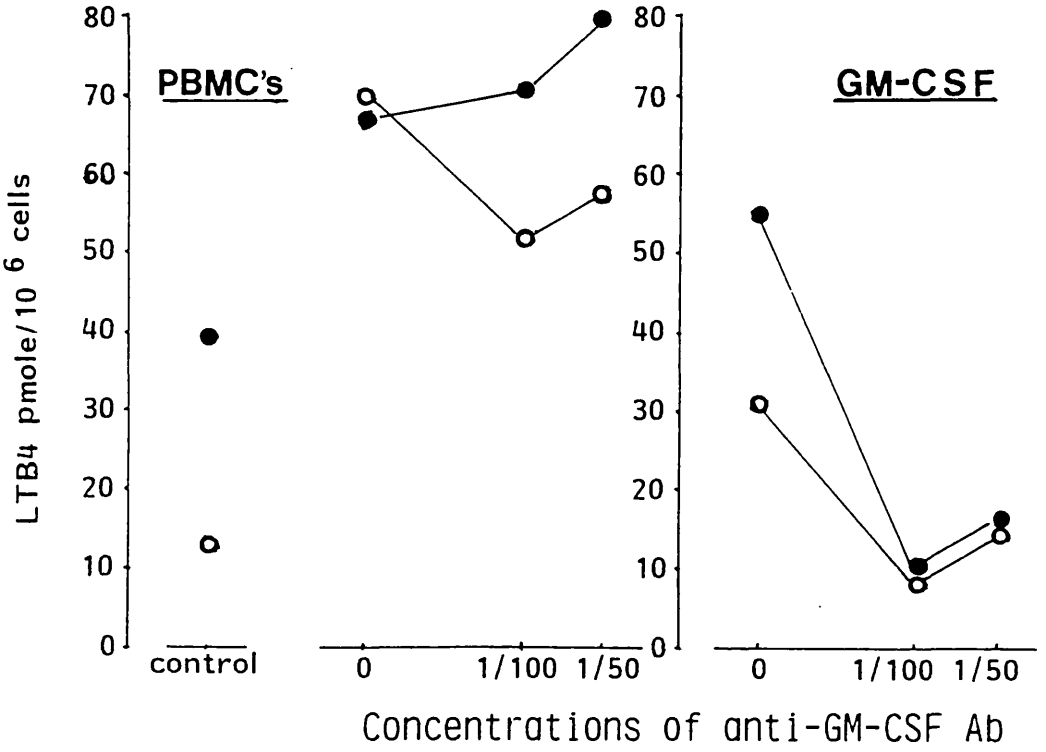

Figure 3.8: Neutralization effect of anti-GM-CSF antibody on the LTB_4 release enhancing activity of PBMC supernatants and GM-CSF. PBMC supernatants and GM-CSF (4 ng/ml) were treated with anti-GM-CSF antibody (60 min, 4°C). After neutralization the supernatants or GM-CSF were incubated with neutrophils (60 min, 37°C) then stimulated with IgG-beads. Cell free supernatants were assayed for LTB_4.

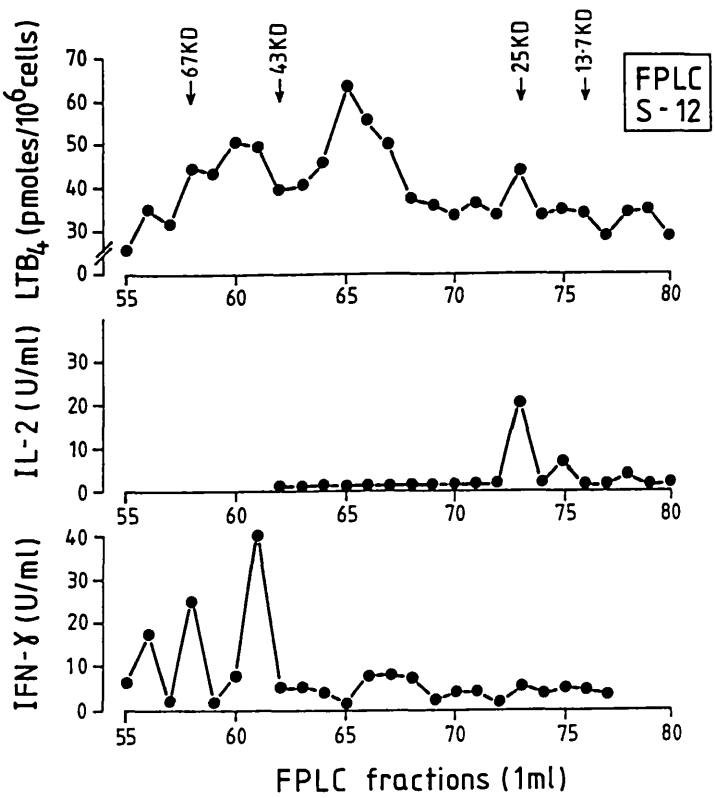

Figure 3.9: Fast protein liquid chromatography (FPLC) analysis of PHA-stimulated PBMC supernatants using Superose-12 prep grade gel filtration. Two millilitres of 10 times concentrated PBMC supernatants were applied to the column, 1 ml of column fractions were collected and tested for the enhancement of LTB_4 released by neutrophils (upper panel), Interleukin-2 content was determined using IL-2 bioassay (middle) and Interferon-gamma content by radioimmunoassay (lower). LTB_4 content in each fraction was determined all less than 1 p moles/ml. Molecular weight markers were indicated. Neutrophil pre-incubated with a buffer fraction produced 24.4 p moles/10^6 cells.

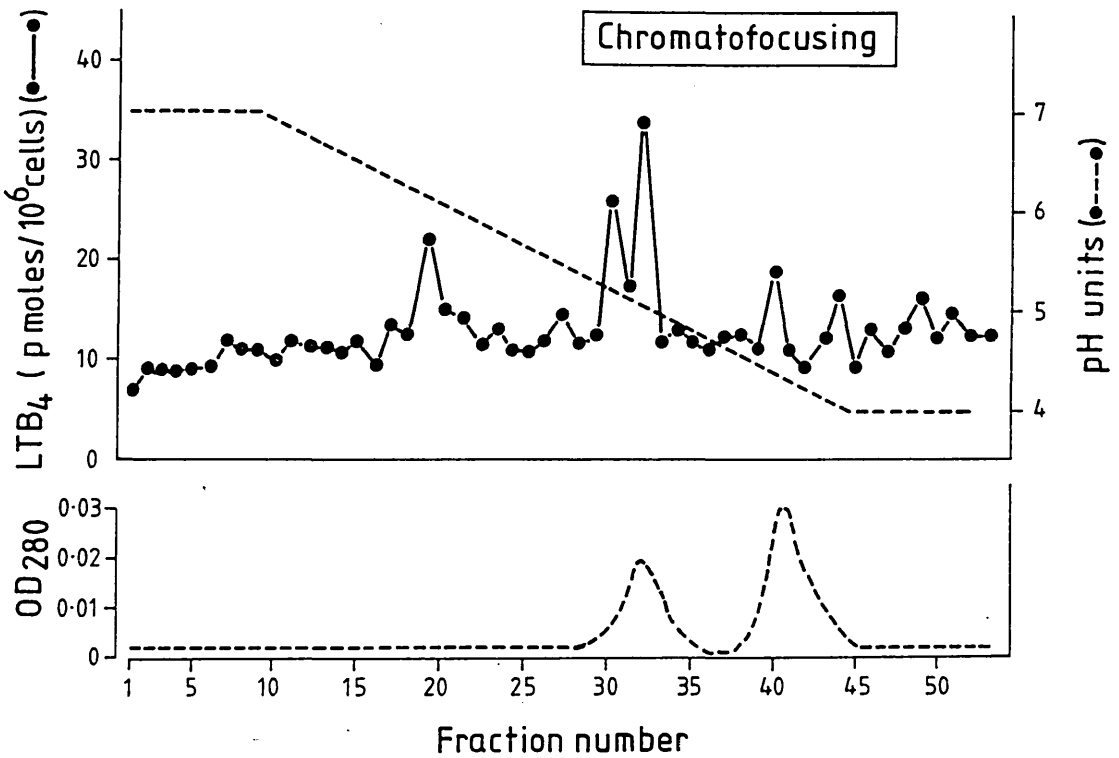

Figure 3.10: Chromatofocusing of PHA-stimulated PBMC supernatants using Mono-P column. The peak of leukotriene release enhancing activity from Superose-12 prep grade FPLC column were pooled (Fractions 65-67) and applied to a Mono-P chromatofocusing column, each fraction was tested for LTB_4 generation by neutrophils, neutrophils pre-incubated with PBS buffer produced 10.4 p moles/10^6cells.

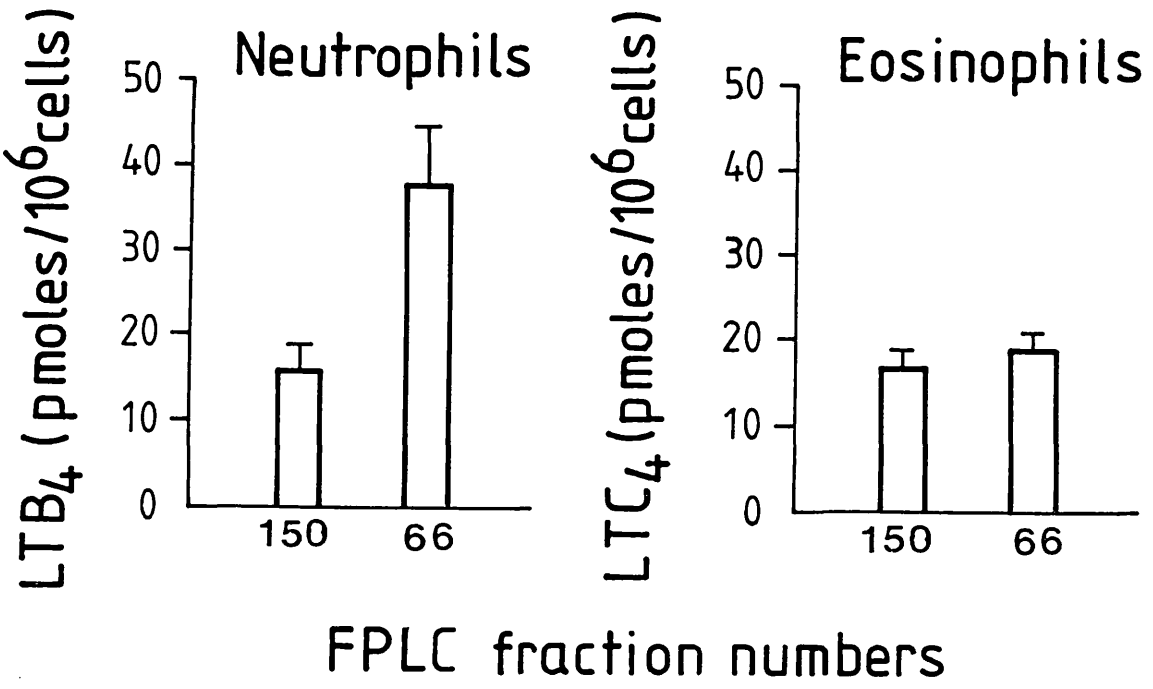

Figure 3.11: The effects of LREF on LTB_4 generation by neutrophils and LTC_4 generation by eosinophils. Neutrophils and eosinophils ($1x10^6$) were pre-incubated (60 min, 37°C) with both active (fraction 66) and non-active (fraction 150) FPLC fractions. IgG-beads ($1x10^6$) were added and incubated for optimal periods (15 min for neutrophils and 45 min for eosinophils). Cell free supernatants were then assayed by specific RIA for LTB_4 and LTC_4, respectively. Results represent mean±s.e.m. values of 6 experiments.

1P

CHAPTER 4.

LREF RELEASE BY SPECIFIC ALLERGEN

4.1 Introduction

A number of studies have suggested that T-lymphocytes may play a role in allergic reactions and bronchial asthma. There are several lines of evidence for this including: 1) lymphocytes from allergen sensitive individuals proliferate in response to specific allergens *in vitro*; 2) the requirement of lymphokines, particularly interleukin 4 (IL-4) for IgE synthesis; and 3) successful immunotherapy was associated with an increase in antigen specific suppressor cells (for a review see chapter 1). Although T lymphocytes from atopic individuals generated lymphokines, such as migration inhibition factor (MIF) and interleukin 2 (IL-2) in response to specific allergen, the mechanisms regulating this response and the relationship between lymphokine generation and allergic inflammation are still unclear.

Recently, much attention has been focused on the role of inflammatory cells in allergic diseases (Atkins & Zweiman 1985; Fleekop et al 1987). It has been demonstrated that neutrophils are the first cells infiltrating the sites of allergic reactions (Kline et al 1932) and that they persist for several hours at such sites (Zweiman et al 1976; Solley et al 1978). Also neutrophil infiltration is a feature of the pathology of asthma (Dunnill 1960). In animal models,

antigen-induced bronchial hyperresponsiveness was associated with neutrophil infiltration (Chung et al 1985; Holtzman et al 1983). In a rabbit model, supernatants derived from activated neutrophils enhanced airway reactivity, and granulocyte depletion prevented the development of airway hyperresponsiveness (Irvin et al 1985; O'Byrne et al 1984). Neutrophils recovered from asthmatic patients possess higher 5-lipoxygenase activity (Mita et al 1985) and have been shown to generate more LTB_4 after exercise challenge (Arm et al 1987).

In the latest studies it has been shown that accumulation of granulocytes and mononuclear cells was prominent and persistent in allergic reaction sites for some hours after antigen challenge (Oertel & Kaliner, 1981; Zweiman et al 1976; Frew & Kay 1988). Both neutrophils and lymphocytes are also prominent in bronchial lavage fluid after allergen challenge (Diaz et al 1986; Gonzalez et al 1987). Furthermore, the potent anti-inflammatory drug prednisolone can abolish allergen induced late phase asthmatic reactions and prevents allergen induced increases in bronchial hyperreactivity (a single dose pre-allergen challenge has no effect on the immediate asthmatic reaction) (Burge 1982). Prednisolone also depresses allergen induced neutrophil infiltration into the skin (Zweiman et al 1976). These observations suggest that

cell-mediated immunity is a feature of the late phase reaction. In order to better understand the mechanisms of the allergic inflammatory reaction, it is of great interest to study allergen induced lymphokine generation. In the previous chapter I have described a LTB_4 release enhancing factor (LREF) in serum-free supernatants of PHA-stimulated human PBMC cultures, which significantly enhanced LTB_4 release by neutrophils under physiological stimulation, in vitro, using IgG-coated Sepharose beads.

In order to assess the relevance of this phenomenon to allergic inflammation, I have attempted to establish whether LREF can be produced by allergen-reactive T-lymphocytes. Experiments were performed by culturing PBMC with allergens to which donor gave a positive skin test, as well as an allergen-reactive long term T cell line (CD4+) which reacted with Dermataphagoides farinae (D. farinae). Supernatants were tested for LREF activity.

4.2 Materials and Methods

4.2.1 Materials

Lyophilized extracts of D. farinae, D. pteronyssinus, 5-grass mix (sweet vernal grass, Rye grass, Timothy grass, cultivated rye and Yorkshire fog), parietaria and timothy grass pollen were generously provided by Pharmacia Diagnostics, Uppsala, Sweden.

4.2.2 PBMC culture

For experiments with specific allergen, subjects with atopic rhinitis and positive skin prick tests to D. pteronyssinus or grass pollen extract were selected from the Allergy Clinic, Brompton Hospital and from laboratory staff. PBMC was obtained from heparinized venous blood, separated over Ficoll-Paque and resuspended at $2x10^6$/ml in RPMI-1640 medium containing 25mM HEPES and 4mM L-glutamine supplemented with 100 IU/ml penicillin and 100 ug/ml streptomycin. Cells were incubated in 12x75 mm plastic cell tissue culture tubes with different concentrations of allergen for varying lengths of time in a 5% CO_2 atmosphere at 37°C and 95% humidity. After incubation the cell suspensions were centrifuged at 400 g for 10 min at 4°C and the sterile cell-free supernatants were aliquoted and stored at -80°C until tested. Cell viability as assessed by trypan blue dye exclusion, was over 90%.

4.2.3 Generation of supernatants from allergen reactive T lymphocyte lines

Allergen reactive T cell lines were isolated by Dr Robyn O'Hehir (O'Hehir et al 1987).

PBMC at $2.5x10^5$cells/ml in RPMI-1640, containing 5% pooled screened human A^+ serum (i.e no inhibitory effect on lymphocyte proliferation), 2mM L-glutamine, 25mM HEPES buffer, 100 IU/ml penicillin/streptomycin, were stimulated with the inducing antigen (D. farinae) for 7 days. Lymphocytes, enriched on Ficoll-Paque were established as a long term line in presence of irradiated (2,500 Rads) autologous PBMC as accessory cell (AC), antigen and IL-2 (Lymphocult T, Biotest Folex; 10% v/v). The lines were maintained with further IL-2 every 3-4 days and irradiated PBMC and antigen were added every 7 days. Before collection of supernatants, the lines were rested for 6-8 days from the last addition of AC and antigen. After washing three times with serum-free RPMI-1640 medium supplemented with 2mM L-glutamine, 100 IU/ml penicillin/streptomycin cells were resuspended to $0.5x10^6$ cells/ml and incubated with equal numbers of irradiated AC and D. farinae for 36 hr. Cell culture fluid was obtained as described for PBMC culture. Control supernatants were from cells treated with irrelevant allergen (grass mix) in the presence of irradiated AC, and from allergen incubated with

irradiated AC.

4.2.4 Gel filtration

Gel filtration was performed on HR 16/50 columns of Superose 12 prep grade in an FPLC system as described in chapter 3.2.6a, except chromatography was performed on three times concentrated PBMC supernatants.

4.3 Results

4.3.1 Allergen stimulated PBMC culture and FPLC analysis of PBMC culture supernatants

Supernatants from PBMC of nine out of ten skin prick test positive subjects cultured with relevant allergens (3 grass mix and 7 D. farinae) showed LREF activity (Table 4.1). As a control, these PBMC were also cultured with irrelevant allergens and no significant amount of LREF activity was generated (Table 4.1).

PBMC from six normal non-atopic individuals (skin tests were negative) were cultured with both D. farinae and grass pollen mix as another control. In these supernatants, LTB_4 generation by IgG-coated Sepharose beads-stimulated neutrophils pretreated with unstimulated PBMC supernatants obtained 19 ± 3.0 p moles/10^6 cells, while pretreated with allergen (10^4 BU/ml) stimulated PBMC supernatants obtained 21.8 ± 1.5 p moles/10^6 cells, there was no significant LREF activity after allergen stimulation. The dose response curve for production of LREF by relevant allergens showed that significant amounts of LREF were generated at allergen concentrations of 10^3BU/ml and 10^4BU/ml. The time course of allergen induced production of LREF was studied with PBMC from three further atopic donors and found to be comparable with

that of LREF production by PHA-stimulated PBMC, maximum activity appearing in the supernatants after 48 hr with no further increases at 72 hr (Fig 4.1).

Supernatants from allergen-stimulated, and unstimulated PBMC cultures were concentrated and applied to a Superose-12 PG column in an FPLC system. A major peak of activity was recovered, corresponding to a molecular size of approximately 40 kD, from the allergen stimulated PBMC supernatants. However, neither the unstimulated PBMC supernatant, nor allergen alone had any peak of activity (Fig 4.2).

4.3.2 Dermatophagoides farinae stimulated DX1 cell line

LREF was present in supernatants generated by a long term CD4+/CD8- human T-cell line specifically reactive to D. farinae, following incubation with antigen and irradiated AC for 36 hr. Only low levels of LREF were produced when the T-cells were incubated with an irrelevant grass-mix allergen extract or when the allergen was incubated with irradiated AC alone (Table 4.2). Six millilitres of D. farinae stimulated DX1 cell line culture supernatant were concentrated and separated by Superose-12 PG column in an FPLC system. Peaks of activity were recovered in the molecular weight range 35-40 kD (Fig 4.3). Maximal enhancement of LTB_4 release by neutrophils was 260% in comparison with the same volume of supernatant

generated by irradiated AC and D. farinae which produced only 88% enhancement compared with a buffer control.

4.4 Discussion

In these experiments, I have demonstrated that leukotriene release enhancing factor (LREF) can be produced by PBMC from allergic subjects after relevant allergen stimulation, but this was less effective than PHA stimulation. It has been reported that in individuals with house dust mite allergy only a small proportion of the circulating lymphocytes are reactive to the specific allergen (Halvorsen et al 1986), suggesting that allergen will trigger relatively few lymphocytes by comparison with PHA. Ninety percent of the atopic subjects in this study showed a T-cell response to antigen *in vitro* and generated LREF activity. A similar proportion of atopic subjects, 85% were responsive to allergen in a study of mitogenic factor generation from allergen stimulated PBMC culture (Rocklin et al 1974). An explanation suggested for this result was that patients were selected on their symptomatology and immedidate skin test reaction which did not necessarily parallel their *in vitro* response.

The production of LREF by the allergen-specific human T-cell line following stimulation by specific antigen suggests that LREF is of T lymphocyte origin. Although AC were present in both allergen and PHA driven culture systems and might conceivably contribute to the generation of LREF through the

action of interleukin-1 or other cytokines, the amounts of LREF recovered in systems using irrelevant antigens were greatly reduced by comparison with those employing specific antigen. These results do not exclude the possibility that other cell types, under appropriate stimulation and culture conditions may also have the capacity to generate LREF activity, as in the case with many other cytokines (Dinarello 1984; Yoshida et al 1976). LREF activity in the allergen stimulated PBMC and cell line cultures was associated in both instances with a molecular weight of 35-40 kD as determined by gel filtration (Fig 4.2 & Fig 4.3) and appeared to be the same as PHA-stimulated PBMC supernatant (Fig 3.9) suggesting that LREF can be generated from PBMC through the activation of either PHA or allergen.

