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THE ROLE OF NEUTROPHIL SECONDARY GRANULES IN INFLAMMATION: MODULATION OF LYMPHOCYTE RESPONSES.

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ABSTRACT.

THE ROLE OF NEUTROPHIL SECONDARY GRANULES IN INFLAMMATION;

MODULATION OF LYMPHOCYTE RESPONSES.

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Human neutrophils, stimulated by phagocytosis of opsonised Candida guilliermondii, were found to release an inhibitor of mononuclear cell proliferation. This inhibitor partially blocked radiolabelled nucleotide incorporation into mononuclear cell cultures stimulated by mitogens (Phytohaemagglutinin and Concanavalin A) or allogeneically in a three-way mixed lymphocyte culture (MLC). The inhibitory factor was identified as the iron binding protein Lactoferrin (Lf), and a requirement for the molecule to be carrying iron was demonstrated.

Inhibition of the MLC by Lf was found to occur only when the mononuclear cells were cultured in crowded conditions by using round bottomed wells. This enforced contact was shown to enhance proliferation dramatically, and it was this additional growth that was affected by Lf. Supernatants produced by crowded cells increased the proliferation of spread cells, whilst the addition of Lf to the cultures used to produce the supernatants reduced this effect. Thus, it appeared that Lf was affecting the production of a growth factor released in response to close cell contact.

The growth factor was subsequently identified as Interleukin 2 (IL-2). The production of IL-2 is dependent on Interleukin 1 (IL-1). However, no IL-1 activity could be detected in MLC supernatants. Under conditions which are known to induce IL-1 secretion, namely stimulation by lipopolysaccharide (LPS), an inhibitory effect of Lf was observed. It is suggested that in the MLC, proliferation is initiated by expression of membrane bound IL-1, and that this is inhibited by Lf, thereby explaining the requirement for crowding the cells in order to obtain an effect by the iron binding protein.

LPS stimulation of mononuclear cells was found to induce rapid secretion of a factor which dramatically enhanced neutrophil chemiluminescence in response to subsequent stimulation. The evidence suggests that this is tumour necrosis factor. Inhibition of this factor by Lf was variable, with some individuals responding and others not.

The combined data indicates a negative feedback role for Lf in the control of inflammation. The effect of Lf on production of pleotropic monokines may provide an explanation for the various reported functions of the protein.

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ABBREVIATIONS

ADCC	Antibody dependant cell mediated cytotoxicity
AEGPC	Acetyl glyceryl ethylphosphatidyl choline
APC	Antigen presenting cell
Apo-Lf	Iron free Lactoferrin
BCDF	B cell differentiation factor
BCGF	B cell growth factor
CC-S	Crowded cell supernatant
Con A	Concanavalin A
CSA	Colony stimulating activity
CSF	Colony stimulating factor
CTDF	Cytotoxic T cell differentiation factor
CTL	Cytotoxic T lymphocyte
DMSO	Dimethylsulphoxide
FBW	Flat bottomed well
FCS	Foetal Calf serum
Fe-Lf	Iron carrying lactoferrin (50% saturated)
fMLP	n-formyl methionyl leucyl phenylalanine
GM-CSF	Granulocyte-macrophage colony stimulating factor
IFN	Interferon
IL	Interleukin
ISC	Immunoglobulin secreting cell
LAK	Lymphokine activated killer cell
Lf	Lactoferrin
LPS	Lipopolysaccharide
LSM	Lymphocyte separation medium

LTB4	Leukotriene B4
MAF	Macrophage activating factor
MFR	Mamosyl fucosyl receptor
MLC	Mixed lymphocyte culture
MLR	Mixed lymphocyte reaction
MNC	Mononuclear cell
MNC-CM	Mononuclear cell conditioned medium
MPRM	Mono-poly resolving medium
NK	Natural killer cell
PAF	Platelet activating factor
PBS	Phosphate buffered saline
PFP	Pore forming protein
PGE_2	Prostaglandin E_2
PHA	Phytohemagglutinin
PMN	Polymorphonuclear neutrophil
PMN-PF	Polymorphonuclear neutrophil priming factor
PNCM	Phagocytosing neutrophil conditioned medium
PWM	Pokeweed mitogen
RBW	Round bottomed well
SAM	Suppresor lymphocyte associated molecule
SC-S	Spread cell supernatant
SIRS	Soluble immune response suppressor
STZ	Serum treated zymosan
TCR	T cell receptor
TdR	Thymidine radionucleotide