My study demonstrates that antigen-specific T lymphocyte cell-lines are a potential source of LREF and suggests that this lymphokine mediated LTB_4 generation from neutrophils might contribute to the exacerbation of allergic inflammatory reaction in vivo. It is tempting to speculate that lymphocyte activation and LREF generation provides a possible means by which lymphocytes may influence the pathogenesis of the allergic response.

4.5 Summary

I have attempted to determine whether leukotriene release enhancing factor (LREF) can be obtained from PBMC after specific allergen stimulation.

LREF was detected in supernatants of PBMC derived from 9 out of 10 skin test positive subjects following culture with extracts of the relevant allergens. The dose response curve for production of LREF by relevant allergens showed that significant amounts of LREF were generated at allergen concentrations of 10^3BU/ml and 10^4BU/ml. In contrast, irrelevant allergens were not active. Time course studies showed that LREF production in response to relevant antigen was maximal between 24 and 48 hours. An established CD4+/CD8- human T cell line (DX1) specifically reactive to *D. farinae*, also generated LREF following incubation with the antigen and irradiated AC for 36 hours.

Supernatants from PBMC and DX1 cultures stimulated with *D. farinae* antigen were analysed by FPLC gel filtration (Superose 12 PG). The allergen-induced LREF was associated with molecules of molecular weight range 35-45 kD, similar to that of the LREF obtained from PHA-stimulated PBMC culture supernatants.

These studies suggest that antigen-specific T lymphocytes are a major cell source of this LREF and that LREF can be released by physiological triggering as well as after lectin stimulation.

Table 4.1

Allergen-induced generation of LREA from PBMC

Pt No.	Allergen		Allergen concentration (B.U.)[5] 0	10^2	10^3	10^4
1	D.f.[1]	(+)	24[6]	30	20	30
	Pariet.[2]	(−)	24	24	28	31
2	D.f.	(+)	23	25	27	13
	Pariet.	(−)	23	23	16	14
3	D.f.	(+)	22	28	32	41
	Pariet.	(−)	22	25	24	29
4	D.f.	(+)	21	25	27	29
	G.M.[3]	(−)	21	16	22	17
5	D.f.	(+)	20	14	39	32
	G.M.	(−)	20	29	19	28
6	G.M.	(+)	18	21	12	16
	D.f.	(−)	18	29	36	36
7	G.M.	(+)	15	18	16	17
	D.f.	(−)	15	13	14	24
8	Timothy[4]	(+)	13	24	20	28
	Pariet.	(−)	13	13	13	14
9	D.f.	(+)	8	14	23	12
	Pariet.	(−)	8	10	9	11
10	G.M.	(+)	4	4	4.5	4
	D.f.	(−)	4	4	5	11
Mean ± S.E.M.		(+)	16.8±2.1	20.6±2.8	24.3±3.2*	25.6±3.3*
		(−)	16.8±2.1	18.3±2.4	16.4±2.3	18.1±2.7

(1) D.f. (D. farinae)
(2) Pariet. (Parietaria Pollen)
(3) G.M. (Grass mix)
(4) Timothy (Timothy grass pollen)
(5) B.U. (Biological Unit)
(6) LTB_4 pmoles/10^6cells
(7) * $p<0.01$
(+) Relevant allergen
(−) Irrelevant allergen

Table 4.2

Leukotriene release enhancing activity from supernatants of a D. farinae stimulated T-cell line (DX1)

Culture constituents	Supernatant dilution		Neutrophil LTB_4 generation (5)
DX1, AC, D.f. (1)	(a) (2)	1:2	17.6
		1:8	14.8
	(b)	1:2	16.4
		1:8	13.2
	(c)	1:2	12.8
		1:8	15.0
Controls			
DX1, AC, G.M. (3)		1:2	7.2
		1:8	9.6
AC, D.f.	(a) (4)	1:2	8.0
		1:8	9.2
	(b)	1:2	6.4
		1:8	9.6
AC, G.M.		1:2	9.2
		1:8	10.0
Culture medium above		-	4.0

(1) Cell line (DX1), accessory cells (AC) and D. farinae (relevant allergen)
(2) Three separate cultures
(3) Grass mix (G.M.) (irrelevant allergen)
(4) Two separate cultures
(5) p moles/10^6 cells

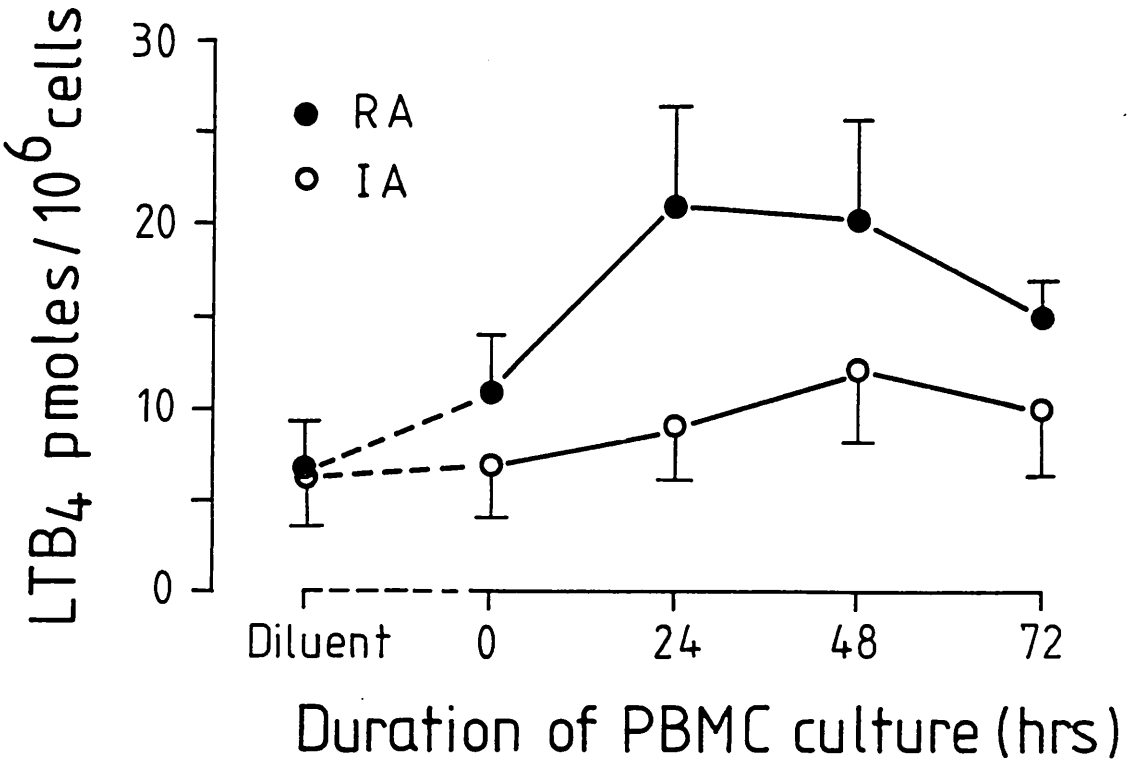

Figure 4.1: The effects of supernatants from PBMC cultured with relevant (RA) or irrelevant allergen (IR) on neutrophil LTB_4 generation. PBMC were cultured for various periods of time with either relevant or irrelevant allergens and neutrophils were incubated with the resulting supernatants (60 min, 37°C) prior to stimulation with IgG-coated Sepharose beads. LTB_4 concentration was measured by radioimmunoassay, and each point represents mean ± s.e.m. of three experiments.

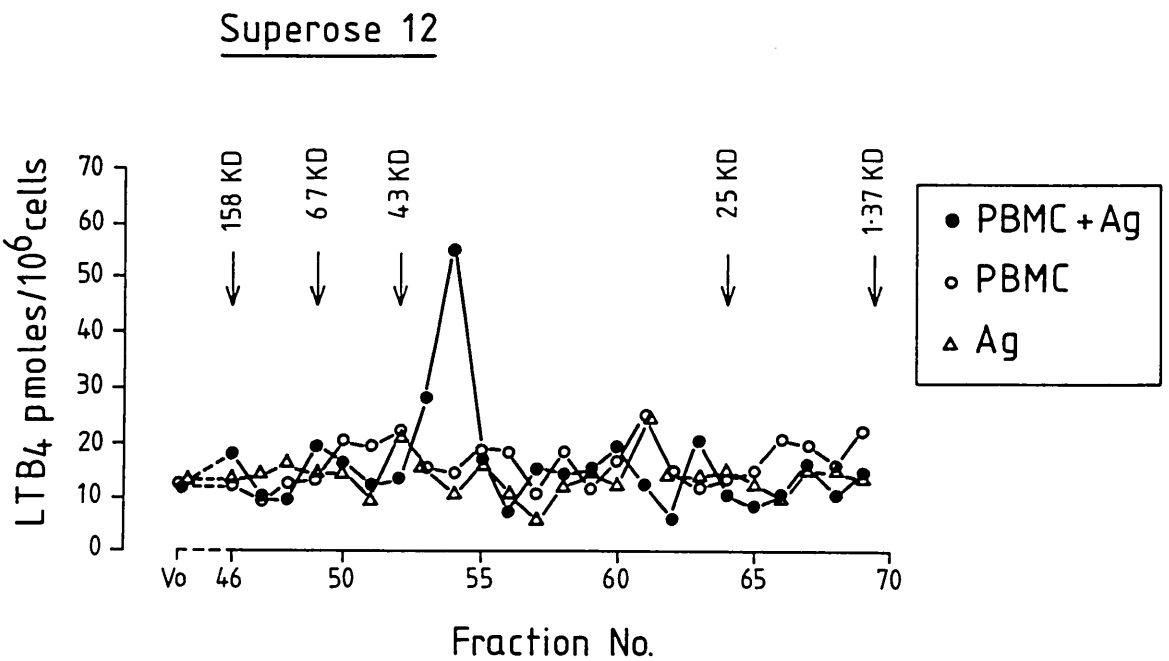

Figure 4.2: Fast protein liquid chromatography (FPLC) analysis of allergen-stimulated PBMC culture supernatant using Superose-12 prep grade gel filtation. Two millilitres of 3-fold concentrated PBMC supernatants were applied to the column. Each column fraction (1ml) was tested for its ability to enhance LTB_4 release from neutrophils. LTB_4 generation by IgG-coated Sepharose beads-stimulated neutrophils pretreated with allergen-stimulated PBMC supernatants (closed circles), unstimulated PBMC supernatants (open circles), and buffer with allergen (open triangles).

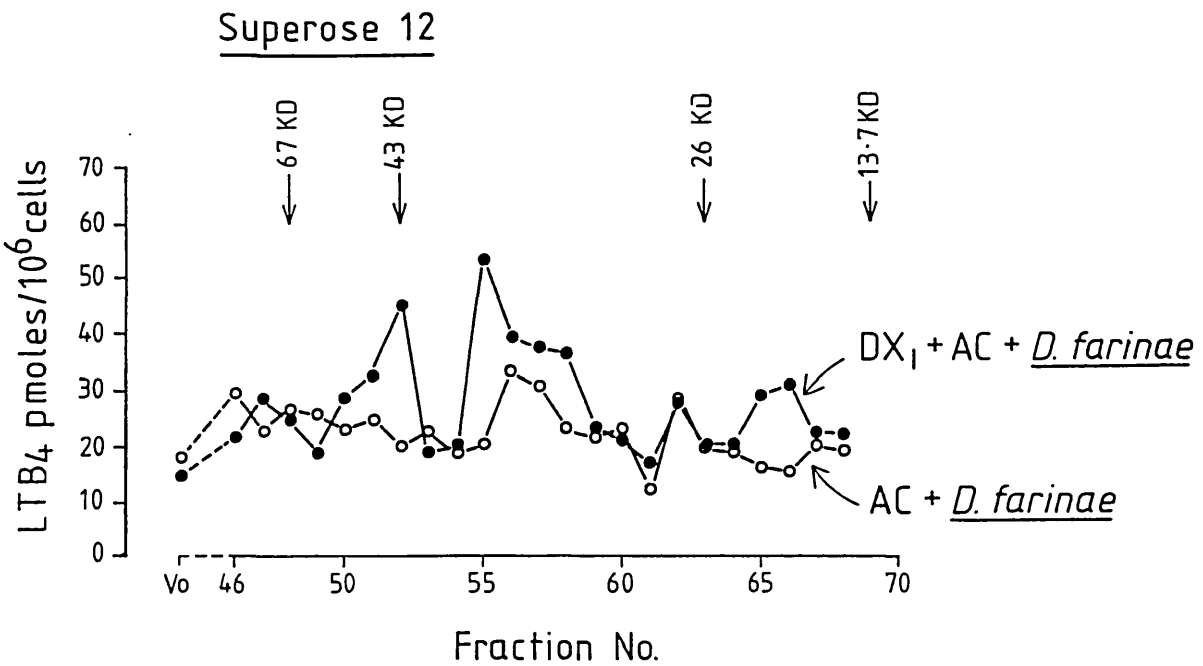

Figure 4.3: Fast protein liquid chromatography (FPLC) analysis of D. farinae stimulated DX1 cell line culture supernatant using Superose-12 prep grade gel filtration. Two millilitres of 3-fold concentrated supernatants were applied to the column Each column fraction (1ml) was tested for its ability to enhance LTB_4 release by neutrophils, under IgG-beads stimulation. Molecular weight markers were as indicated. LTB_4 generation by IgG-coated Sepharose beads-stimulated neutrophils pretreated with D. farinae stimulated DX1 cell line culture supernatant (closed circle) and D. farinae stimulated irradiated accessory cell culture supernatant (open circle).

CHAPTER 5.

CELL SOURCE OF LEUKOTRIENE RELEASE ENHANCING ACTIVITY

5.1 Introduction

As described in the preceeding chapters leukotriene release enhancing activity (LREA) was produced by mononuclear cells after mitogen stimulation using PHA. PHA is a polyclonal activator which stimulates T cells although other cell types may also be reactive. Recently, the T-cell antigen receptor has been identified as a 90 kD heterodimer molecule on the surface membrane of cloned T lymphocytes (Meuer et al 1983). This receptor is linked to the CD3 (T3) molecules recognized by OKT3 monoclonal antibody to form the T-cell antigen recognition complex (T3/Ti complex) (for a review see chapter 1). The CD3 molecule has been shown to be physically associated with the T-cell antigen receptor for which it appears to act as a signal transducer (Chang et al 1981; Reinherz et al 1982). Although the molecular mechanism is not yet known, triggering the CD3 molecule with OKT3 MoAb appears to activate T lymphocytes in a way similar to that of antigen. Thus OKT3 MoAb, when placed with T-cells in the presence of adherent cells induces proliferation (Van Wauwe & Goosens 1981) and stimulates the production of lymphokines such as GM-CSF (Platzer et al 1985) and interferon-gamma (Von Wussow et al 1981; Chang et al 1982).

Human T lymphocytes can be purified by rosetting

with sheep red blood cells (E rosettes). Historically this was a widely accepted means of identifying human T lymphocytes, although the interaction is now known to be due to interaction of CD2 molecules on the T cell surface with LFA-3 molecules present on many cell types, including sheep erthrocytes (Selvaraj et al 1987). An improved method resulting in enhanced binding of SRBC-A to human T-lymphocytes is to treat SRBC with a sulfhydryl reagent causing erythrocyte membrane alterations. In this study I have assessed the effects of supernatants from PHA- and anti-CD3 MoAb-stimulated PBMC and PHA-stimulated non-adherent ER+ lymphocytes for their effects on LTB_4 generation by neutrophils. The effect of IL-2 on LREF generation from PHA-stimulated ER+ lymphocytes was also evaluated.

5.2 Materials and Methods

5.2.1 Anti-CD3 stimulated PBMC culture

Human PBMC were separated and resuspended at a concentration of $2x10^6$ cells/ml. One millilitre of cell suspension in 12x75 mm tissue culture tubes was incubated with different concentrations of anti-CD3 MoAb (OKT3, Ortho Pharmaceutical Co., Raritan, N.J., U.S.A.) for 48 hr in a 5% CO_2 atmosphere at 37°C and 95% humidity. Controls included cells cultured a) with medium alone, b) with PHA and c) with anti CD4, CD5 and CD8 MoAb (anti-Leu-3a, anti-Leu-1 and anti-Leu-2a, Becton Dickinson, Sunnyvale Inc., Ca, U.S.A.)

5.2.2 Gel filtration

Six millilitres of anti-CD3 stimulated PBMC supernatant were pooled, concentrated to 2 ml and fractionated by gel filtration using Superose-12 PG in an FPLC system as described in Chapter 3.2.6a.

5.2.3 Preparation and culturing of erythrocyte rosette positive (ER+) lymphocytes

SRBC-A were prepared from fresh sheep red cells (SRBC) by washing five times in isotonic saline at room temperature and treating with the sulfhydryl reagent S-(2-aminoethyl) isothiouronium bromide (AET) (Adrich Chemical Co. Inc., Milwaukee, WIS, U.S.A.). AET was prepared as a 0.143M solution in deionized water (adjusted to pH 9.0 with 4N NaOH) and used in a

ratio of one volume of washed packed SRBC to four volumes of AET solution. The suspension was mixed thoroughly and incubated at 37°C for 20 min. Thereafter, the cells were washed 5 times with Hanks balanced salt solution (HBSS) (Gibco) (1000 g, 10 min, 20°C), and resuspended in RPMI-1640.

Mononuclear cells were suspended in 5 ml RPMI-1640, deposited on a tissue culture petri dish for 60 min at 37°C (5% CO_2 and 95% humidity atmosphere). The non-adherent cells were washed off with pre-warmed RPMI-1640, and readjusted to 10^7cells/ml. For each 1 ml volume of cell suspension 0.5 ml heat-inactivated foetal calf serum (Gibco) and 2 ml of 2% SRBC-A were added. The cell mix was centrifuged gently (130 g, 10 min, 4°C) and left at 4°C for 60 min. Cells were then gently suspended layered over Percoll (Percoll was prepared by mixing 9 part percoll with 1 part 10x PBS, then took 65 ml percoll/PBS mixture diluted with 35 ml normal saline and adjusted to pH 7.4) and centrifuged at 300 g, 4°C for 30 min. Cell pellets were lysed with NH_4Cl lysis buffer. Monocyte contamination was determined by non-specific esterase stain.

The ER+ cells were readjusted to $2x10^6$cells/ml and cultured with or without PHA 5 ug/ml for 48 hr. In some experiment IL-2 was added to the culture medium to test its effect on LREF generation.

Supernatants were collected by centrifugation and stored at -80°C until tested.

Cell proliferation assays were performed in several experiments, as previously described (2.2.3).

5.3 Results

5.3.1 CD3+ cells in the PBMC cultures as the source of LREF

LREF activity was found when PBMC were cultured with anti-CD3 MoAb. This stimulation produced an activity comparable with that present in PHA-stimulated PBMC supernatants. There was a dose-dependent increase of LREF activity when anti-CD3 MoAb was increased from 10 ng to 100 ng. PBMC stimulated with anti-CD5 or anti-CD4/CD8 generated insignificant amounts of LREF comparable with that produced by unstimulated PBMC supernatants (Table 5.1). When the MoAb were tested for a direct effect on the neutrophil LTB_4 generation, there were no differences from the buffer controls.

Six millilitres of supernatants from PBMC stimulated by anti-CD3 MoAb were concentrated and separated by Superose-12 PG column in an FPLC system. As with the PHA and allergen-stimulated cultures, a peak of LREF activity was detected, which was associated with a molecular weight range of 35-45 kD (Fig 5.1).

5.3.2 ER+ lymphocytes as the source of LREF

ER+ lymphocytes (containing less than 1% monocytes) were cultured for 48 hr with or without PHA. LREF activity (110% enhancement) was detected in the PHA-stimulated lymphocyte supernatants. Some

activity (approximately 40%) was detected in unstimulated ER+ lymphocyte supernatants (Table 5.2).

When IL-2 (10 units/ml) was added to the ER+ lymphocyte culture, LREF activity was detected only in the presence of PHA and the amount of activity was similar to that of either obtained from PHA stimulated ER+ lymphocyte or from PHA stimulated PBMC. The activities obtained from unstimulated ER+ lymphocyte culture supernatants (either with or without IL-2) were similar. In the same experiment, cell proliferation assay showed that in the presence of PHA both PBMC and ER+ lymphocytes had increased ^{3}H-TdR uptake. IL-2 alone did not induce cell proliferation (Fig 5.2).

5.4 Discussion

In these experiments I have shown that LREF is produced by PBMC incubated with PHA or anti-CD3 MoAb and by non-adherent ER+ lymphocytes incubated with PHA.

Both PHA and anti-CD3 MoAb are mitogenic and are specifically activate T lymphocytes (Mills et al 1985; Williams et al 1985a). In anti-CD3 MoAb stimulation systems, several cytokines (IFN-gamma and GM-CSF) have been shown to be released, but only in the presence of monocytes (Platzer et al 1985; Chang et al 1982). Also some authors claim that both PHA and anti-CD3 MoAb are insufficient stimuli for T cell proliferation (Lis & Sharon 1986). However it seems that the monocyte role in these cultures is to act as an inert support for the lectin or MoAb since anti-CD3 MoAbs which are bound to Sepharose beads will activate T cells (complete internalization of the CD3 T-cell receptor complex by antigen or antibody is associated with tolerization and delivers a suppressive signal to T cells). Furthermore, anti-CD3 isotypes which do not bind to human Fc receptors (IgM & IgG 2b) are not mitogenic in solution (Clark & Ledbetter 1986).