TNF	Tumour necrosis factor
TRF-1	T cell replacing factor 1
UdR	Uridine radionucleotide
ug	micro gram
ul	micro litre
uM	micro Molar
ZAS	Zymosan activated serum

CHAPTER ONE

INTRODUCTION

Inflammation has been described by civilisations dating back to the Greeks, who referred to the process as phlogosis. Later the Romans used the term inflammatio. Both civilisations recognised inflammation clinically and described the outward manifestations of the condition : rubor (redness), tumor (swelling), calor (heat) and dolor (pain). However, the first systematic observations were not made until the 16th century. John Hunter (1728-1793) was the first to recognise that the redness of inflammation was due to an increase in the blood flow through dilated blood vessels, and described the extravasation of plasma and "small globules". The next major advances in the understanding of the inflammatory response were made by Julius Cohnheim (1839-1884). He described micro-vascular events in vivo, notably hyperaemia followed by localised slowing of the blood. Cohnheim also noted margination and migration of leucocytes through vessel walls.

Elie Metchnikoff (1845-1916) followed by demonstrating phagocytosis and the elimination of the injurious agents of inflammation by phagocytic cells. With the work of Paul Ehrlich (1854-1915) on humoral immunity and that of Louis Pasteur (1822-1895) and Robert Koch (1843-1910) on the role of bacteria and other micro-organisms in disease processes, the foundations for the current understanding of the inflammatory response were set. Current understanding of inflammation has revealed a complex scenario involving cellular and humoral events which combine to cause the eradication of infectious

agents and removal of damaged tissue.

Briefly, the inflammatory response occurs following the release of soluble chemical mediators by the invading micro-organisms or by damaged tissues. This leads initially to arteriolar dilatation followed by an increased rate of blood flow through the micro-vasculature. The increased blood flow is responsible for bringing large numbers of leucocytes, plasma proteins, oxygen and nutrients to the affected area. As capillary dilatation occurs, vascular permeability increases, resulting in an extravasation of fluid (oedema) from the vessels to the tissues. This results in an increase in the concentration of red cells within the capillaries which slows down the blood flow. This enables the leucocytes to adhere to the vascular endothelium and to migrate towards the inflammatory site. The neutrophils are considered to be the first cells to migrate followed by monocytes and then lymphocytes. The leucocytes then kill invading organisms, remove the debris and may also mediate subsequent repair of the injured tissues.

Inflammatory sites, therefore, contain both inflammatory cells (neutrophils and monocytes) and immunocytes (lymphocytes). These cells interact in a variety of ways and communicate with each other by liberating various molecular signals. The following review will focus on the cells involved in inflammation with particular reference to the protein molecules with which they communicate, namely the cytokines.

1.1. Cells Involved In Inflammation

1.1.1. Polymorphonuclear Leucocytes

The neutrophil or polymorphonuclear leucocyte (PMN) is the most abundant circulating white cell in man, accounting for approximately 70% of circulating leucocytes. A major proportion of PMN in the circulation (60%) are marginated to the blood vessel walls. The circulating pool, however, represents only 5% of the body's total, the remainder being located within the bone marrow reserve pool.

Development

Development of neutrophils within the bone marrow takes approximately 14 days. During the first 7.5 days, the cell undergoes mitosis from the myeloblast through the promyelocyte to the myelocyte. During the next 6.5 days, post-mitotic development occurs from the metamyelocyte, to the band cell which leads to the mature neutrophil (Bainton *et al.* 1971).