The question of whether or not monocytes play a part in mitogen induced cell proliferation and differentiation remains unresolved. Some reports show

that IL-2 alone is sufficient for the growth and differentiation of lectin-stimulated cytolytic T lymphocytes precursors (Erard et al 1985; Vohr & Hünig 1985), while several other reports showed that macrophages participate in lectin-induced mitogenic stimulation by producing a soluble factor IL-1, which maybe necessary for the production and release of IL-2 (Mizel 1982). My results showed that PHA-driven ER+ lymphocyte culture generate comparable amounts of LREF activity to that generated by PHA-driven PBMC culture, suggesting that lymphocytes are the major source of LREF. The possibility that monocytes may contribute to LREF generation cannot be excluded, even though there was still less than 1% of monocyte contaminated in the lymphocyte preparation but definitive answer will only appear when studies of gene expression are undertaken.

In the classical model of lymphocyte proliferation the sequential activation of macrophages and T lymphocytes is required. After macrophage activation, or in the presence of exogenous IL-1, T lymphocytes express IL-2 receptors and secreted IL-2. This suggested that T cell proliferation depends on continued T cell activation and IL-2 secretion. As expected, my data indicated that exogenous IL-2 did not cause ER+ lymphocyte proliferation and LREF generation (Fig 5.2). The LREF activity obtained from

unstimulated ER+ lymphocytes, either with or without IL-2 was similar to unstimulated PBMC supernatant. These results are consistent with the view that IL-2 itself does not cause T cell activation unless T cells have been pre-activated.

Interleukin-2 was added to PHA stimulated ER+ lymphocytes to ascertain whether or not it augmented LREF generation. The results showed no enhancement of LREF activity although cell proliferation was increased in the presence of IL-2. The failure of IL-2 to enhance LREF generation might be because the high dose of PHA (5 ug/ml) causes maximal LREF release which cannot be augmented by additional IL-2. PHA 5 ug/ml is the optimal concentration for cell proliferation and LREF generation, and substantial amounts of IL-2 were detected in PHA stimulated PBMC supernatants (Fig 3.9). This may explain why the addition of IL-2 to the culture had no marginal benefit for LREF generation. The use of lower concentrations of PHA with purified T-cells and added IL-2 might enable us to clarify the role of IL-2 in PHA-induced LREF generation.

Thus LREF activity can be generated by ER+ lymphocytes under the stimulation of PHA or by PBMC stimulated by anti-CD3 MoAb. The generation of LREF by PHA-stimulated ER+ lymphocytes was not augmented by IL-2. IL-2 itself was not capable of inducing LREF

generation, indicating that T cell activation was necessary for the generation of LREF. Since the monocyte contamination was less than 1%, it is suggested that lymphocytes were the major source of LREF.

5.5 Summary

Experiments were performed in order to demonstrate the cell source of leukotriene release enhancing factor (LREF). LREF was elaborated by mononuclear cells stimulated directly with anti-CD3 MoAb, and also by T-lymphocytes isolated from these cells by ER+ rosetting and stimulated with lectin.

The LREF produced in anti-CD3 stimulated cultures was characterised by FPLC and was shown to have the same molecular weight as the LREF produced by PHA-stimulated PBMC cultures. These experiments suggest that T-lymphocytes were the major source of LREF.

Table 5.1

Identification of CD3+ cells as the major source of LREF

Treatment	LTB_4 (p moles/10^6 cells)	% Enhancement
PHA (5 ug/ml)	56 ± 9*	167 ± 43
Anti-CD3 (100 ng/10^6 cells)	47 ± 3	123 ± 14
Anti-CD3 (10 ng/10^6 cells)	34 ± 2	63 ± 11
Anti-CD5 (100 ng/10^6 cells)	30 ± 4	43 ± 19
Anti-CD4/Anti-CD8 (100 ng/10^6 cells)	27 ± 4	28 ± 19
RPMI 1640 buffer	25 ± 5	19 ± 25

* Results are expressed as mean±s.e.m. (n=3).

Baseline LTB_4 generation (neutrophils + IgG beads) 21 ± 2 p moles/10^6 cells.

Table 5.2

Identification of ER+ cells as the major source of LREF

Treatment	LTB_4 (p moles/10^6cells)	%Enhancement
Lymphocytes (ER+) incubated with PHA	26.0±2.8*	110±11
Lymphocytes (ER+) incubated without PHA	17.5±1.5	40±9

* Results are expressed as mean ± s.e.m. (n=3)
baseline LTB_4 generation (neutrophil + IgG beads)
12±2.1 p moles/10^6 cells

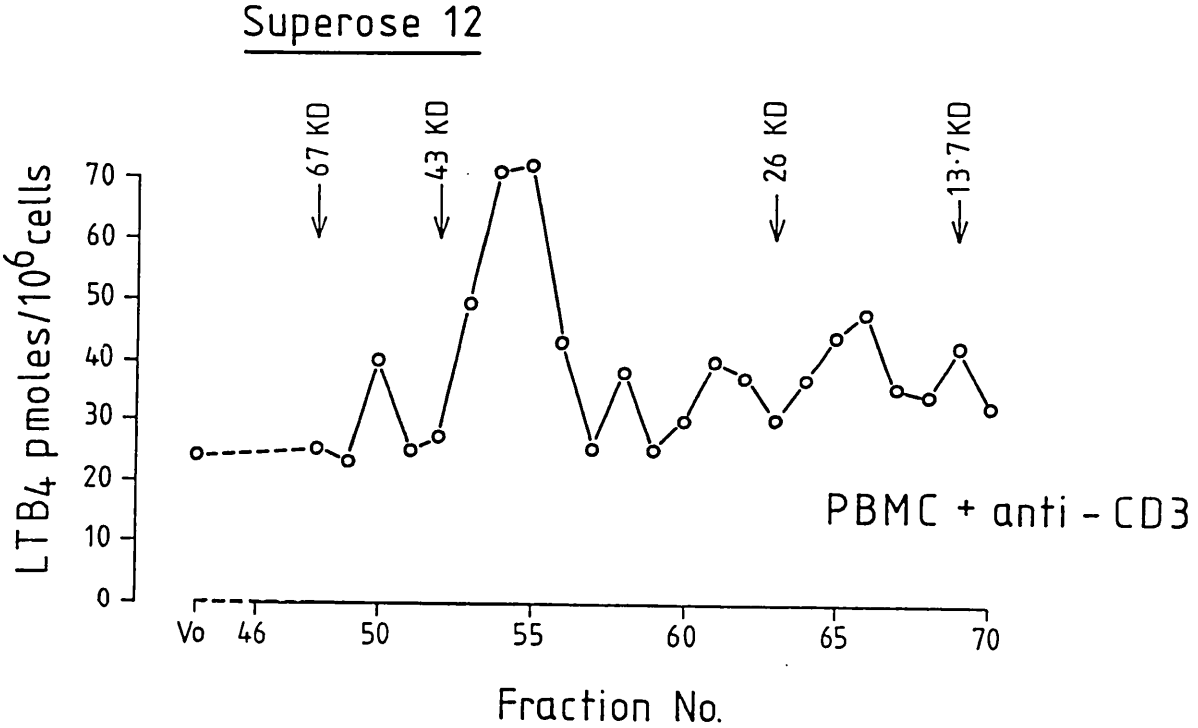

Figure 5.1: Fast protein liquid chromatography (FPLC) analysis of anti-CD3 stimulated PBMC culture supernatant using Superose-12 prep grade gel filtration. Two millilitres of four fold concentrated supernatant from anti-CD3 stimulated PBMC culture were applied to the column, fractions (1ml) were collected from Vo to 70 and assayed for the ability to enhance IgG-dependent LTB_4 released by neutrophils. Molecular weight markers were as indicated.

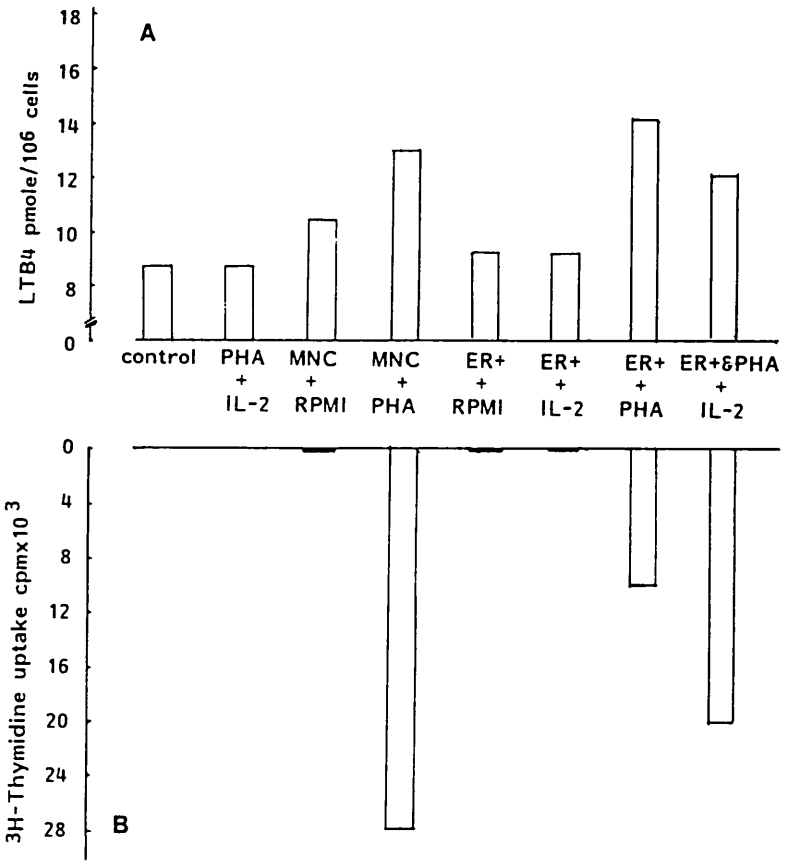

Figure 5.2: Identification of ER+ lymphocytes as the source of LREF. PBMC ($2x10^6$ cells/ml) were separated with or without SRBC, then stimulated with PHA (5 ug/ml) for 48 hrs. Supernatants were collected and tested for the LTB_4 release enhancing activity. IL-2 (10 U/ml) were added in some culture to modulate the LREF activity. (A) Each column represents the effect of supernatant on the ability to enhance IgG-dependent LTB_4 generation by neutrophils. (B) Each column represented the cell proliferation (expressed as ^{3}H-TdR incorporation) at each culture condition. PHA and IL-2 were also used to test their direct effect on the IgG-dependent LTB_4 generation from neutrophils. RPMI-1640 was used as a negative control.

CHAPTER 6.

THE EFFECTS OF MEDIATORS AND DRUGS ON LREF GENERATION

6.1 Introduction

LTB_4 has been shown to be a potent activator of many granulocyte functions and also has regulatory effects on mononuclear cells, augmenting monocyte chemokinesis (Palmer et al 1980), chemotaxis of mixed T-lymphocytes (Payan & Goetzl 1981) and reducing PHA driven proliferation (Rola-Pleszczynski 1985). This effect on proliferation appears to be primarily on the CD4+ (helper/inducer) T cell subset. Cytokine production has also been reported to be modulated by LTB_4 (Rola-Pleszczynski & Lemaire 1985; Johnson & Torres 1984) (for a review see chapter 1).

The cytokines GM-CSF and TNF have been shown to prime granulocytes resulting in generation of increased amounts of leukotriene under calcium ionophore stimulation (Dessein et al 1986; Roubin et al 1987), but production of these cytokines has not been reported to be influenced by LTB_4.

Data presented in the previous chapters demonstrates that LREF can be generated after T-lymphocyte activation by the mitogenic lectin (PHA), anti-CD3 MoAb or relevant allergens. In order to characterise the mechanisms regulating LREF it is of interest to ascertain whether anti-allergic drugs or immunoregulatory mediators can affect LREF generation.

In this study, I have investigated the effects of

LTB_4, gamma-interferon (IFN-gamma), dexamethasone (DEX) and disodium cromoglycate (DSCG), on PHA-induced PBMC proliferation and LREF generation.

6.2 Materials and Methods

6.2.1 PBMC cultures

PBMC ($2x10^6$cells/ml) were incubated with or without PHA (5 ug/ml) in the presence of different concentrations of LTB_4 (Miles Scientific, Slough, UK.), recombinant gamma-interferon (IFN-gamma) (Biogen Res Corp., Cambridge, MA.), dexamethasone (Sigma Chemicals Co. Ltd., Poole, Dorset) or DSCG (Fisons plc, Pharmaceutical Division, Loughborough, England) for 48 hr in a 5% CO_2 atmosphere at 37°C and 95% humidity. After incubation the cell suspensions were centrifuged at 400g for 10 min at 20°C and the sterile cell free supernatants were collected by centrifugation and stored at -80°C until tested. Cell viability, as assessed by trypan blue dye exclusion, was > 90% after 48 hr culture. Controls included a) medium alone, b) cell supernatants incubated in the absence of PHA. Cell proliferation was assayed in all experiments as previously described. In LTB_4 culture experiments, all the supernatants were dialysed against PBS for 16 hr to remove the remaining LTB_4.

6.2.2 Effect of dexamethasone and PBMC supernatants on LTB_4 generation by neutrophils

The direct effect of dexamethasone, on neutrophil LTB_4 generation was tested at the same concentrations as in the PBMC cultures. Neutrophils were pre-treated with different concentration of dexamethasone for

60 min at 37°C, then IgG-coated beads were added and incubated for a further 15 min. The cell free supernatants were collected by centrifugation and stored at -80°C.

6.2.3 Gel filtration

Twenty millilitres of PHA-stimulated PBMC (cocultured with or without LTB_4) were concentrated to 2ml and fractionated by gel filtration using Superose-12 PG in an FPLC system as described in Chapter 3.2.6a.

6.3 Results

6.3.1 LTB_4 effect on the generation of LREF activity by PBMC

The introduction of LTB_4 with PHA at the onset of incubation resulted in reduced generation of LREF activity that was significant at the concentrations of 10^{-8}M and 10^{-9}M ($p < 0.05$) (Fig 6.1). LTB_4 had no inhibitory effect when cocultured with unstimulated PBMC; LREF activity obtained from the supernatant of unstimulated PBMC cultured with LTB_4 showed no statistical difference by comparison with that of unstimulated PBMC cultured without LTB_4.

In parallel experiments, I measured the effect of LTB_4 on ^{3}H-TdR incorporation in PBMC cultures (Table 6.1). LTB_4 caused significant inhibition of PHA-induced cell proliferation, whereas LTB_4 had no significant effect on ^{3}H-TdR incorporation in unstimulated PBMC cultures.

The PBMC supernatants were dialysed against PBS and LTB_4 concentrations in the dialysed supernatants were determined and were found to be less than 1 p mole/ml in all cases.

6.3.2 Gel filtration

I have previously shown that LREF was associated with a molecular weight of 35-40 kD as determined by gel filtration (Fig 3.9). To test whether the inhibitory effect of LTB_4 on LREF generation was a

direct effect on LREF production or an indirect effect involving generation of inhibitory proteins, concentrated PBMC supernatant was fractionated by Superose-12 PG column in the FPLC system, and each fraction was assayed for LREF activity. The maximal enhancement of LTB_4 generation was 410% at the molecular weight region of 35-40 kD from the PHA-stimulated PBMC supernatant. This region of activity was inhibited by co-culturing PHA-stimulated PBMC with LTB_4 and thus the inhibition of LREF generation appears to be due to reduced LREF production (Fig 6.2) and not to the production of inhibitory proteins.

6.3.3 DEX effect on the generation of LREF activity by PBMC.

LREF activity in PHA-stimulated PBMC supernatants was reduced by DEX in a dose dependent manner ($p < 0.05$ at all tested doses) (Fig 6.3). In the proliferation assay, DEX inhibited PHA-induced cell proliferation (Table 6.2). There was no difference in LREF production between the unstimulated PBMC culture in the presence or absence of DEX.

The possibility that DEX might have a direct effect on LTB_4 generation by neutrophils was tested. Neutrophils were pre-treated with DEX for 60 min, then added to IgG-coated beads. This experiment showed that LTB_4 generation was not inhibited by DEX in my

IgG-beads stimulation system (Fig 6.3).

6.3.4 IFN-gamma effect on the generation of LREF activity by PBMC

The generation of LREF activity in PHA-stimulated PBMC cultures was not significantly reduced by IFN-gamma at all concentrations tested, though the ^{3}H-TdR incorporation was inhibited in a dose dependent manner (Table 6.3).

When unstimulated PBMC were cultured with IFN-gamma, LREF generation showed no difference from buffer control (Fig 6.4), and there were no differences in ^{3}H-TdR uptake at the various concentrations of IFN-gamma.

6.3.5 DSCG effect on the generation of LREF activity by PBMC

The generation of LREF activity in PHA-stimulated PBMC cultures was not inhibited by DSCG at all concentrations tested. In addition, the cell proliferation assay was not inhibited by DSCG as examined by ^{3}H-TdR uptake (Table 6.4). When unstimulated PBMC were cultured with DSCG, LREF generation again showed no difference from buffer control (Fig 6.5).

6.4 Discussion

The inhibitory effect of LTB_4 on PHA-stimulated T-cell proliferation is well known (Gualde et al 1985), and I have confirmed this observation. Similarly, dexamethasone has been reported to inhibit PHA-stimulated blastogenesis (Blomgren 1974; Blomgren & Andersson 1976), and the production of several cytokines (Snyder & Unanue 1982; Ralph et al 1978). In this investigation I have shown that LTB_4 and dexamethasone reduced LREF generation from PHA-stimulated human PBMC, as well as attenuating the proliferative response to PHA. Using gel filtration, there was no evidence that an inhibitory regulatory protein was induced by LTB_4, and it appears that this reduction in LREF activity is due to reduced production of the 35-45 kDa leukotriene release enhancing factor (LREF) which I have previously characterised. Although this does not exclude the possibility that an inhibitory protein might migrate with the LREF.

It might be suggested that LREF generation was directly related to cell proliferation. However the relationship between the two does not appear to be a simple one, since the degree of suppression of LREF activity was not parallel to the degree of suppression of proliferation by LTB_4, and IFN-gamma reduced T cell proliferation but had no detectable effect on LREF

generation.

The reduction in LREF is unlikely to be attributed to carry over of LTB_4 from the cultures since no LTB_4 was detectable in the supernatants after dialysis treatment. These results are consistent with the previously proposed immunoregulatory role of LTB_4 (Rola-Pleszczyznski 1985).

IFN-gamma has been identified as the lymphokine that mediates LTB_4-induced regulation of IL-1 production by monocytes (Rola-Pleszczyznski et al 1987). LTB_4 is also able to enhance IFN-gamma production of T4+ cells and to inhibit IFN-gamma generation by T8+ cells (Rola-Pleszczynski et al 1987). Thus it might be suggested that LTB_4 inhibition of LREF generation is mediated by IFN-gamma. However, in my system the addition of IFN-gamma did not affect the generation of LREF, though cell proliferation was inhibited by IFN-gamma in the parallel experiments. These results suggest that the effect of LTB_4 on LREF generation was not through the action of IFN-gamma. Furthermore the inhibition of cell proliferation was not due to a cytotoxic effect of LTB_4 or IFN-gamma, since cell viability after culture was always greater than 90%. It remains possible that IFN-gamma and LTB_4 affect different T cell subsets, leading to differential effects on LREF production and proliferation.

It has been reported that LTB_4 can augment monocyte function and enhance IL-1 and tumor necrosis factor (TNF) generation (Rola-Pleszczynski & Lemaire 1985; Gagnon et al 1987), however, LREF generation is reduced. Therefore it seems unlikely that LREF is generated from monocytes since LTB_4 enhances monokine generation and inhibits LREF generation. Taken together with my previous findings that LREF can be generated from human T4+ cell lines and from monocyte depleted ER+ lymphocytes (Chapter 4&5) it seems unlikely that LREF is derived from monocytes.

LTB_4 generation depends on the activation of phospholipase A2 which cleaves arachidonic acid from membrane phospholipids so providing substrate for the 5-lipoxygenase pathway. Glucocorticoids can induce a phospholipase A2 inhibitory protein in neutrophils (Hirata et al 1980). My results showed that the capacity of neutrophils to generate LTB_4 was not inhibited by the pre-treatment of neutrophils with DEX for up to 60 min. This is not entirely surprising since new protein synthesis in response to DEX usually takes several hours (Plaut 1987).

Since LREF can be generated by allergen--stimulated PBMC cultures (chapter 4), I examined the effects of the anti-allergic drug DSCG on LREF generation by PHA-stimulated PBMC. In these experiments DSCG had no effect on LREF generation nor

did it have any effect on cell proliferation at all concentrations tested (range of 10^{-5}M to 10^{-8}M). Although DSCG has been reported to have an immunoregulatory effect (at very high concentration) (10^{-3}M) (Wang & Wang 1986), the effect of DSCG on cell mediated immunity remains ill defined.

In conclusion, LTB_4 can down regulate LREF generation by T-cells, representing a form of negative feedback control which may influence the magnitude of the inflammatory response. This inhibition appears to involve specific suppression of the production of LREF and in contrast to other LTB_4 driven regulatory systems, does not appear to be mediated by IFN-gamma.

6.5 Summary

The regulation of production of LREF was studied by culturing PBMC with PHA and various concentrations of LTB_4, dexamethasone (DEX), disodium cromoglycate (DSCG) and gamma-interferon.