This development arises from a progenitor cell common to monocytes which has been described in *in vitro* culture systems as the granulocyte-macrophage colony forming unit (GM-CFU). This arises from the pluripotent stem cell in response to colony stimulating factors produced by macrophages, lymphocytes, endothelial cells, and fibroblasts (Metcalf 1985). The production of neutrophils during myelopoiesis occurs at a massive rate, with a turnover of approximately 1.6×10^9 cells per kilogram per day (Athens *et al.* 1961).

Mature PMN have a very short half life in the circulation lasting about 6.5 hours. The cells then migrate to the tissues, where they are thought to remain functional for around 1-2 days. The fate of the neutrophils following this remains a mystery. However, a large number of PMN are excreted in the faeces and evidence is emerging that they are phagocytosed by macrophages.

Structure

The mature neutrophil is characterised by a lobed nucleus and by the presence of numerous cytoplasmic granules. The cell is 10-14 um in diameter, about twice the size of a normal erythrocyte, and is a typical end stage cell. It does not replicate and is capable of only limited protein synthesis, as indicated by condensed hetero-chromatin, no nucleolus and a small number of ribosomes and mitochondria. The cell contains microtubules and microfilaments which are involved in cell mobility and maintenance of cell shape. The microtubules are composed of tubulin and form the cytoskeleton of the PMN. The microfilaments are composed of actin and myosin and play an essential role in cell movement during chemotaxis, and also in the flow of membrane around a particle during phagocytosis. Glycogen particles are randomly distributed throughout the cytoplasm and appear to provide the energy source for anaerobic metabolism which produces ATP required for phagocytosis.

Primary (Azurophil) Granules

The primary granules develop during the promyelocyte stage of PMN maturation. They are essentially lysozomes containing hydrolytic enzymes involved in the killing and digestion of ingested organisms and particles. During phagocytosis their membranes fuse with that

of the phagocytic vacuole and their contents are discharged within.

Secondary (Specific) Granules

Secondary granules are formed during the myelocyte stage of development. At this point PMN division ceases and consequently the secondary granules outnumber the primary granules by approximately 2:1 due to the dilution of the latter during division. The secondary granules are smaller than the primaries. Like primary granules they are discharged during phagocytosis but, as will be discussed in detail below, the majority of secondary granules are released extracellularly.

Recently it has been suggested that the secondary granules are the source of various receptors which are translocated to the cell surface during activation of the PMN. O'Shea et al. (1985) suggested that the receptor for C3bi (CR3) is present in the secondary granule. C3bi is an opsonic fragment of the 3rd component of complement which binds to the surface of micro-organisms during the complement cascade. This receptor has been shown to act synergistically with receptors for the Fc portion of IgG to enhance adherence and phagocytosis of serum opsonised micro-organisms (Mantovani 1975; Goldstein et al. 1976 and Arnaout et al. 1983). Also, the secondary granule has been proposed as the intracellular location of the receptor for n-formyl-methionyl-leucyl-phenylalanine (fMLP) (Fletcher and Gallin 1983; Gardner et al. 1986). Components of the enzymatic complex that produces hydrogen peroxide have also been associated with the secondary granules (Borregaard and Tauber 1984; Ohno et al. 1985).

Neutrophils deficient in secondary granules fail to increase surface membrane expression of CR3, fMLP receptors and components of the hydrogen peroxide generating system as do normal neutrophils after stimulation. These observations support the concept that secondary granule membranes (possibly in addition to tertiary granule membranes) serve as an intracellular reserve for these and perhaps other components that can be mobilised to the cell surface during priming in the circulation, and sustain activation in the tissues (Gallin 1984). However, recent data reported by Berger et al. (1988) may question this assumption (see section 1.2.2.).

Tertiary Granules

Zonal centrifugation of PMN lysates has shown the existence of a third population of cellular storage particles, smaller than the other particles but of the same density as the secondary granules (Dewald et al. 1982). Like the secondary granules, they may be a major intracellular storage site for the CR3 receptor and for the cytochrome B_{245} (Petrequin et al. 1987; Mollinedo and Schneider 1984). At present it is difficult to know whether these tertiary granules should be distinguished from secondary granules, as there is considerable overlap in their components. However, it appears that there is a population of granules which is released very rapidly as a result of mild stimulation, such as warming the cells from $0^{o}C$ to $37^{o}C$ or by very low concentrations of fMLP. It has been suggested that this occurs early in cell activation and primes the cells for further activity as described above for secondary granules.