LREF production was decreased in a dose-dependent manner by LTB_4 (10^{-8} to 10^{-11}M). This decrease in LREF activity was shown by gel filtration to be associated with a reduction in the amount of 35-45 kD protein previously characterised as LREF. A similar reduction in LREF generation was observed with DEX. DEX had no direct effect on LTB_4 production by neutrophils in this assay system. In contrast, gamma-interferon reduced PBMC proliferation but did not affect LREF generation. DSCG had no effect on both LREF generation and PBMC proliferation.

These findings indicate that the regulation of LREF production is not simply correlated with PBMC proliferation and that negative feedback by LTB_4 is a possible regulatory mechanism. These experiments provide further evidence for interactive regulation between human neutrophils and PBMC, through the mediators LTB_4 and LREF.

Table 6.1

Effect of LTB_4 on PHA-stimulated PBMC proliferation

LTB_4 (M)	PHA (5 ug/ml)	c.p.m.
0	+	31342 ± 6588a
10^{-11}	+	31850 ± 6647
10^{-10}	+	29636 ± 7195
10^{-9}	+	29136 ± 7379b
10^{-8}	+	27742 ± 6573b
0	-	343 ± 36
10^{-11}	-	364 ± 65
10^{-10}	-	323 ± 37
10^{-9}	-	400 ± 75
10^{-8}	-	507 ± 81

a. PBMC were cultured with or without PHA in the presence of various concentrations of LTB_4 for 48 hour and proliferation was assessed by addition of ^{3}H-TdR for the final 6 hour of the culture
Data represents the mean ± s.e.m. of four experiments

b. T cell mitogenesis was significantly inhibited by LTB_4 at 10^{-9} and 10^{-8}M when compared with the control culture with no added LTB_4 ($p < 0.05$).

Table 6.2

Effect of dexamethasone (DEX) on PHA-stimulated PBMC proliferation

Dex (M)	PHA (5 ug/ml)	c.p.m.
0	+	41301 ± 6808a
10^{-8}	+	15550 ± 4367b
10^{-7}	+	6066 ± 1284b
10^{-6}	+	4639 ± 1463b
10^{-5}	+	3122 ± 463b
0	-	537 ± 36
10^{-8}	-	165 ± 21
10^{-7}	-	352 ± 18
10^{-6}	-	155 ± 33
10^{-5}	-	198 ± 54

a. PBMC were cultured with or without PHA in the presence of various concentrations of DEX for 48 hours and proliferation was assessed by addition of ^{3}H-TdR for the final 6 hour of the culture
Data represents the mean ± s.e.m. of four experiments

b. T-cell mitogenesis was significantly inhibited by DEX from 10^{-8} to 10^{-5} when compared with the control culture with no added DEX ($p<0.01$).

Table 6.3

Effect of IFN-gamma on PHA-stimulated PBMC proliferation

IFN-gamma (U/ml)	PHA (5 ug/ml)	c.p.m.
0	+	31301 ± 7139[a]
1	+	32402 ± 9240
10	+	29746 ± 10079
100	+	28259 ± 8883
1000	+	23060 ± 7671
0	-	385 ± 44
1	-	545 ± 79
10	-	327 ± 11
100	-	314 ± 82
1000	-	751 ± 32

a. PBMC were cultured with or without PHA in the presence of various concentrations of IFN-gamma for 48 hours and proliferation was assessed by addition of ^{3}H-TdR for the final 6 hour of the culture.
Data represents the mean ± s.e.m. of three experiments.

Table 6.4

Effect of DSCG on PHA-stimulated PBMC proliferation

DSCG (M)	PHA 5ug/ml	c.p.m.
10^{-5}	+	48827±29a
10^{-6}	+	44795±13
10^{-7}	+	43509±56
10^{-8}	+	44662±350
0	+	49061±163
0	-	205±82

a. PBMC were cultured with or without PHA in the presence of various concentrations of DSCG for 48 hours and proliferation was assessed by addition of ^{3}H-TdR for the final 6 hour of the culture.
Data represent the mean ± s.e.m. of four experiments.

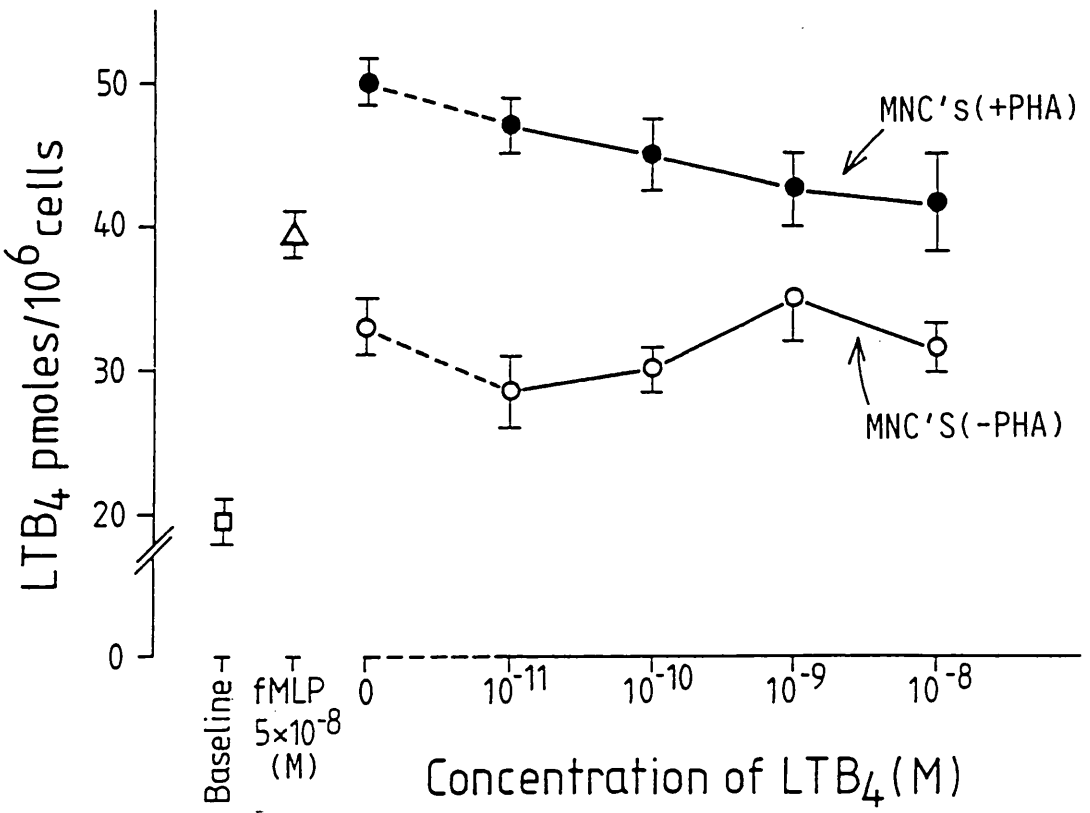

Figure 6.1: Effect of LTB_4 on the generation of LREF from PHA-stimulated PBMC cultures. PBMC were treated with various concentrations of LTB_4 in the presence (closed circle) or absence (open circle) of PHA for 48 hours. Cell free supernatants were collected and dialysd against PBS, and then tested for the LREF activity. Each point represents mean ± s.e.m. of four experiments.

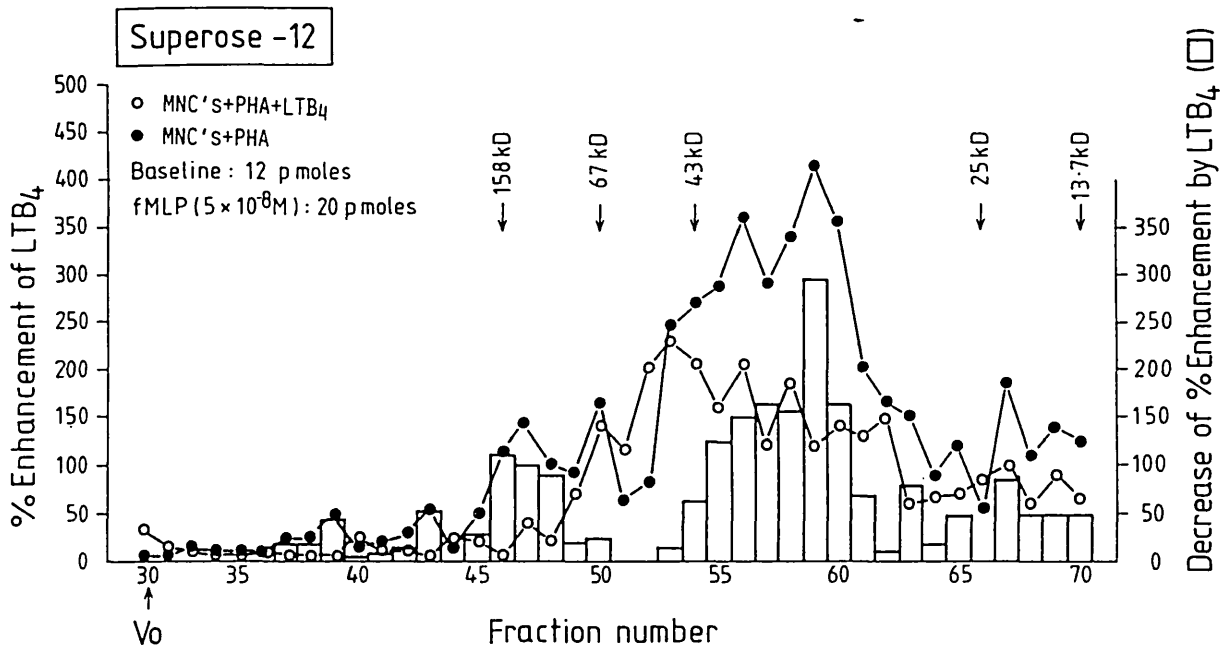

Figure 6.2: Comparison of LREF recovered by FPLC gel filtration of the supernatants from PHA-stimulated PBMC cultures in the presence or absence of LTB_4. Two millilitres of 10 fold concentrated PBMC supernatants from PHA-stimulated PBMC cultures (either with (closed circle) or without (open circle) LTB_4) were applied to the column, 1 ml of column fractions were collected and tested for enhancement of LTB_4 generation by neutrophils in response to IgG beads. Data presented as % enhancement of LTB_4 as compared with buffer control. The decrease in LREF generation attributable to LTB_4 in the culture system is represented by the open columns. Molecular weight markers were indicated at the top of the figure. In this experiment, buffer control in 12 p moles/10^6 cells, fMLP ($5x10^{-8}$M) obtained 20 p moles (=67% enhancement).

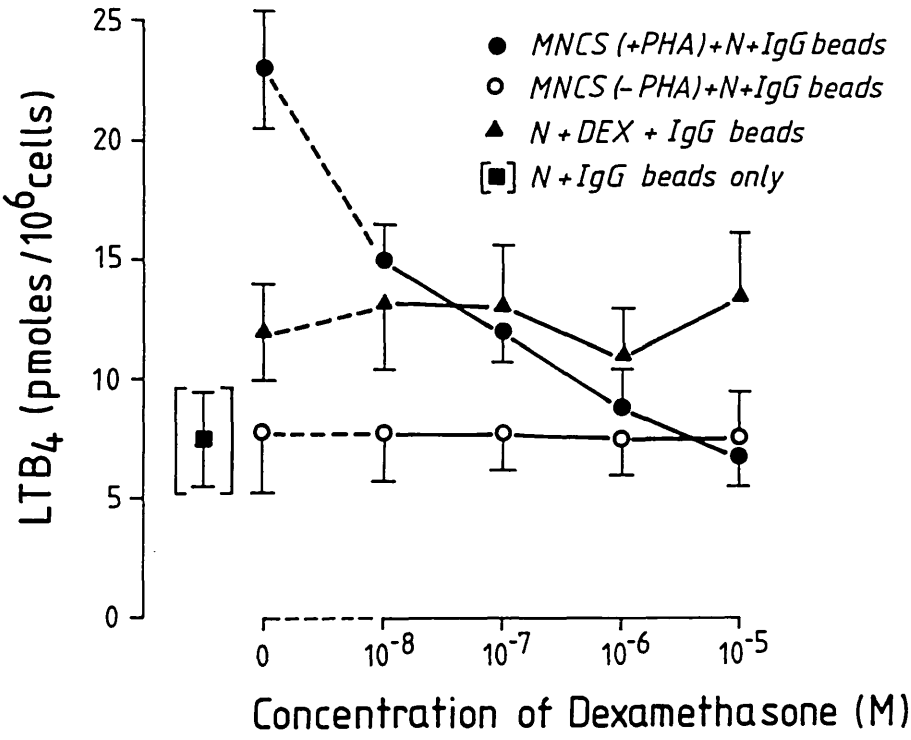

Figure 6.3: Effect of dexamethasone (DEX) on the generation of LREF from PHA-stimulated PBMC cultures. PBMC were treated with various concentrations of DEX in the presence (closed circle) or absence (open circle) of PHA for 48 hours. Cell free supernatants were collected and tested for the LREF activity. The direct effect of DEX on LTB_4 generation by neutrophils (N) in response to IgG-beads was also measured (closed triangle). Each point represents mean ± s.e.m. of four experiments.

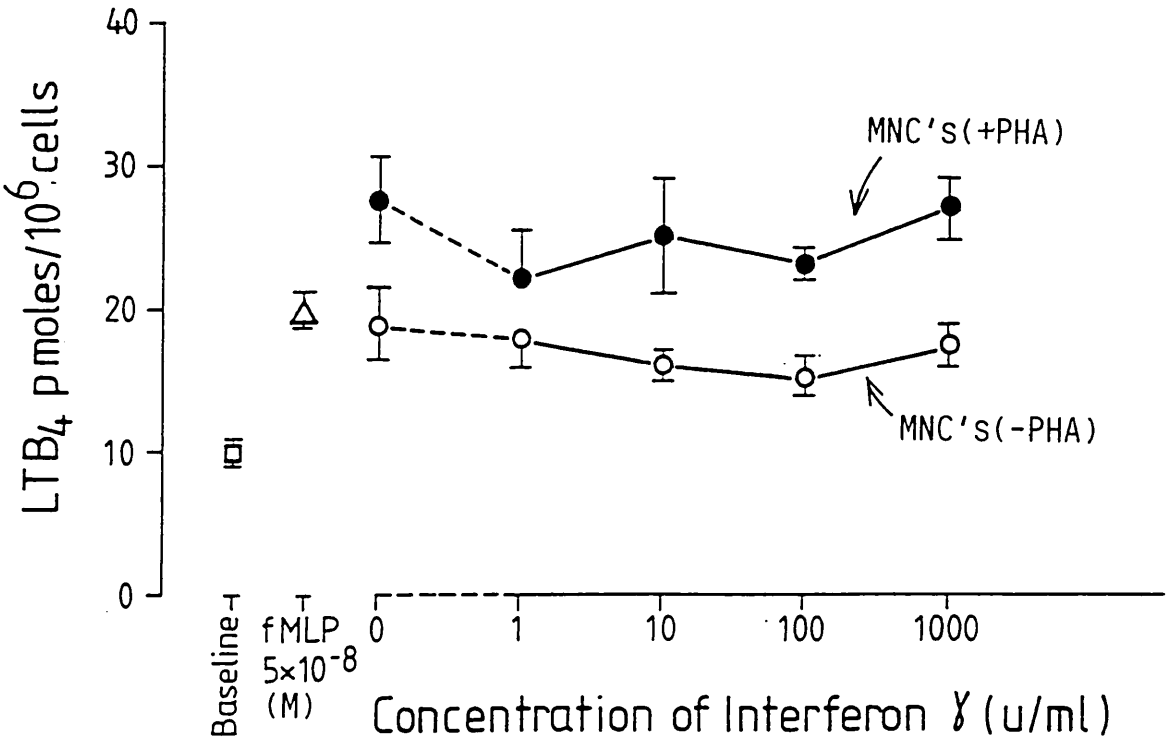

Figure 6.4: Effect of IFN-gamma on the generation of LREF from PHA-stimulated PBMC cultures. PBMC were treated with various concentrations of IFN-gamma in the presence (closed circle) or absence (open circle) of PHA for 48 hours. Cell free supernatants were collected and tested for the LREF activity. Each point represents mean ± s.e.m. of three experiments.

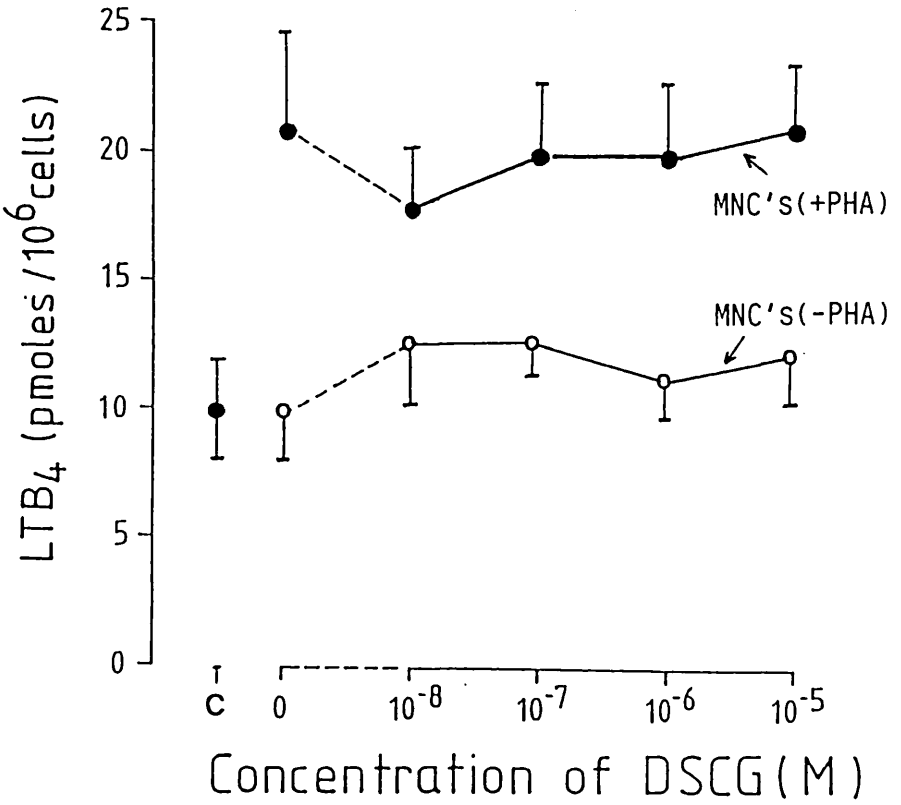

Figure 6.5: Effect of DSCG on the generation of LREF from PHA-stimulated PBMC cultures. PBMC were treated with various concentrations of DSCG in the presence (closed circle) or absence (open circle) of PHA for 48 hours. Cell free supernatants were collected and tested for the LREF activity. Baseline LTB_4 generation (C) obtained 10 ± 2 p moles/10^6cells. Each point represents mean $\pm$ s.e.m. of four experiments.

CHAPTER 7.

PURIFICATION AND CHARACTERIZATION OF LREF

7.1 Introduction

I have described an LTB_4 release enhancing factor (LREF) in serum-free supernatants of PHA-stimulated human PBMC cultures, which significantly enhances LTB_4 release by neutrophils in vitro, following stimulation with IgG-coated Sepharose beads. My initial characterization showed LREF was associated with a molecular weight of 35-45 kD by gel filtration and a pI of 5.0-5.2. In this study I have refined the methodology in order to purify LREF from PHA-stimulated PBMC bulk cultures.

One of the major problems in the purification of lymphokines is the difficulty in obtaining large amounts of material. It is apparent that LREF is generated in very small amounts by antigen or mitogen stimulated human lymphocytes. Recently leukapheresis has permitted the recovery of large numbers of PBMC (Feige & Sorg 1984), which are functionally comparable with PBMC isolated by conventional methods. Leukapheresis is commonly used for treatment of paraproteinemia, T cells from such patients are thought to behave normally in functional assays (Feige & Sorg 1984). Moreover lymphokine generation has been studied in leukemic-lymphoma cell lines (Meshulam et al 1982). I therefore attempted to purify LREF from PBMC recovered from leukapheresis blood packs.

In this chapter I describe a method for the

purification of LREF by the sequential use of gel filtration, chromatofocusing, and anion exchange chromatography which has resulted in the production of a homogenous product. Protein iodination was used to facilitate assessment of the efficacy of the purification steps. From 1.2 litre of PBMC supernatant 2 ug of protein were recovered. The molecular weight and isoelectric points were fully certified by two methods, recovery of biological activity from column fractions and identification of a protein band on isoelectric focusing and SDS-PAGE gels. The purified material was also tested for esterase activity, by treatment with serine esterase inhibitors such as phenylmethylsulphonyl fluoride (PMSF), in order to assess the relationship to LIF (Rocklin & Rosenthal 1977).

7.2 Materials and Methods

7.2.1 PBMC cultures

PBMC supernatant was prepared from the cells recovered from the leukapheresis packs of 7 different donors. Cells were incubated with PHA for 2 hr, washed once, and cultured for a further 48 hr in PHA-free RPMI-1640. The supernatants were pooled and concentrated 100-fold using a hollow fibre cartridge (HIP 5-20) with a molecular weight cut-off of 5000 daltons (MWCO 5000), followed by Diaflo ultrafiltration on a YMCO 5000 (Amicon Ltd., U.K.).

7.2.2 Gel filtration

Gel filtration chromatography was performed as previously described (3.2.6a). Each fraction was tested for neutrophil LTB_4 release enhancing activity. The viability of neutrophils after incubation with FPLC fractions was also evaluated using trypan blue dye exclusion.