Function

PMN are among the first cells to arrive at the site of inflammation. This occurs in response to a series of signals, resulting in the mobilisation of the marginated pool and the marrow reserve pools and an increase in myelopoiesis and PMN production. As a result, the number of PMN in the peripheral circulation rises. The increased numbers of PMN then adhere to the capillary endothelium near the site of infection, emigrate into the perivascular tissues and then migrate towards the infection. The infecting particles are identified by interaction between their coating of opsonins and specific receptors on the surface of the PMN. They are then phagocytosed, killed and digested. This sequence of events is controlled by a series of regulatory molecules for which there are specific receptors on the PMN surface. The result of their binding is stimulation of cell adherence, aggregation, movement, degranulation and the respiratory burst. The regulatory molecules include products from the activation of complement, the coagulation cascade, bacteria, the oxidation of arachidonic acid and cytokines from mononuclear cells.

The receptors for these ligands are expressed in small numbers on the surface of circulating, unstimulated PMN. Upon stimulation, the receptor expression is increased from intracellular pools within the PMN granules as previously discussed. Low concentrations of stimuli are chemotactic and cause partial degranulation with increased expression of receptors for C3bi, fMLP, leukotriene B4 (LTB4) and probably C5a des arg. Higher concentrations lead to more extensive discharge of secondary granules, with translocation

of the cytochrome B_{245} to the cell surface and activation of the NADPH oxidase and the respiratory burst. The result is a progressive up regulation or priming of the cell, with increased chemotactic responsiveness and oxygen radical production as shown by exudate PMN compared with circulating PMN (Zimmerli et al. 1986).

Cellular Emigration

The PMN begin to adhere to the capillary endothelium near the site of insult. Both PMN and monocytes then extend pseudopods which force their way between adjacent endothelial cells. The mechanism of this process still remains obscure. It is not known whether the leucocyte mechanically breaks the junction, or whether enzymatic cleavage occurs. Once the pseudopod has been passed, the entire cell follows. Once through the endothelium, the cell passes through the basement membrane and into the perivascular connective tissues.

The adhesion of PMN to vascular endothelium involves a group of related leucocyte adhesion proteins which include LFA-1, P150,95 and CR3, known collectively as the Mac-1 complex (Anderson et al. 1986). Each member of the Mac-1 complex consists of a common beta sub-unit of 95 KDa and a specific alpha sub-unit of 150 to 180 KDa. The importance of these adherence proteins is illustrated in a rare autosomal recessive disorder in which there is a failure to synthesize the normal beta sub-units. These patients suffer severe bacterial and fungal infections. In addition, they have a high peripheral blood PMN count, which is probably due to the lack of a marginated pool (Anderson and Springer 1987). Anderson and Springer also reported in vitro defects in adhesion related

functions, including aggregation of activated PMN, adherence to endothelial surfaces, chemotaxis and phagocytosis of opsonised particles.

Chemotaxis

Chemotaxis is the unidirectional movement of cells up a concentration gradient of a chemotactic substance. This differs from chemokinesis, which is the random enhanced movement of cells in response to a chemotactic substance of constant concentration. During inflammation, the principal chemotactic cells are neutrophils and monocytes.

Both endogenous and exogenous chemotaxins have been described. The endogenous chemotaxins are either cell or plasma derived. Exogenous chemotaxins have been used extensively to investigate the phenomenon and are usually synthetic compounds related to procaryotic cell products.

The principal plasma derived endogenous chemotaxins are C5a and C5a des arg. C5a is a more potent chemotaxin than C5a des arg, with activity in the range 10^{-10} M to 2.4 x 10^{-8}M. This was found to be about tenfold more potent than C5a des arg (Fernandez et al. 1978). C5a des arg results from the rapid cleavage of the C terminal argenine from C5a by serum carboxypeptidase N. Therefore, C5a des arg is the most likely complement component to induce chemotaxis in vivo (Webster et al. 1980).

The remaining principle endogenous chemotaxins are cell derived. These are tumour necrosis factor (TNF), LTB4 and platelet

activating factor (PAF). TNF will be discussed in greater detail in the later sections. LTB4 is produced by mature neutrophils and was shown to be equipotent to C5a *in vitro* (Goetzl and Picket 1981). However, *in vivo*, LTB4 was considered less potent than C5a des arg in attracting neutrophils when injected intradermally (Movat *et al.* 1984). PAF, which is acetyl glycyl ethylphosphatidylcholine (AGEPC), induces LTB4 synthesis by neutrophils (Gorman *et al.* 1983), and this may be its *in vivo* significance.

Of the exogenous chemotaxins, formylmethionine tripeptides (eg. fMLP) have received considerable interest. There is a body of evidence which suggests that these compounds are of physiological importance; for example, *Eschericia coli* commences protein synthesis with N formylmethionine, whereas eucaryotic cells do not. Receptors for N formylated tripeptides have been found on neutrophils, as discussed earlier. It is therefore likely that these peptides act as procaryotic products which could be distinguished from host products. The best studied of these compounds is fMLP, which has been shown to attract neutrophils to an inflammatory site, stimulate adhesion and induce aggregation, activate the respiratory burst and stimulate degranulation.

Neutrophils migrating down a chemotactic gradient exhibit a characteristic morphology with a blunt leading front. Release of granules appears to occur at the leading edge, resulting in the highest concentration of receptors at the cell front and a gradient of receptors across its trailing surface. This could

explain the movement of the cell in a gradient of chemotactic factor resulting in directed movement of the cell towards a site of inflammation (Niedel et al. 1979). Bound receptors are probably internalised, the ligand digested and the receptor returned to the cell surface (Nunoi et al. 1985).

Degranulation

It is now clearly established that neutrophils exhibit both intracellular and extracellular degranulation, and that the types of granule have different functions during degranulation.

During intracellular degranulation, adjacent granules fuse with the membrane of the forming phagocytic vacuole, converting the phagosome into a phagolysozome. Since granule formation ceases beyond the myelocyte stage, degranulation irreversibly depletes the mature cell of these organelles. Electron microscopic cytochemical techniques on rabbit heterophils, using myeloperoxidase as a marker for primary granules and alkaline phosphatase for the secondaries, have shown that both granule types empty into the phagocytic vacuole. Alkaline phosphatase is seen in the phagocytic vacuole 30 seconds after the initiation of phagocytosis; however, myeloperoxidase does not appear until 1-3 minutes (Bainton 1973). Bainton suggested that the more rapid release of the secondary granules was due to them being less dense than the primaries, thus increasing the rate of active random movement and hence the probability of collision with the vacuole. This rapid release of the secondary granules may account for the increased concentration of secondary granule constituents in the extracellular medium following phagocytosis, due to the protein leaking before

completion of the vacuole. However, there is now a considerable volume of data which suggests that the secondary granules have a secretory role and that the contents function extracellularly (Leffel and Spitznagel 1974; Wright et al. 1977). In general, primary granules fuse with phagosomes and do not release their contents into the extracellular environment except after stimulation with high concentrations of chemotactic secretagogues (Wright 1982; Gallin 1984).

Extracellular release of neutrophil granule contents may occur actively or passively. Passive release occurs as a result of cell death and lysis. The lysis may occur as a result of extracellular environmental factors such as extremes of pH, temperature and osmolarity, or in response to toxins such as streptococcal and staphylococcal endotoxins. A number of circulating granule contents have been equated with neutrophil turnover. Hansen and Andersen (1973) showed that lysozyme in the plasma and urine reflects neutrophil turnover. Similarly, lactoferrin has been used as a marker of neutrophil turnover and activation (Harle et al. 1984; Lash et al. 1984; Hansen et al. 1975).