7.2.3 Chromatofocusing

Three millilitres of the peak of neutrophil LTB_4 release enhancing activity obtained from Superose 12 PG were concentrated to 1 ml, dialysed against 0.025M bis-tris-iminodiacetic acid buffer, pH 7.1 (16 hr, 4°C), and applied to a Mono-P HR 5/20 column (Pharmacia) equilibrated in the same buffer. A 10% solution of polybuffer 74, adjusted to pH 4 with iminodiacetic acid, was used for elution at a flow

rate of 0.5 ml/min. A linear pH-gradient of 40 ml from pH 7 to 4 was generated and 1 ml fractions were collected. The fractions were dialysed and subsequently tested for LREA. The column was regenerated with 2M iminodiacetic acid disodium salt and re-equilibrated with the starting buffer before each experiment.

7.2.4 Isoelectric focusing (IEF)

IEF was performed in polyacrylamide gels containing Pharmalyte (R) carrier ampholytes using the Phast system (Pharmacia). Half millilitre of the FPLC fractions containing LREA were dialysed against distilled water (Spectra/por 1 MWCO 6000-8000, Spectrum Medical Industries Inc., Los Angeles, U.S.A.) and lyophilised. Buffer and inactive column fractions were used as controls. Samples were resuspended in 20 ul of 0.9% normal saline and separated on Phast Gel IEF 3-9. Gels were stained by a protein silver stain method (Bio-Rad Laboratories Ltd., U.K.). Isoelectric point calibration standards in the range of pI 3-10, were run on each occasion: amyloglucosidase 3.50, soybean trypsin inhibitor 4.55, beta-lactoglobulin A 5.20, bovine carbonic anhydrase B 5.85, human carbonic anhydrase B 6.55, horse myoglobin 6.85, methyl red (dye) 6.75, horse myoglobulin 7.35, lentil lectin 8.15, lentil lectin 8.45, lentil lectin 8.65, trypsinogen 9.30 (Pharmacia).

7.2.5 Protein iodination

Protein was labelled by conjugation with the hydroxy-succinimide ester of 3-(4-hydroxy,5-[^{125}I] iodophenyl) propionic acid (Amersham International plc, Amersham, Bucks, U.K.) according to the method described by Bolton and Hunter (Bolton & Hunter 1973). One millilitre of the pooled fractions containing LREF obtained from the Mono-P separation was dialysed against 0.005M borate buffer, pH 8.5, and concentrated to 50 ul (3 ug). This was added to the dried iodinated ester and the reaction mixture agitated for 15 min at 0°C. Unreacted ester was reacted with 0.5 ml of 0.2M glycine in 0.1M borate buffer, pH 8.5, for 5 min at 0°C to avoid subsequent conjugation to carrier proteins. The iodinated LREF was separated from the other labelled products of the conjugation reaction, glycine conjugate and 3-(4-hydroxyphenyl) propionic acid by gel filtration using Sephadex G-50 (fine grade) (Pharmacia) (0.9x25 cm column). To minimize the loss of labelled protein by absorption, the Sephadex was equilibrated and eluted with 0.05M phosphate buffer, pH 7.5, containing 0.25% w/v gelatin.

7.2.6 Anion exchange chromatography

One millilitre of LREF recovered from Mono-P column fractions or labelled LREF was dialysed for 24 hr at 4°C against 20mM bis-tris-HCl buffer, pH 6.5

(buffer A), and applied to a Mono-Q HR 5/5 column (Pharmacia) which had previously been equilibrated with the same buffer. A bis-tris-HCl buffer containing 1.0M NaCl, pH 6.5, was used for elution (buffer B) at a flow rate of 1 ml/min. The molar concentration gradient was increased at a rate of 17.5mM/ml with three intervals at 100mM and 250mM and 500mM NaCl. The column was washed with buffer B and re-equilibrated with buffer A. One millilitre fractions were collected and dialysed for 24 hr against PBS (pH 7.3) and subsequently tested for LREF activity and protein concentration.

7.2.7 SDS-PAGE

Polyacrylamide gel electrophoresis was performed in the presence of sodium dodecyl sulphate (SDS-PAGE) using the Phast system (Pharmacia). Fractions (0.5 ml) from a Mono-P column run or labelled LREF were dialysed against distilled water and lyophilised. Samples were resuspended in 20 ul of buffer containing 2.5% SDS, 10mM tris/HCl, 1mM EDTA, pH 8.0, with or without 5% β-mercaptoethanol, and heated for 10 min (100°C). Bromophenol blue was added at approximately 0.01%. Any insoluble material was removed by centrifugation (10,000g for 5 min: Beckman Microfuge) to prevent streaking patterns in the developed gel.

Samples were separated on SDS-Phast gels (8-25% acrylamide gradient). The gels were stained by a

protein silver stain method (Bio-Rad). Phosphorylase (94 kD), bovine serum albumin (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD), Soybean trypsin inhibitor (20.1 kD) and alpha-lactalbumin (14.4 kD) were used as molecular markers. The gels were placed on a filter paper, dryed and, when appropriate, exposed to X-ray film at -70°C for three days for autoradiography.

7.2.8 Treatment of LREF with serine esterase inhibitor PMSF

PMSF (phenylmethylsulphonyl fluoride) was obtained from Sigma Chemicals and dissolved in 100% isopropanol at a concentration of 0.1M. The purified LREF and its control fractions were incubated with 10^{-4}M PMSF for 30 min at 37°C. After incubation, the supernatants were dialysed extensively against 0.15M PBS for 18 hr and then aliquoted and stored at -70°C until tested. 0.1% isopropanol and PBS were used as negative controls.

7.3 Results

7.3.1 Purification of LREF by gel filtration, chromatofocusing and anion exchange chromatography

LREF was progressively purified from 100 times concentrated PBMC supernatants using a combination of Superose-12 PG gel filtration, chromatofocusing, and anion exchange chromatography. This gave a homogenous preparation of LREF as judged by SDS-PAGE analysis.

Concentrated PBMC supernatants were fractionated by gel filtration using Superose-12 PG in an FPLC system. In the first set of experiments, the column was eluted with PBS buffer. After testing for LREA the fractions (7 ml) associated with the activity were pooled (MW range 35 to 70 kD) and applied to the same column equilibrated with 20mM NH_4HCO_3 buffer. The highest peak of enhancement of LTB_4 release of this sample, had a molecular weight of 40 kD to 60 kD. (Fig 7.1). The viability of neutrophils after incubated with each FPLC fraction showed all above 90%.

LREF was further purified and characterized in terms of its isoelectric point by chromatofocusing on a Mono-P column in the FPLC system. Samples were focused on a pH gradient between 7.1 and 4.0. The highest peak of specific activity was recovered between pH 5.0 and 5.2 (Fig 7.2)

These samples were further purified by anion exchange chromatography (Mono-Q) column in FPLC system. Preliminary experiments using continuous sodium chloride gradients failed to resolve various protein species and two gradient steps were introduced to improve resolution. Two peaks of LREF activity were observed in the material which was eluted with 200mM and 300mM NaCl in 20mM bis-tris pH 6.5. Most of the activity was detected in the first peak and some in the second. When the salt gradient was continuously applied, no further activity was observed (Fig 7.3).

Protein concentration in each fraction was determined by Bio-Rad protein microassay using bovine-gamma-globulin as a standard (Bio-Rad Laboratories Ltd., U.K.). The specific activity (% Enhancement of LTB_4 generation per microgram of protein) was summarized in Table 7.1.

7.3.2 Analysis of the purity of LREF by isoelectric focusing and SDS-PAGE

Samples containing the highest LREF were analysed by Phast Gel IEF 3-9. Several protein bands were detected, all having an isoelectric point (PI) between 4.6 and 5.2 (Fig 7.4). Samples containing the highest LREF specific activity were separated by Phast Gel SDS gradient 8-25. Only one protein band was detected, which was associated with a molecular weight of 60 kD

(Fig 7.5). When the samples were prepared in reducing conditions and separated by SDS-PAGE, the protein band was found at 60 kD (Fig 7.6). These samples were also used for protein iodination. Several steps were used to study the purity of ^{125}I-labelled protein. Firstly, ^{125}I-protein was separated by Phast Gel SDS gradient 8-25 and only one protein band associated with a molecular weight of 60 kD (Fig 7.7) was detected by autoradiography. Secondly, ^{125}I-protein was applied to anion exchange chromatography and radioactivity was recovered in two peaks (fractions 19 & 20, and fractions 32 & 33), which were eluted with 200mM and 300mM NaCl in 20mM bis-tris pH 6.5 (Fig 7.8a). Subsequent chromatography on a Superose-12 PG column showed that the radioactivity in both Mono-Q column fractions was associated with a molecular weight of 40 kD (Fig 7.8b).

7.3.3 PMSF effect on LREF activity

LREF preparations were treated with PMSF for 30 min at 37°C, extensively dialysed to remove the inhibitors, and each preparation was assayed for residual activity. Results showed that 10^{-4}M PMSF can only abolish some LREF activity but this did not reach a significant level when compared with that of diluent control. Isopropanol had no inhibitory effect on LREF activity (Fig 7.9).

7.4 Discussion

The availability of large quantities of PBMC from leukapheresis permitted the development of a protocol to purify LREF. From 1200 millilitres of cell free supernatant, 2 ug of protein with LREF activity was obtained by the sequential use of gel filtration, chromatofocusing and anion exchange chromatography.

Gel filtration can separate proteins on the basis of molecular size using porous beads with a defined pore size. Small molecules are distributed in the aqueous solution both inside the beads and between them, whereas large molecules are excluded from the beads and confined to the solution between them and therefore flow more rapidly through the column. Superose-12 PG can give a good resolution of proteins in the molecular weight range 1-300 kD. Using this method and amicon ultrafiltration (MWCO 5000) we can exclude lower molecular weight protein contamination.

Chromatofocusing and isoelectric focusing (IEF) gel can separate proteins on the basis of their isoelectric point (pI). The pI of a protein is the pH at which its nett charge is zero. Each protein will move until it reaches a position in the gel at which the pH is equal to the pI of the protein. Anion exchange chromatography can separate proteins on the basis of their nett charge. When sample components at pH above their pI, negatively charged components bind

to such columns containing quaternary amines and then be eluted by increasing the concentration of sodium chloride in the elution buffer. SDS-PAGE can separate protein on the basis of mass electrophoresis in a polyacrylamide gel under denatured conditions (all non-covalent bonds are disrupted by SDS and the disulphide bonds can be further reduced by adding β-mercaptoethanol). The negatively charged SDS-protein complex migrate in the direction of the anode. Using polyacrylamide gel gradient also have molecular sieving effect to separate proteins. Small proteins move rapidly through the gel, whereas large ones remain at the top.

After purification, LREF was found to have a molecular weight of 35-45 kD as determined by gel filtration and 60 kD as determined by SDS-PAGE under both reducing (with β-mercaptoethanol) and non-reducing conditions. The isoelectric point was in the region 5.0-5.2 as determined by each of two methods. Firstly, chromatofocusing, using a Mono-P column lead to recovery of LREF activity in fractions eluted at pH 5.0-5.2; secondly, isoelectrofocusing gels revealed several protein bands (pH 5.0-5.2) after treatment with silver stain.

The purification was monitored using iodinated material obtained from sequential gel filtration and chromatofocusing. Two peaks of LREA recovered after

Mono-Q separation and these coeluted with radio-labelled LREF with molecular weights of 40 kD as determined by gel filtration. This suggests that LREA may be composed either of two different molecular species which can only be separated by anion exchange chromatography or a single protein with different percentages of glycosylation. The multiplicity of bands obtained on IEF also suggests that the protein may exist in several glycosylation states.

Molecular weight determination by SDS-PAGE and gel-filtration are often different especially those of glycoproteins which may behave anomalously on the SDS-system. Gel-filtration may conceivably underestimate molecular weight, due either to partial absorption of the protein to the column packing material or to a particularly globular protein structure, conversely SDS-PAGE can give an overestimate of molecular weight because of the reduced level of SDS binding by glycosylated, as compared with non-glycosylated, proteins (the lower SDS binding results in a decreased charge-to-mass ratio, consequently, a decreased mobility is observed during electrophoresis, and hence the glycoprotein travels with a higher molecular weight (Poduslo 1981). Molecular weights based on SDS-PAGE are probably a more accurate estimate than those of molecular size in gel filtration, but should only be regarded as

preliminary estimates. Definitive estimation of the molecular weight must await compositional analysis of the isolated component and amino acid sequencing.

The molecular weight and pI of LREF was similar to that of the leukocyte inhibitory factor (LIF) which has been reported to be a serine esterase (Rocklin & Rosenthal 1977), whose activity can be blocked by treatment with inhibitor such as diisopropyl phosphofluoride (DFP) or phenylmethlysulfonyl fluoride (PMSF). It was therefore of interest to determine whether LREF was sensitive to esterase inhibitors. The results indicated that LREF can only partially be inactivated by the esterase inhibitor PMSF. This implied that either esterase activity found in LIF was not present in my LREF preparation or esterase activity is not the biological functional site for LREF activity. At this time, it has still not been possible to distinguish between LIF and LREF by using enzyme inactivation method.

This study has shown that large amounts of LREF can be obtained and generated from leukapheresis blood pack. Studies making use of these sources should help in the further characterization and understanding of the roles of lymphokines in the pathogenesis of allergic inflammation.

7.5 Summary

In this study I attempted to purify LREF from PBMC recovered from leukapheresis blood packs. Approximately 2 ug of LREF was obtained from 1.2 litre of PHA-stimulated PBMC bulk culture supernatants, by sequential use of ultrafiltration, gel filtration, chromatofocusing and anion exchange chromatography. After each step, LREF activity and protein concentration were determined. Protein iodination was employed to monitor and facilitate the purification. The molecular weight of LREF was 60 kD as determined by SDS-PAGE. The pI was 5.0-5.2 as determined by gel IEF and chromatofocusing. These studies indicate that LREF can be generated by bulk cultures of PBMC from leukapheresis blood packs.

The protease inhibitor PMSF was used to determine whether the purified LREF was identical to the previously described lymphokine LIF. Since LREF activity was resistant to PMSF, it appears at present that LREF is biologically distinct from LIF, though their molecular size and pI are very similar.

Table 7.1

Progressive Purification of LREF

	Volume (ml)	Mean enhancement of LTB_4 generation (%)	Mean protein concentration (ug)	Mean specific activity (% enhancement/ug)
Superose 12 (PBS)(1)	7	103	41.3	2.1
Superose 12 (NH_4HCO_3)(1)	3	63	19.7	11.8
Mono-P(2)	3	76	3.2	92.4
Mono-Q(3)	3	92	2.6	140.0

(1) gel filtration
(2) chromatofocusing
(3) anion exchange chromatography

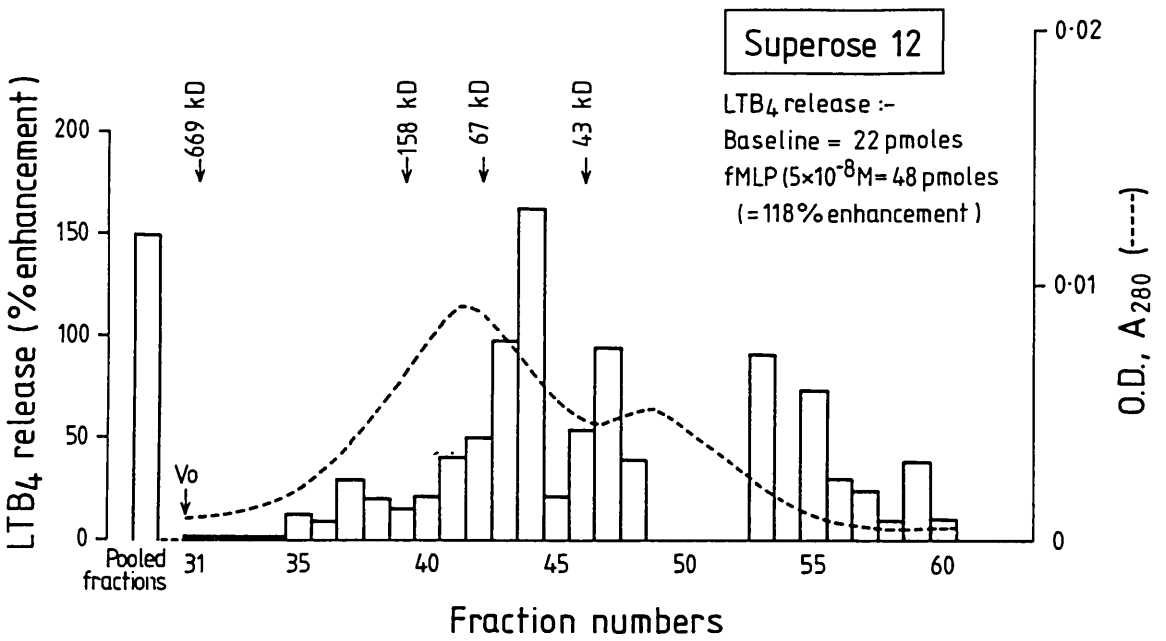

Figure 7.1: Gel filtration (FPLC, Superose-12 prep grade) of concentrated PHA-stimulated PBMC supernatants. Two millilitres of 100 fold concentrated PBMC supernatants were chromatographed on Superose 12 PG in PBS, and fractions with LREF activity (35-70 kD) were pooled and concentrated to 2 ml. These samples were applied to a second Superose 12 PG column equilibrated with an ammonium bicarbonate buffer. LREF activity was measured in each fraction (this figure), and the percentage enhancement of LTB_4 generation is shown with respect to a buffer control. Neutrophils incubated with a buffer control generated 22 p moles $LTB_4/10^6$cells. Incubation with $fMLP_6$ ($5x10^{-8}$M) resulted in generation of 48 p moles $LTB_4/10^6$cells (=118% enhancement). The elution volumes of molecular weight markers are indicated at the top of figures.

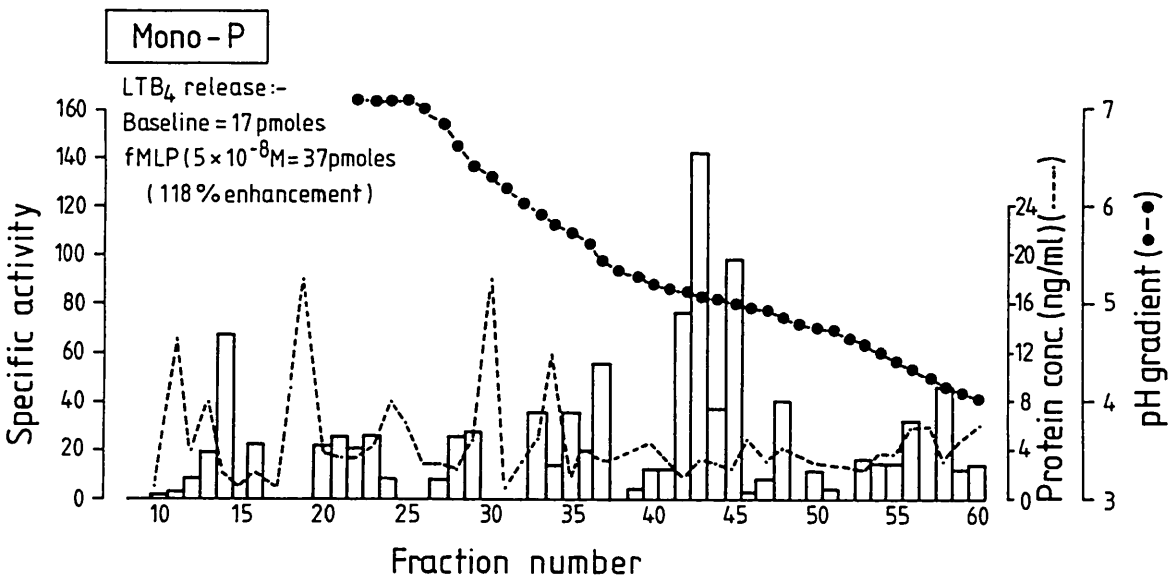

Figure 7.2: Chromatofocusing of concentrated PHA-stimulated PBMC culture supernatants. The samples subjected to this procedure were partially purified from FPLC gel filtration (Figure 7.1 fraction 43-45). Three millilitres of column fractions were pooled and dialysed against 0.025 M bis-tris buffer pH 7.1. Then applied to a Mono-P column. Fractions (1 ml) were collected and LREF activity was measured. Specific activity (open column) refers to percent enhancement of LTB_4 generation per microgram of protein. The pH gradient is represented by the closed circle. In this experiment, buffer control was 17 p moles $LTB_4/10^6$ cells, fMLP ($5x10^{-8}$M) gave 37 p moles/10^6 cells (=118% enhancement).

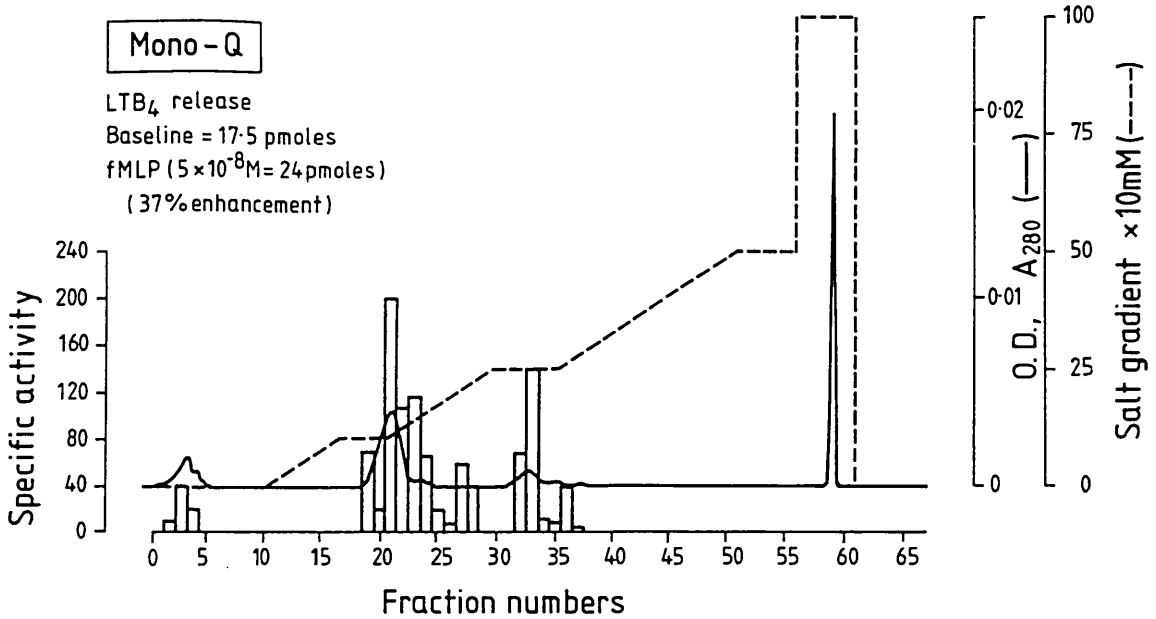

Figure 7.3: Anion exchange chromatography of concentrated PHA-stimulated PBMC culture supernatants. The samples subjected to this procedure were partially purified LREF from chromatofocusing (Figure 7.2 fractions 43-44). Fractions were pooled and dialysed against 20 mM bis-tris buffer pH 6.5 then applied to a Mono-Q column. Fractions (1 ml) were collected and LREF activity was measured. Specific activity (open column) represents percentage enhancement of LTB_4 generation per microgram of protein. Salt gradient was represented as dash line. Buffer control was 17.5 p moles $LTB_4/10^6$ cells, and with fMLP ($5x10^{-8}M$) LTB_4 generation was 24 p moles/10^6 cells (=37% enhancement).

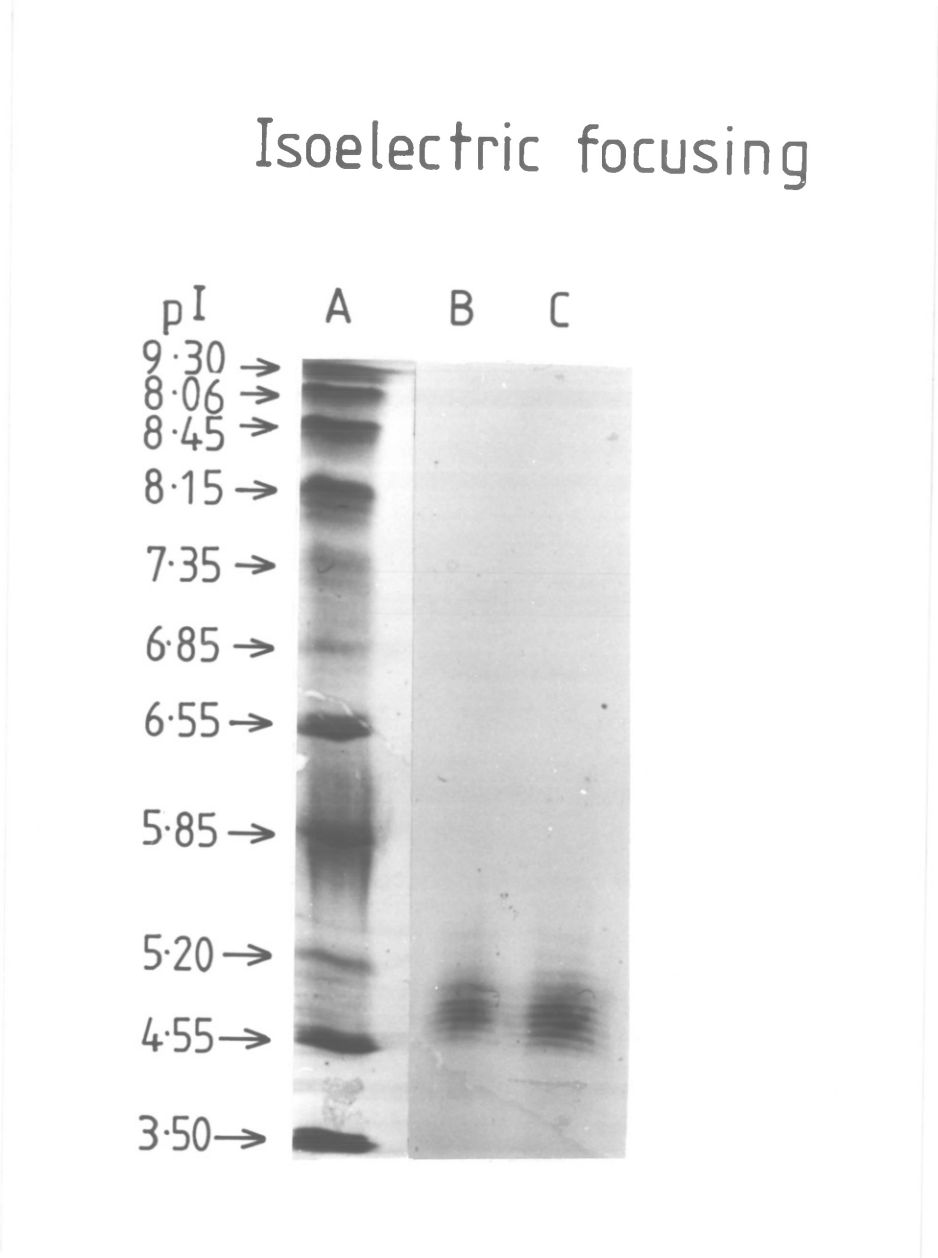

Figure 7.4: Isoelectric focusing of partially purified LREF from gel filtration on IEF gel using Phast Gel electrophoresis system. The samples subjected to this procedure were partially purified LREF from gel filtration (Figure 7.1 fractions 44 & 45). Fractions were dialysed against distilled water and lyophilised. After resuspension in normal saline, samples were separated on Phast gel IEF 3-9. Gel was stained with a protein silver stain method. Lane A: isoelectric point markers, Lane B: fraction 44 and Lane C: fraction 45.

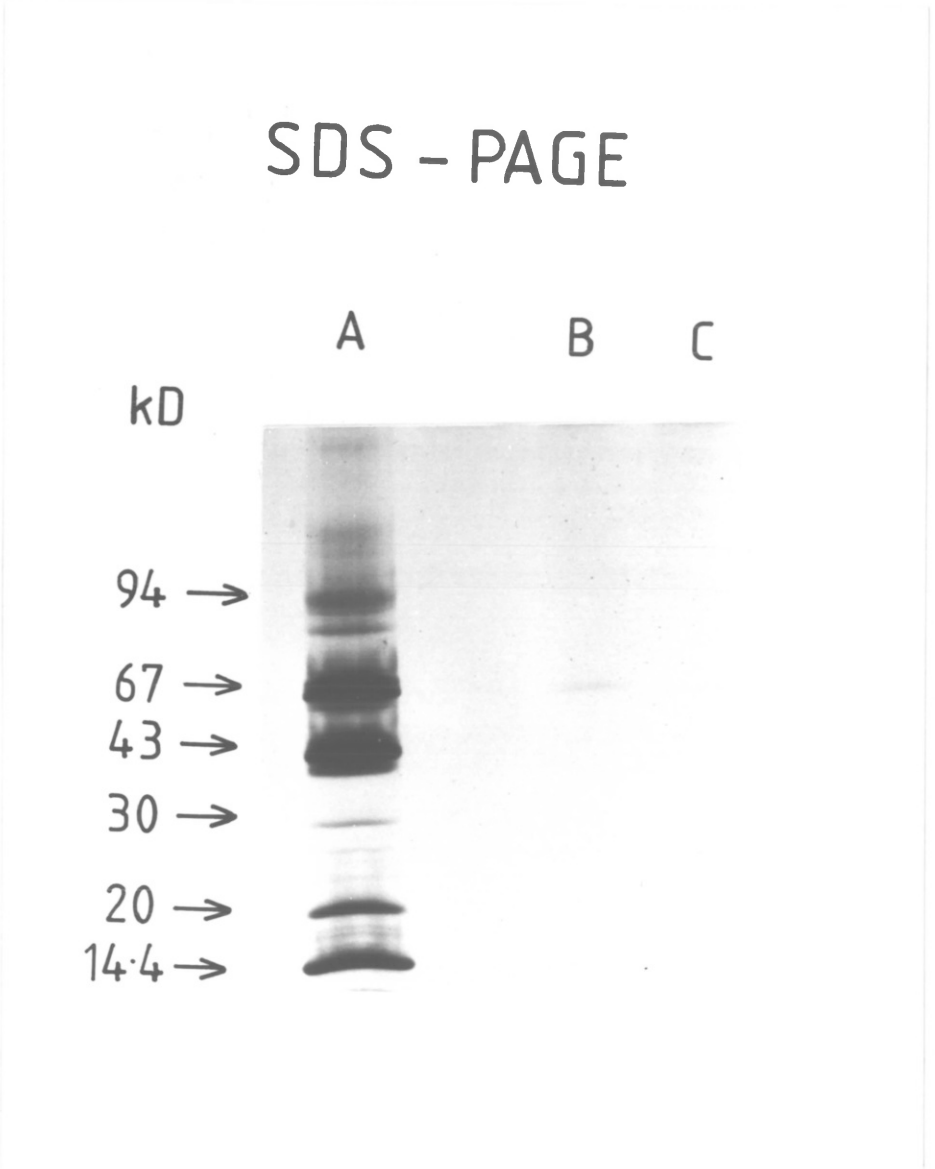

Figure 7.5: Molecular weight determination of LREF by SDS-PAGE using Phast gel electrophoresis system (non-reducing condition). Samples applied to the Phast gel were partially purified LREF from PHA-stimulated PBMC supernatants after FPLC gel filtration and chromatofocusing. (Figure 7.2, fractions 43 & 44). Fractions were dialysed against distilled water and lyophilised. After resuspension in Phast gel buffer, samples were separated on Phast SDS-PAGE (gradient 8-25%). Gel was stained with a silver stain. Lane A: molecular weight markers. Lane B: fraction 44 and Lane C: fraction 43.

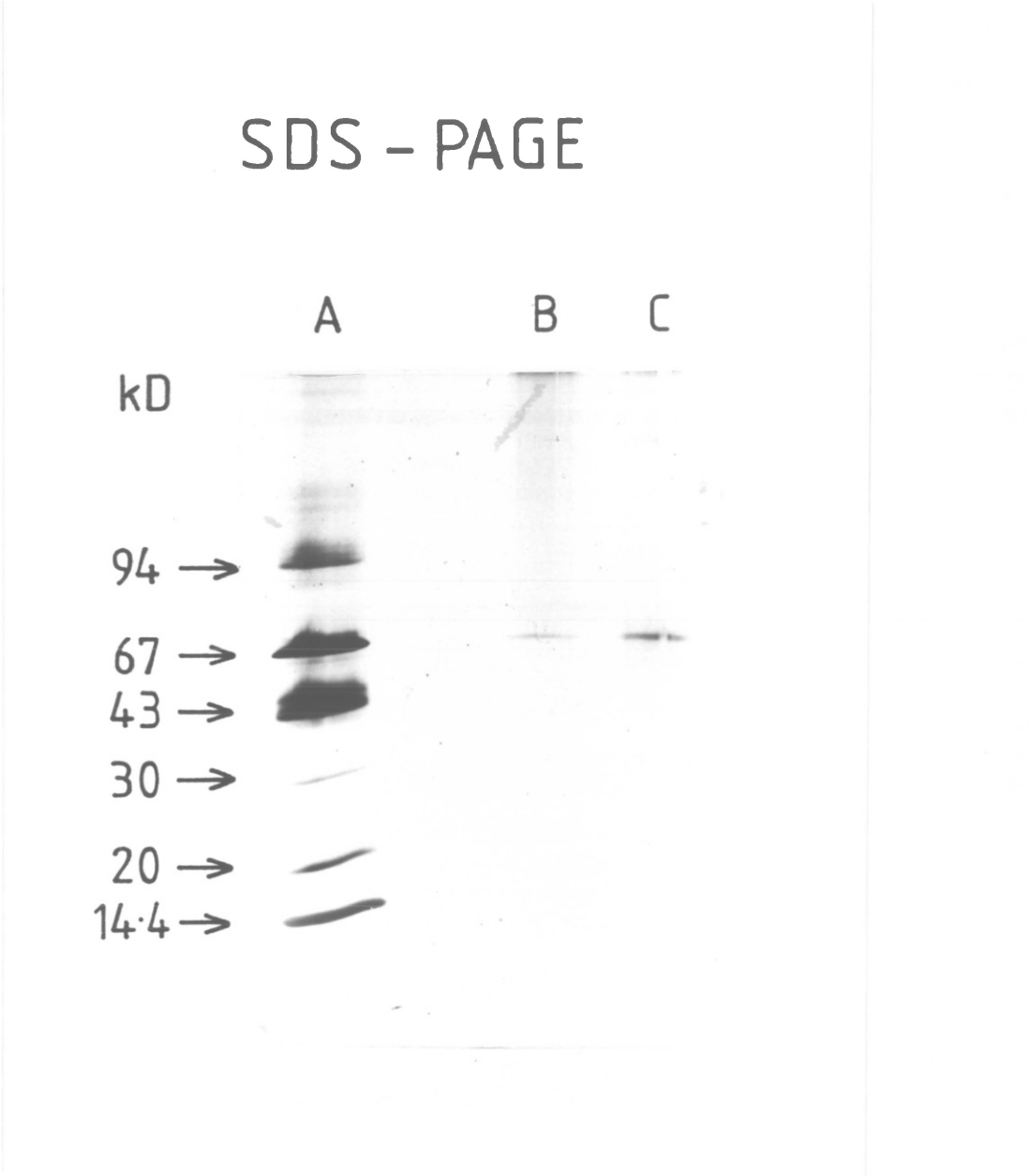

Figure 7.6: Molecular weight determination of LREF by SDS-PAGE using Phast Gel electrophoresis system (reducing condition). Samples applied to the Phast gel were partially purified LREF from PHA-stimulated PBMC supernatants after FPLC gel filtration and chromatofocusing. (Figure 7.2, fraction 43 & 44). Fractions were dialysed against distilled water and lyophilised. After resuspension in Phast gel buffer (containing 5% β-mercaptoethanol), samples were separated on Phast SDS-PAGE (gradient 8-25%). Gel was stained with a silver stain. Lane A: molecular weight markers, Lane B: fraction 44 and Lane C: fraction 43.

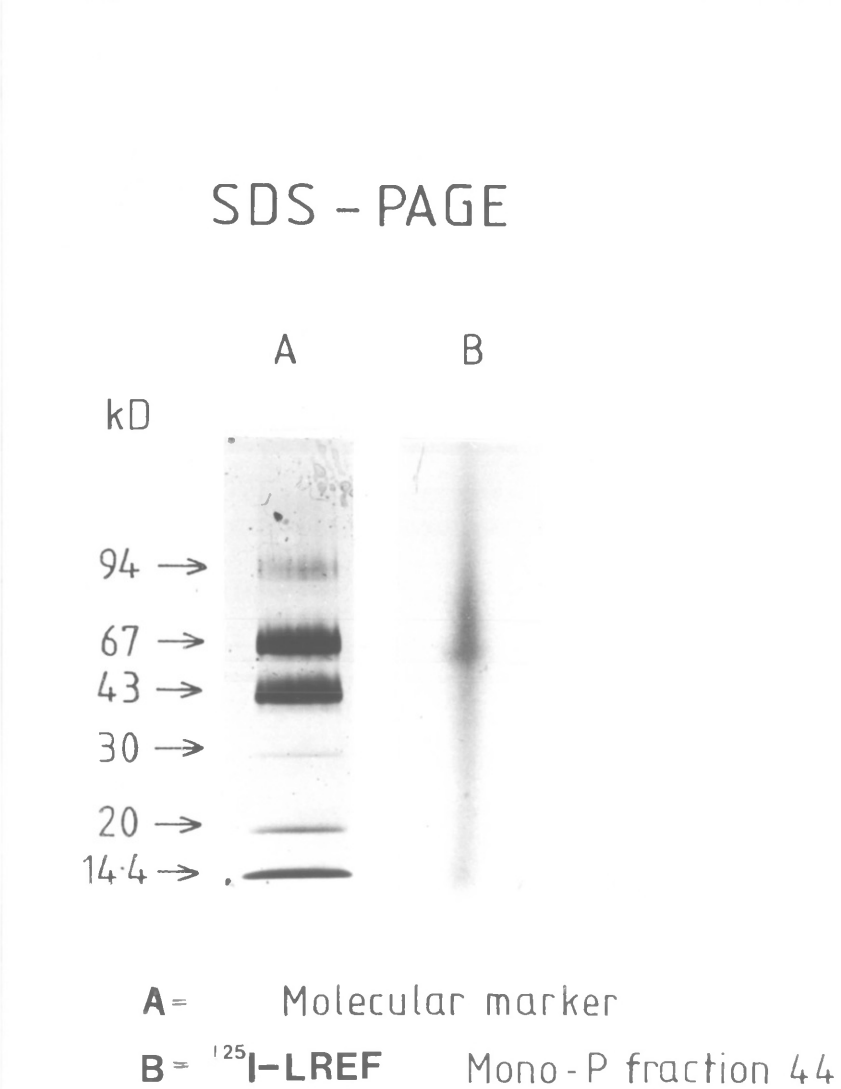

Figure 7.7: Molecular weight determination of ^{125}I-LREF by SDS-PAGE. Samples applied to the Phast gel were ^{125}I-labelled LREF from PHA-stimulated PBMC supernatants after gel filtration and chromatofocusing (Figure 7.2, fractions 43 & 44). Fractions were pooled and labelled with ^{125}I. These materials were dialysed against distilled water and lyophilised. After resuspension in Phast gel buffer, samples were separated on Phast SDS-PAGE (gradient 8-25%). The gel was exposed to X-ray film for 3 days for autoradiography.

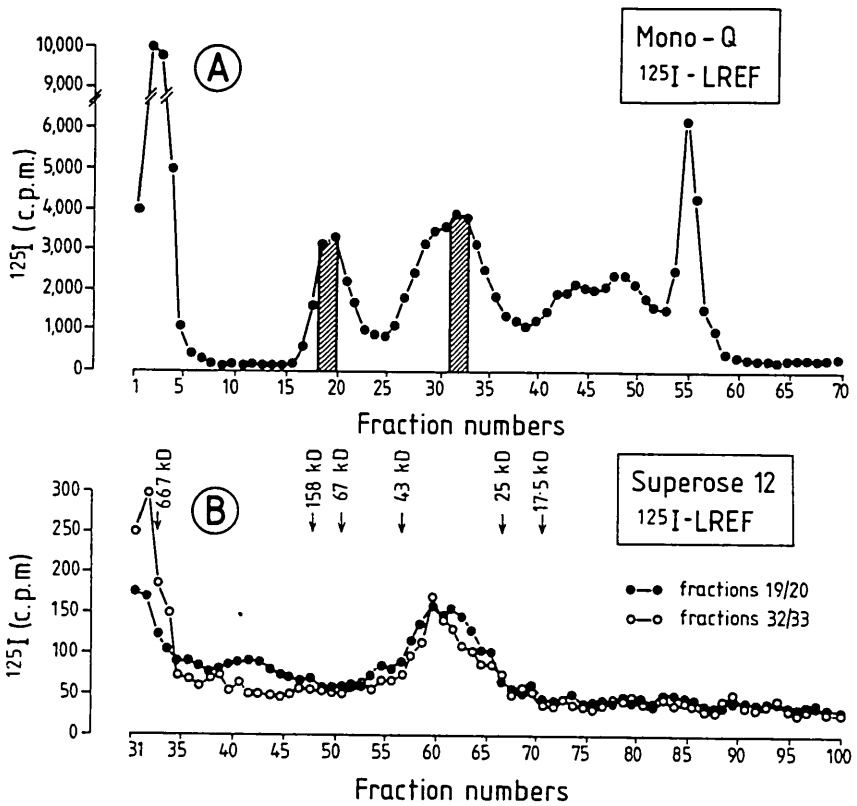

Figure 7.8: Anion exchange chromatography and gel filtration of ^{125}I-LREF. (A) Anion exchange chromatography of ^{125}I-LREF. Samples applied to the procedure was ^{125}-labelled LREF which was from purified PHA-stimulated PBMC supernatant after gel filtration and chromatofocusing. After labelling, samples were dialysed against 20 mM bis-tris buffer pH 6.5 before being applied to the column. Each fraction was collected and radioactivity measured with a gamma-counter. (B) Gel filtration of ^{125}I-LREF. Samples applied to the procedure were ^{125}I-LREF, which were the peaks of radioactivity recovered in anion exchange chromatography (fractions 11 & 20 fractions 32 & 33, Figure 7.8a). ^{125}I-LREF was fractionated by gel filtration chromatography and radioactivity was recovered and counted with the gamma-counter.

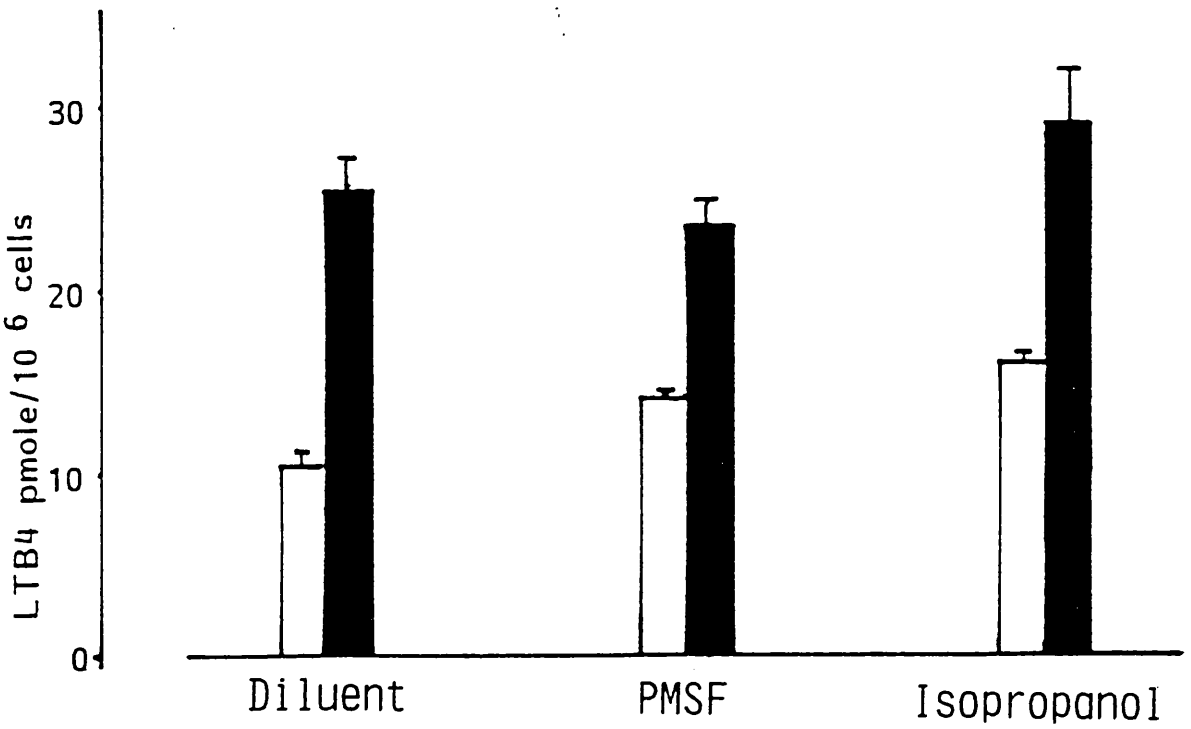

Figure 7.9: The effect of the protease inhibitor PMSF (phenylmethylsulfonyl fluoride) on the LTB_4 release enhancing activity of purified LREF. LREF, after the treatment of PMSF ($1x10^{-4}$M, 37°C for 30 min), were dialysed against PBS and tested for the LTB_4 generation from neutrophils (closed columns), diluent were used as a negative control (open columns). PBS (0.12 M) and 0.1% isopropanol were used as negative treatments. Data represented as mean±s.e.m. of three experiments.

CHAPTER 8.

OTHER BIOLOGICAL ACTIVITY OF PURIFIED LREF

8.1 Introduction

LREF was initially described as a cytokine which had a priming effect on neutrophil leukocytes increasing generation of LTB_4 in response to IgG bead stimulation. It is of interest to know the underlying mechanisms of this phenomenon.

In this IgG bead stimulation system, neutrophil activation could be reflected in either increased IgG Fc receptor expression or increased neutrophil adhesive and phagocytic capacity towards the beads.

Several bioassays have been devised to evaluate neutrophil adhesive capacity and phagocytic activity. Adhesion to a nylon wool column is one of the most widely used methods to evaluate neutrophil adhesive capacity (MacGregor et al 1974); whilst the phagocytic capacity of neutrophils can be associated using opsonized target parasites such as the schistosomula of Schistosoma mansoni (Anwar et al 1979).

More recently, neutrophil function has been evaluated using monoclonal antibodies against cell surface receptors. Several leukocyte adhesion glycoproteins are present on neutrophils. The adhesion mechanism of neutrophils has been reported to be associated with expression of the adherence glycoprotein family, comprising Mac-1 (CD11a/CD18), LFA-1 (CD11b/CD18) and p150.95 (CD11c/CD18), which share an identical beta subunit but distinct alpha

subunits. Adherence glycoprotein family is crucial to adherence and cell-to-cell aggregation (Trowbridge & Omary 1981; Sanchez-Madrid et al 1983; Weight et al 1983). In vitro studies have shown that patients with CD11/CD18 receptor deficiency have impaired neutrophil adhesion capacity (Anderson et al 1984). Also, anti-CD11 antibody is able to inhibit neutrophil adhesion capacity (Diener et al 1985). The Mac-1 glycoprotein appears to be identical to the type 3 complement receptor (CR3) (Gamble et al 1985).

Two distinct IgG Fc receptors are found to be present in resting human neutrophils. Both can be recognized and differentiated by various monoclonal antibodies (3G8 & IV3) (Looney et al 1986). The overlapping distribution of Fc receptors and CR1 (C3b) receptors on neutrophils has also been reported (Jack & Fearon 1984).

In this study I have evaluated the effect of LREF on neutrophils in terms of their adherence to nylon wool, phagocytic capacity to S. Mansoni and complement receptor (CR1 & CR3) expression (by FACS analysis). The chemotactic activity of LREF were also evaluated.

8.2 Materials and Methods

8.2.1 LREF preparation

LREF (PBMC supernatants followed by amicon ultrafiltration and gel filtration) were prepared as described in Chapter 7. In order to compare other possible neutrophil directed biological activities of LREF with its leukotriene release enhancing effect, 60 min incubation with LREF, the optimal period for LTB_4 enhancement, was used in all tests.

8.2.2 Neutrophil adherence assay

The neutrophil adherence assay was based on the method described by MacGregor (MacGregor et al 1974). Briefly, scrubbed nylon fibre (50 mg) was packed into Pasteur pipettes and the length of the packed columns adjusted to exactly 15 mm, extending from the midpoint of the pipette shoulder. The pipettes were warmed to 37°C, and 1 ml of neutrophils ($5x10^6$ cells/ml) pretreated with various concentrations of fMLP or LREF for 60 min at 37°C, were added to the top of pipette and allowed to filter through the column over 5-10 min.

Neutrophils in the effluent were counted and data were expressed as % enhancement of adherence as compared with that of buffer treatment.

8.2.3 Schistosomula killing assay

The details of schistosomula killing was based on the methods described by Anwar (Anwar et al 1979).

Briefly, the assay was performed in triplicate in flat-bottomed microtitre plates with neutrophils (50 ul, $4x10^6$/ml) to which were added LREF, fMLP ($1x10^{-7}$M) or control buffer (50 ul). Plates were incubated at 37°C, 5% CO_2, 95% humidity for 60 min prior to the addition of autologous fresh serum (50 ul, final dilution of 1:4) as a source of complement plus schistosomula (50 ul). The numbers of dead schistosomula were determined by trypan blue dye exclusion after 40 hr at 37°C.

8.2.4 Chemotaxis assay

Neutrophil chemotaxis was assayed using a modified Boyden technique (Boyden 1962) with a 48 well microchemotaxis assembly (Neuro-Probe Inc., MD, U.S.A.) and nitrocellulose filters (Sartorius membrane filters, 8 um pore size; 24 Gottingen, W. Germany). Neutrophils (purity > 95%) were suspended in HBSS (Gibco Ltd) containing 0.4% ovalbumin (type IV Sigma Chemical Co.). Test solutions and controls (25 ul) were placed in the lower compartment of the chemotaxis chamber, and the neutrophil suspension (50 ul at $5x10^6$ cells/ml) were placed in the upper compartment. Following incubation at 37°C for 90 min the filters were removed, fixed and stained as previously described by Turnbull (Turnbull & Kay 1976). Neutrophils that had migrated through the entire thickness of the filter were counted and results

expressed as the total number of cells in 10 random high-power fields. Samples were assayed blind in triplicate with an intra-assay variation of <20%.

8.2.5 Immunofluorescent staining and FACS analysis

Neutrophils were treated with LREF, fMLP (10^{-7}M), or buffer for 60 min, 37°C prior to staining for CR1 or CR3. Irrelevant IgG1 and IgG2a mouse myeloma proteins were used for the baseline control of CR1 and CR3.

Neutrophils (50 ul, $5x10^5$ cells) were incubated with anti-CR1 or anti-CR3 moAb for 15 min at 4°C. Cells were washed once with 2 ml of PAB (PBS with 0.5% BSA and 0.1% NaN_3), and incubated with 20 ul of RAM-FITC for 15 min. Cells were washed once, fixed with 1% paraformaldehyde, resuspended in 0.5 ml of PBS containing 0.1% w/v sodium azide and stored at 4°C prior to analysis.

FACS analysis was performed on a Becton-Dickinson FACS Analyser interfaced with a Hewlett-Packard Consort 30 computer. 10,000 cells were analysed in each sample.

8.3 Results

8.3.1 Effect of LREF on neutrophil adherence to nylon wool

The capacity of neutrophils to adhere to nylon wool was increased by LREF in a dose-dependent manner. Statistical significance was only reached at the concentrations of 6 ug/ml and 3 ug/ml of LREF when compared with buffer control ($p<0.05$, n=3) (Fig 8.1). In these experiments, fMLP ($1x10^{-6}M$) produced a 92±24% increase of adherence, baseline adherence were 24±4%. When this activity was compared with LTB_4 release enhancing activity, 100% increase of LTB_4 release was equivalent to 20-31% enhancement of adherence.

8.3.2 Effects of LREF on neutrophil cytotoxicity against opsonized schistosomula of S. mansoni

The effects of LREF on complement dependent neutrophil cytotoxicity are shown in Fig 8.2. Enhancement of schistosomula killing by LREF was concentration dependent over the range 0.75 to 6 ug/ml. The enhancement reached a significant level only when LREF was tested at the concentration of 6 ug/ml and 3 ug/ml ($P<0.05$, n=3). In these experiments, baseline killing was 19±2.6%, fMLP ($1x10^{-7}M$) produced 116±31% enhancement. When the activity was compared with LTB_4 release enhancing activity. The activity containing 100% increase of LTB_4 generation was equivalent to 37-50% enhancement

of cytotoxic capacity.

8.3.3 Chemotactic activity of LREF

LREF had only minimal neutrophil chemotactic activity when compared with fMLP in the two experiments performed. The major part of neutrophil chemotactic activity from FPLC column fraction corresponded to a molecular size of approximately 10 kD and only small peaks of activity were observed of molecular weights above 40 kD (Table 8.1).

8.3.4 LREF effects on neutrophil complement CR1 and CR3 receptor expression

Expression of CR1 and CR3 receptors on neutrophils was found to increase in numbers following incubation with LREF (Fig 8.3). LREF increased CR1 expression by 40% to 129% , while in the same experiments 30% to 79% increase was produced by fMLP ($1x10^{-7}M$) when compared with buffer control (Mean fluorescence intensity 10-12) (Table 8.1).

The percent increase of CR3 expression by LREF ranged from 69 to 78% and in the same experiments fMLP ($1x10^{-7}M$) produced a 67% to 153% increase when compared with buffer control (mean fluorescence intensity 92-125) (Table 8.1).

8.4 Discussion

Purified LREF enhanced neutrophil adherence to nylon wool, enhanced neutrophil cytotoxic capacity against schistosomula of S. mansoni, and increased expression of complement receptors (CR1 & CR3). LREF showed no direct toxic effect towards neutrophils.

My results show that LREF is capable of enhancing neutrophil CR3 receptor expression and increasing neutrophil adhesion to nylon wool, suggesting the possibility that the enhancing effect of LREF upon LTB_4 release may be due to LREF inducing increased expression of adhesive glycoproteins (CR3/Mac-1) and thus rendering the neutrophils more adhesive to IgG-coated beads. The enhancement of CR1 expression by LREF might also suggest that the increased generation of LTB_4 by neutrophils may also be via the enhancement of neutrophil Fc receptor expression since Fc and CR1 receptors are coexpressed during neutrophil activation (Jack & Fearon 1984).

Previous reports have indicated that LTB_4 can enhance neutrophil cytotoxic capacity and CR3 receptor expression (Moqbel et al 1983; Ford-Hutchinson 1983). Thus it was important to consider whether the present observations could be accounted for by LTB_4 in culture supernatants. LTB_4 should be removed by the dialysis step during LREF preparation and in addition, there was no detectable LTB_4 in the chromatography fractions

(Fig 3.9). Thus, it is highly unlikely that in the culture supernatants could account for the effects reported in this chapter.

Despite its effects on adhesion LREF only possessed minimal neutrophil chemotactic activity. GM-CSF also shows disassociation between these two activities and LIF, enhances neutrophil phagocytic capacity but inhibits neutrophil migration in vitro (Gasson et al 1984; Arnaout et al 1986; Borish & Rocklin 1987a,b). It is possible that these lymphokines slow down the movement of neutrophils by enhanced cell adhesion secondary to increased surface expression of certain leukocyte adhesion molecules.

8.5 Summary

In this chapter I have studied the effects of LREF on a wide range of neutrophil functions.

Purified LREF enhanced neutrophil adherence to nylon wool, enhanced neutrophil cytotoxic activity against schistosomula of S. mansoni, and induced increased expression of the complement receptors CR1 and CR3 on the cell surface. LREF showed no direct toxic effect towards neutrophils. LREF possessed only minimal neutrophil chemotactic activity.

These results suggest that LREF may modulate the activity of neutrophils either by upregulating cell surface receptors such as those for IgG and complement or by increasing 5-lipoxygenase activity, thereby increasing LTB_4 production.

Table 8.1.

Biological effects of LREF on neutrophils

Protein conc. of LREF (ug/ml)	LTB_4 generation[1]	Chemotactic response[2]		CR1 receptor expression[3]	CR3 receptor expression[3]
		Exp 1	Exp 2		
Diluent	7.5	14	18	10.8±0.6[a]	114±11[a]
3	22.5	102	100	ND	ND
6	36.5	210	86	19.8±4.0	187±8.5*
fMLP	15.0	1095	510	18.5±2.7	216±15.6

(1) p moles/10^6 cells

(2) cells/10 high power field

(3) mean fluorescence

a. mean ± s.e.m. of three experiments

* $p < 0.05$

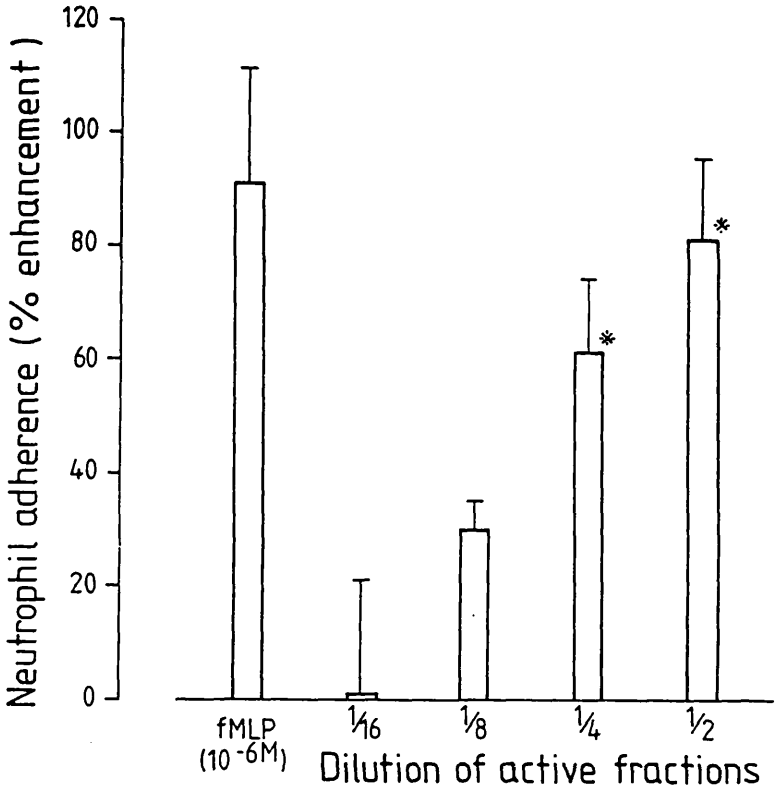

Figure 8.1: Effect of LREF on the neutrophil adherence to nylon wool. Neutrophils ($5x10^6$ cells/ml), pretreated with various concentrations of LREF (37°C, 60 min), were tested for their adherence capacity to nylon wool. In these experiments, baseline adherence were 24±4.1% (n=3). fMLP ($1x10^{-6}$M) obtained 92±24% enhancements.

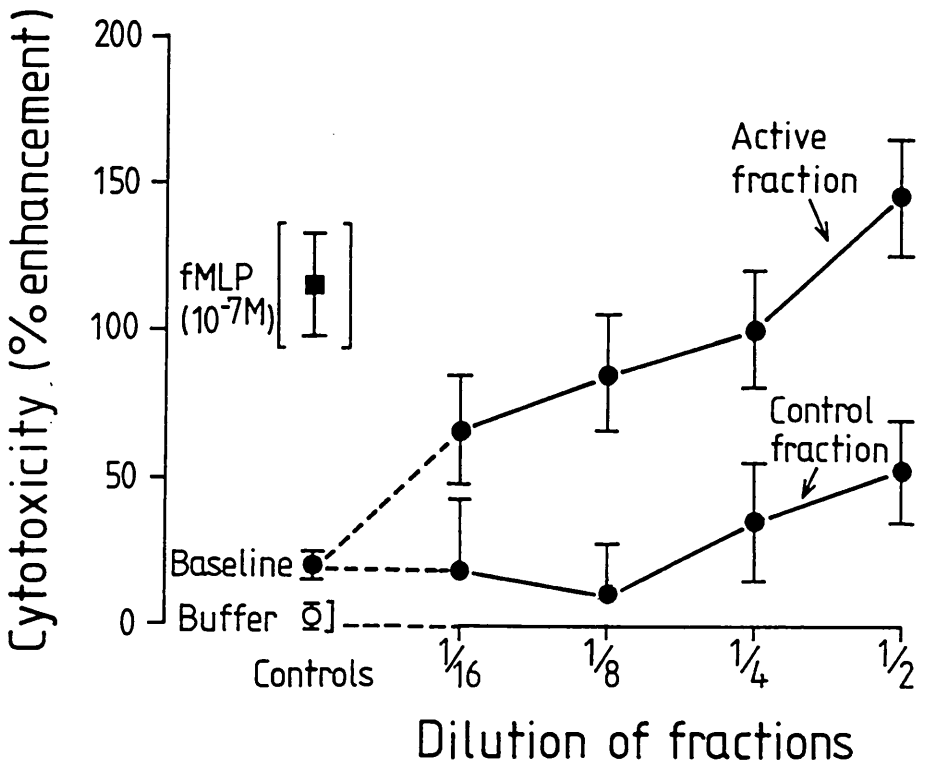

Figure 8.2: Effect of LREF on the cytotoxic capcity of neutrophils against opsonised schistosomula of S. mansoni. Neutrophils ($4x10^6$ cells/ml), pretreated with various concentrations of LREF (37°C, 60 min), were tested for their cytotoxic capacity against opsonized S. mansoni. In these experiments, baseline cytotoxicity were 19±2.6% (n=3). fMLP ($1x10^{-7}$M) obtained 116±31% enhancements.

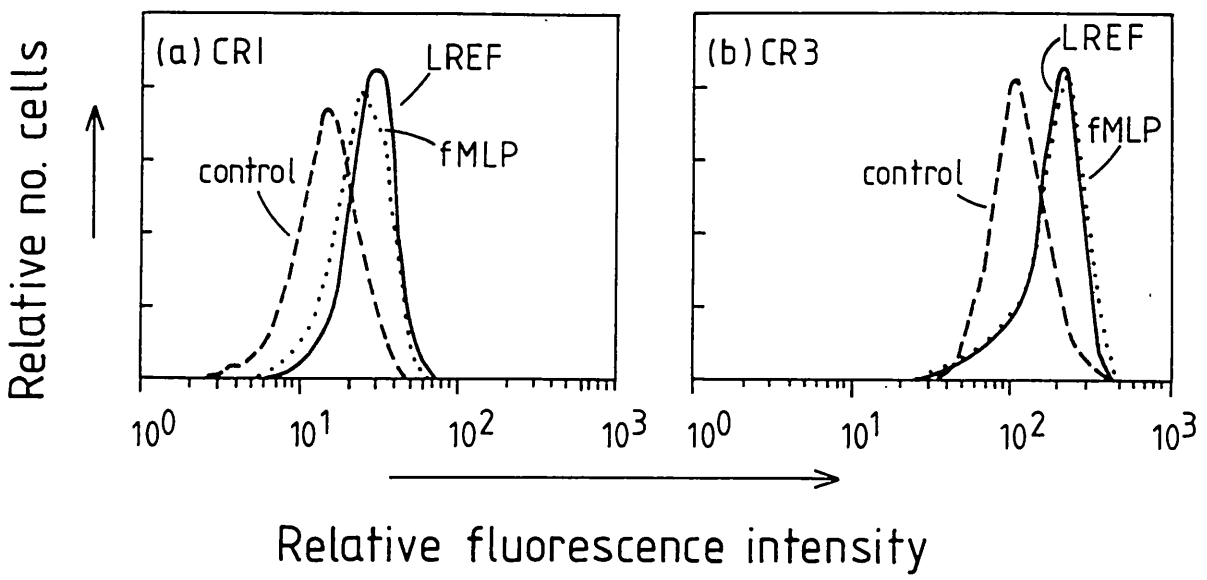

Figure 8.3: Effect of LREF on the surface expression of the complement receptor CR1 and CR3. Neutrophils ($1x10^7$/ml), pretreated with LREF (6 ug/ml) for 60 min, 37°C, were stained for their CR1 or CR3 expression. 10,000 cells were analysed in each case using FACS IV flow cytometer. (A) Quantitative expression of CR1 antigens on the neutrophils. The mean fluorescence was converted into a logarithmic value, as indicated in parenthesis. LREF or fMLP induced a rise in surface expression, obtained 27.5 and 21.5 mean fluorescence intensity, when compared with buffer control 12.0. (B) Quantitative expression of CR3 antigens on the neutrophils. The mean fluorescence was converted into a logarithmic value, as indicated in parenthesis. LREF or fMLP induced a rise in surface expression, obtained 195 and 192 mean fluorescence intensity, when compared with buffer control 125.

CHAPTER 9.

GENERAL DISCUSSION AND CONCLUSION

9.1 Summary of results

In this thesis I have described a lymphokine which could be generated either by mitogenic stimulation using PHA, or by non-specific T cell receptor activation using the monoclonal antibody OKT3, or by specific T cell receptor stimulation using specific allergen (house dust mite). The lymphokine primed neutrophils for increased amounts of LTB_4 generation in response to stimulation by IgG-coated Sepharose beads. For this reason, it has been termed leukotriene release enhancing factor (LREF). However, the purified protein was shown to have additional effects on neutrophils in that it enhanced complement receptor (CR1 & CR3) expression, increased their cytotoxic capacity towards schistosomula of S. mansoni and enhanced neutrophil adherence to nylon wool. LREF generation was shown to be inhibited when lymphocytes were cultured in the present of dexamethasone or LTB4, but not with DSCG and IFN-gamma. After progressive purification, LREF activity was found to be associated with proteins of molecular weight 35-45 kDa as determined by gel filtration and 60 kDa shown by SDS-PAGE.

9.2 Mechanism(s) of mediator production and release

"Lymphocyte activation" consists of three distinct phases. Firstly complexing of a ligand with the corresponding cell membrane receptor and signal

transduction to the interior. This may be associated with aggregation of the complexes and ingestion of the complexes or alternatively shedding into the ambient medium. The second step is protein synthesis and the third is DNA synthesis followed by blast transformation and proliferation. Most lymphokine production is associated with protein synthesis (Granger & Williams 1968, Remold et al 1972, Gifford et al 1971). Several of my experiments, indicated that LREF production did not correlated with DNA synthesis, as determined by ^{3}H-TdR incorporation. Firstly, LREF activity was detectable in PBMC supernatant after 12 hr culture with mitogen (PHA) and after 24 hr culture with relevant allergen, and in both instances LREF appeared several hours before DNA synthesis (Fig 3.2 & Fig 4.1). Secondly, IFN-gamma inhibited cell proliferation, but did not reduce LREF generation in PHA-stimulated PBMC cultures (Fig 6.4). Thirdly, IL-2 enhanced cell proliferation, but did not enhance LREF generation in PHA-stimulated ER+ lymphocyte cultures (Fig 5.2). These kinetics are similar to those reported for lymphotoxin (TNF-beta) production, which starts as early as 2-3 hr after lymphocyte stimulation and shows no correlation with later blast transformation (Shack & Granger 1971). Similarly, MIF is produced within the first 2-3 hr and peaks by 24 hr (David & David 1972). MIF is also

produced by sensitized lymph node cells exposed to antigen in the presence of concentrations of cytosine arabinoside or vinblastine which completely inhibit DNA synthesis and mitosis (Bloom et al 1972). These findings suggest that lymphokine generation is not necessarily correlated with DNA synthesis.

Serum-free culture conditions were used in all experiments to reduce protein contamination and to facilitate the purification of LREF. It might be argued that these conditions are unphysiological. Human serum contains various immunosuppressive proteins such as alpha-foetal protein (Yachin 1975) C-reactive protein (Mortensen & Gewurz 1976) and IFN-gamma (Blomgren et al 1974) all of which might interfere with the expression of LREF activity. Human complement fragment C5a is also chemotactic for neutrophils, and might influence the biological assay systems I have used (Cooper 1984). The next best alternative, foetal calf serum, has been reported to have variable suppressive effects on the growth of cell lines (Loo et al 1987). All the cultures in this work were short term (up to 3 days), and cell viability was always above 90% as determined by trypan blue dye exclusion. Other evidence for the well-being of the cells comes from the 3H-TdR incorporation assay which showed good DNA synthesis. In addition, there were substantial amounts of IL-2 and IFN-gamma in the

culture supernatant, suggesting that the culture system was indeed appropriate for lymphokine generation. Finally, several other lymphokines, such as LIF (Rocklin 1974) and HRF (Thueson et al 1979a) have also been generated in serum-free culture conditions.

9.3 Identification of T-lymphocytes producing LREF and the role of macrophage in LREF generation

While lymphocytes provide the specificity of the immune response, their differentiation and function is governed at most levels by macrophages, acting either as antigen-presenting cells or by release of mediators (IL-1, TNF etc) which affect lymphocyte function.

In this study, several lines of evidence suggest that LREF generation is through the activation of T helper cells. Firstly, LREF generation was seen in three different systems (PHA, OKT3 and D. farinae), which all operate through T cell antigen receptor stimulation. Although PHA has been considered to be mainly a T-cell mitogen, some reports show that it can also stimulate human B-cell proliferation (Lis & Sharon 1986). The precise role of the macrophage in the triggering of T-cells by PHA remains unclear. An early report demonstrated a requirement for macrophages in PHA-stimulated production of lymphotoxin (TNF-beta) by human T-cells (Walker et al 1976). It has been suggested that macrophages

participate in PHA-induced mitogen stimulation by providing a soluble factor, IL-1, which is necessary for the production and release of lymphokines from T cells (Lis & Sharon 1986). In the OKT3 stimulation system, the monoclonal antibody activates T cells by a direct action on the CD3 molecule. There are several reports suggesting that accessory cells (AC) are required for OKT3 to crosslink surface CD3 molecules (Williams et al 1985a). However, proliferation can also be induced by OKT3 immobilised solid phase (Clark & Ledbetter 1986), implying that the requirement for AC in other systems is only to act as a support for the monoclonal antibody. In the D. farinae specific CD4+ cell line, LREF generation is seen in the presence of D. farinae and irradiated AC, while supernatants from irradiated AC and D. farinae alone show only minimal LREF activity, suggesting that T cell activation is necessary for the generation of LREF. Antigen recognition by T lymphocytes appears to involve simultaneous presentation of both MHC molecules and antigen on the AC surface membrane.

Secondly, LREF was generated by PHA-stimulated lymphocyte cultures which were selectively depleted of macrophages. Finally, there is the suppressive effect of LTB_4 on LREF generation. LTB_4 can augment monocyte function and enhance IL-1 and TNF generation (Rola-Pleszczynski & Lemaire 1985; Gagnon et al 1987),

while in my LTB_4 co-culture experiments, LREF generation was inhibited. If IL-1, TNF and LREF were all released from stimulated monocytes, it would seem unlikely that both IL-1 and TNF should be increased by coculture with LTB_4 whilst LREF generation was reduced.

9.4 Purification and characterisation of LREF

The purification and characterization of soluble lymphokines are required if discrimination studies are to be carried out either in vitro or in vivo. Yet such purification is technically difficult because of the complexity of the cell populations used to produce active supernatant, the multiplicity of factors produced even in simplified in vitro systems and the extremely low concentration of any particular factor of interest. In general, the best purification studies have started by attempting to simplify the culture system. To eliminate the need for separation of mediators from serum proteins, cells are cultured in serum-free media. In some systems, insolubilized antigen or mitogen are used to stimulate the cells. I chose short term culture culture (48 hr to 72 hr) in serum free medium to generate LREF, which was optimal in both PHA and allergen stimulation systems.

One of the major problems in the purification of LREF is the difficulty in obtaining large amounts of lymphocytes, beside which there is a wide variation in

the ability of PBMC from different donors to generate the lymphokine LREF. During these studies PBMC from 10 different subjects were stimulated with mitogen (PHA) under identical conditions and LREF activity in the supernatant was assayed on neutrophils from one donor. Although the lymphocytes had comparable 3H-TdR incorporation, there was a wide range of LREF activity in their supernatants (Fig 3.4).

Similar considerations applied in the allergen stimulated PBMC cultures, though these were less easy to interpret because 3H-TdR uptake was generally quite low. Further complicating features are the known differences in epitope recognition between individuals (Herzenberg et al 1983) and differences in the number and activity of regulatory T lymphocytes (Rocklin et al 1974). Similar variability of lymphokine generation between individuals is well documented for IL-2 generation (Gearing & Bird 1987).

In this work I have used standard protein chemistry techniques, including Amicon membrane and hollow fibre ultrafiltration, gel filtration, chromatofocusing and anion exchange chromatography to purify the lymphokine. After each step, protein concentration and LREF activity were measured. SDS-PAGE and isoelectricfocusing were used to confirm molecular weight and pI. Protein iodination was used to facilitate evalution and monitoring the

purification. Although only small amounts of protein were recovered, these experiments helped to develop a scheme for the further purification of LREF.

Another problem is that of comparing LREF with other cytokines and distinguish between them. Of the recombinant cytokines tested, two (GM-CSF and TNF) were shown to have the capability of enhancing LTB_4 generation in my assay system. However, it seems improbable that LREF could be accounted for by production of either GM-CSF or TNF, firstly because the treatment of PBMC supernatants with anti-GM-CSF antibody did not block the LREA activity and secondly because GM-CSF and TNF have much lower molecular weights and different pIs' from the purified LREF.

Two lymphokines which have similar molecular weight and PI to LREF are the leukocyte inhibitory factor (LIF) described by Rocklin which inhibits neutrophil migration (Rocklin et al 1974), and phagocytosis-inducing factor (PIF) which enhances phagocytosis of IgG-coated ox red cells by U937 cells (monocyte cell line) (Margolick et al 1986). The effects of LIF and PIF on LTB_4 generation have not been studied and we have been unable to obtain purified LIF or PIF to compare with our material. In the absence of purified material and sequence analysis, it is difficult to state whether LREF, PIF and LIF are the same or distinct molecules. Because

of their similarities in gel filtration, complete characterization of PIF, LIF and LREF will be necessary before their precise relationship can be determined.

A further problem is that of ascertaining the molecular weight of LREF. The determination of molecular weight of cytokines is a notoriously difficult process. Various methods are used to identify the molecular weight of proteins which exploit different characteristics of the molecule. Analysis by gel filtration depends on the Stokes radius of the protein molecules which is dependent upon their tertiary structure. The remaining conditions of the SDS-PAGE technique result in partial desaturation of the proteins with unfolding of the molecules and loss of much of the tertiary structure and the effects of charged amino acids. If performed under reducing conditions, that is with β-mercaptoethanol or dithioerythritol, disulphide bridges are disrupted with loss of quaternary structure, and subunits run independently.

On SDS-PAGE LREF gave a single band at 60 kDa under both reducing and non-reducing conditions indicated that the molecular weight is 60 kD.

Since the Mono-P purified LREF appeared to be a single band on SDS-PAGE, but separated into two fractions using anion exchange chromatography, it is

possible that LREF is a single protein with variable glycosylation. This is suggested by the fact that isoelectric focusing on polyacrylamide gels revealed numerous bands in the pI range 5.0 to 5.2. The use of multiple donors to generate LREF may contribute to this problem. One possible way to reduce this problem is to use a lymphocytic leukaemia cell line, as described for other lymphokines such as LIF (Meshulam et al 1982).

9.5 Biological role of LREF and its specificity

The effect of LREF was relatively specific for neutrophils, in that it could prime cells to generate more LTB_4 after IgG-bead stimulation, while it had little effect on eosinophil generation of LTC_4 using the same conditions (Fig 3.11). LREF appeared to have other biological effects on neutrophils including enhancing adhesive capacity to nylon wool and increasing cytotoxic capacity against schistosomula of S. mansoni (Fig 8.1 & Fig 8.2). These biological effects overlap with other cytokines, such as TNF and LIF, and might thus ensure effective functioning on neutrophils by providing a compensatory or "back-up" system. The fact that LREF has multiple effects on neutrophils is not unique, being seen with other inflammatory cytokines (e.g. LIF, TNF and GM-CSF)

The mechanisms by which lymphokine stimulation leads to 'activation' and/or directed movement of

neutrophils has yet to be determined. This is due in part to the fact that characterisation of lymphokine receptors has lagged behind the characterisation of the lymphokines themselves.

The increased expression of CR1 and CR3 receptors by LREF suggest its action on neutrophils is primarily a membrane effect. If there are indeed surface receptors for LREF, the inter individual variation in the effects of LREF may be due to variation in the relative numbers of available receptors (Williams & Granger 1973; Rosenau & Tsoukas 1976).

The mechanisms whether LREF is acting through its own receptors on neutrophils, or through other receptors (CR1 & CR3) were still undetermined. When sufficient quantities of purified materials become available it will be possible to radiolabel LREF, to establish a binding assay and to clarify the nature of LREF receptors on neutrophils.

There is good evidence that the immune response can be modulated by biological active mediators (e.g. LTB_4 or IFN-gamma) or by drugs such as dexamethasone. In my system, a clear inhibitory effect of LTB_4 on LREF generation was seen (Fig 6.1). Gel filtration analysis of supernatants from PHA-stimulated PBMC co-cultured with LTB_4 indicated that the decrease of LREA was due to a reduction in the 35-40 kDa protein which was previously characterized as LREF (Fig 6.2).

The addition of IFN-gamma did not affect LREA generation, although it had the same degree of inhibition of the cell proliferation as LTB_4 in the PHA-stimulated PBMC cultures. These findings suggest that the effect of LTB4 on the inhibition of LREF generation is not due to IFN-gamma production. However it remains unclear whether the reduction in LREF generation is due to a direct effect of LTB_4 on the T cells which produce LREF, or to an indirect effect, acting through monocytes or other cell types which suppress the T cells generating LREF.

Dexamethasone had no direct effect on neutrophil LTB_4 generation for up to 60 min incubation, confirming that the decrease in LTB_4 generation was due to the action of dexamethasone on T cells.

9.6 Could LREF be LIF?

Since LIF appears to be one of the most important lymphokines which can activate neutrophils, it is important to consider whether LREF is LIF. Several points suggested that LREF may be LIF. Firstly, both lymphokines possessed similar molecular weight (58 to 60 kDa) and pI (5.0 - 5.2)(Fig 7.1 & 7.2). Secondly, both lymphokines are generated from T lymphocytes under the stimulation of PHA and can be inhibited by co-culturing PHA-stimulated PBMC with LTB_4 (Fig 6.1). Thirdly, both lymphokines can enhance neutrophil cytotoxic activity (Fig 8.2). One of the major

characteristics of LIF is its enzymatic activity. LIF possesses esterolytic activity (Rocklin 1974; Rocklin & Rosenthal 1977; Lomnitzer et al 1975), and its action is inhibited by soybean trypsin inhibitor and by typical trypsin substrates, such as BAEE (benzyl anginine ethyl ester) (Bendtzen 1977a,b). The action of LIF on neutrophils is blocked by the serine esterase inhibitors DFP and PMSF (Bendtzen 1977a), and cAMP or dibutyryl cAMP partially block the effect of LIF on migration (Bendtzen 1978). LREF activity can only be partially inhibited by PMSF (Fig 7.9), but I cannot fully exclude the possible enzymatic activity of LREF without performing a study of a wider range of likely substrates. At the present time, it does not appear likely that LREF is LIF, but until such time as the amino acid sequences of both lymphokines are determined the matter cannot be resolved.

9.7 Relation of in vitro observation to in vivo events

Almost all studies of lymphokines have been carried out in cultures of more or less well defined cell populations. We tend to assume that what is observed in vitro also occurs in vivo and represents a significant part of the evolution of the immune response or of cell-mediated inflammation. Inferences have to be justified by convincing demonstrations of at least some of the following points: (1) that the

factor is produced and released in vivo, where its role is postulated, in physiologically effective concentration and at the correct time; (2) that injection of the factor reproduces the phenomena attributed to it; (3) that failure of factor production, e.g. in desensitized or immunologically deficient individuals, is correlated with failure of the associated phenomena; (4) that appropriate use of an antibody or specific pharmacologic antagonist inhibits its action in vitro and results in failure of the associated in vivo phenomena.

However, the application of these criteria has only been successfully performed for a few lymphokines such as MIF, IFN-gamma and IL-1.

At this early stage of LREF study, I can only speculate that LREF might play a role in some in vivo situations, especially in those showing mononuclear cell and neutrophil infiltration, such as allergic inflammation, acute and hyperacute allograft rejection, and rheumatoid arthritis (Robbin & Angell 1976). To confirm its role in vivo it is obviously necessary to raise an antibody against LREF and to use it to detect LREF in body fluids in appropriate situations.

What are the possible biological advantages in lymphokine regulation of IgG dependent LTB_4 release from neutrophils? The local production of LREF by

lymphocytes after cell activation might be important in trapping neutrophils at inflammatory sites and enhancing their cytotoxic functions. Systemic production of LREF, on the other hand, may contribute to leukoaggregation and control of bacterial infection. The fact that surface bound IgG-containing immune complexes stimulate the release of LTB_4 from neutrophils suggests that this mechanism could result in LTB_4 generation *in vivo* in any situation where specific IgG antibodies have bound to surface antigen, either foreign or host derived.

9.7 Summary hypothesis (Fig 9.1).

Many forms of acute and subacute inflammation show mixed neutrophil and mononuclear cell infiltration. The mechanisms of recruitment and activation of neutrophils are still the subject of some speculation, but acute phase proteins, bacterial products and, in certain circumstances, chemotactic mediators generated by mast cells and other cell types may all contribute to these events.

My working hypothesis is that neutrophil function is regulated by additional factors including T cell products. Once lymphocytes have been activated by specific antigen they produce a variety of lymphokines which can recruit and activate neutrophils. This property is shared with a number of mediators including LTB_4, C5a and PAF. Neutrophil activation can be demonstrated in terms of increased surface receptor expression. One lymphokine in particular, LREF, can prime neutrophils to enhance their capacity to generate LTB_4 in response to physiological stimuli such as an IgG-coated target.

IFN-gamma is a potent inducer of Ia antigen on human endothelial cells, this might endow endothelium in inflammation sites with the capacity to primed T lymphocytes and participate in the binding of other inflammatory cells. In the presence of LYNAP (LDNCF) neutrophil directional migration will be induced,

whereas cytokines such as LIF and GM-CSF will counter this effect and may act to retain the neutrophils in the vicinity of the target. Neutrophil activation may then be achieved by factors such as LREF and LIF and the cells will then deliver a maximum response at their target. Monokines may also participate in this activation process. Neutrophils may be attracted by MDNCF and TNF-alpha, then activated by TNF-alpha and IL-1. The primed neutrophils will release more LTB_4 after interacting with IgG-coated targets and this will exacerbate the inflammation through recruitment of further neutrophils and increased vascular permeability. This system also provides a possible autoregulatory feedback loop in that LTB_4 can inhibit lymphocyte activation, down-regulate lymphokine generation by lymphocytes and reduce LTB_4 generation by neutrophils.

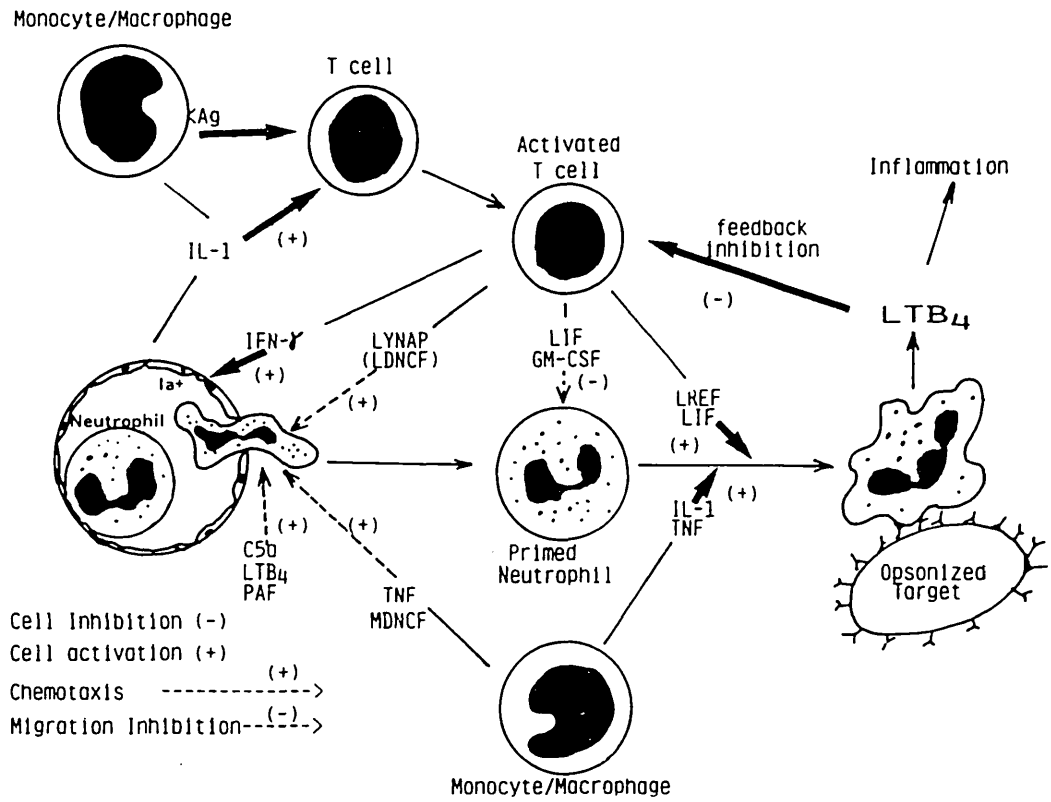

Figure 9.1: Schematic representation of the factors considered important in the cell-cell interaction and inflammation.

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