Phagocytosis

Once the PMN reaches the site of infection, it recognises the particle to be phagocytosed by an interaction between its surface receptors and opsonins bound to the invading organisms. The early investigations into the role of opsonins in phagocytosis revealed that serum contained heat stable and heat labile components. The heat stable opsonins were found to be antibodies and the labile

factors found to be complement components. These are now known to be specifically the Fc portion of IgG, particularly subclasses IgG1 and IgG3, and two derivatives of C3, namely C3b and C3bi. The receptors for these opsonins are FcR1 and FcR3 (IgG1 and IgG3 respectively) and CR1 and CR3 (C3b and C3bi). The immunoglobulin and complement opsonins have separate roles during phagocytosis. CR3, as discussed earlier, is a member of Mac-1 adhesion complex and is involved in the binding of opsonised particles to the PMN. The immunoglobulins are capable of stimulating the whole range of neutrophil responses, and ligation of the FcR results in ingestion and intracellular events involved in microbial killing (Newman and Johnson 1979).

Ingestion depends upon the sequential reaction between opsonin and receptor as the neutrophil flows around the particle. Binding of Fc to its receptor causes a release of actin binding protein within the cytoplasm, which induces actin filaments to form a solid gel. The gel in turn contracts in response to myosin in the presence of ATP. Production of further actin binding protein as a result of sequential ligand binding causes the pseudopod to form around the phagocytosed particle (Smolen and Boxer 1983). The energy for this process is derived from glycolysis and does not require oxygen. Sequential binding between receptors and opsonins leads to a "zipper effect" so that the particle becomes surrounded by a plasma membrane which forms the phagocytic vacuole (Wright 1985).

The Respiratory Burst

Mature neutrophils are metabolically rather dormant; they have few mitochondria and little endoplasmic reticulum. However,

perturbation of the plasma membrane by contact with a micro-organism results in a marked increase in metabolic activity, termed the " Respiratory Burst". This phenomenon was first described by Baldridge and Gerard (1933), who showed a rapid increase in neutrophil oxygen consumption following challenge with micro-organisms. This increase in O_2 consumption is accompanied by a rapid increase in glucose oxidation via the hexose monophosphate shunt (Sbarra and Karnovsky 1959). This surge in metabolic activity is used by the neutrophils to provide energy for phagocytosis and killing. The oxygen consumption is used by the cell's oxidase system to produce a number of highly reactive oxygen species involved in the killing of micro-organisms.

The nature of the oxidase has remained a subject of controversy for many years. It is now understood that the oxidase system is located on the plasma membrane. Thus as the membrane invaginates during phagocytosis, toxic oxygen radicals can be produced directly into the phagosome. The oxidase is now considered not to be one discrete enzyme, but rather an electron transport chain. This utilises NADPH as an electron donor and contains a novel B cytochrome (B_{245}) (Segal and Jones 1978), an FAD containing flavo protein (Cross et al. 1982) and possibly a ubiquinone (Crawford and Schneider 1982).

The oxidase system of the neutrophil can be activated by a wide variety of particulate and soluble stimuli. These are very diverse in nature, ranging from the physiological such as opsonised micro-organisms, immune complexes and complement components, to synthetic

compounds such as calcium ionophores and soluble tumour promoters. The physiological stimuli exert their effects by binding to specific receptors on the neutrophil's surface as previously discussed.

The superoxide produced by the neutrophil's oxidase system is rapidly converted to hydrogen peroxide and hydroxyl radicals, which provide most of the microbicidal oxidative activity within the phagosome. Neutrophil myeloperoxidase within the primary granules in the presence of hydrogen peroxide and halide is responsible for further catalysing formation of additional oxidants such as hypochlorous acid and free chlorine during the respiratory burst.

The products of the respiratory burst are toxic not only to the micro-organisms but also to the cell. Therefore the PMN is provided with a number of enzymes which protect against radicals that may leak from the phagosome. Hydrogen peroxide is broken down to water through oxidation of glutathione, which is then reduced by glutathione reductase to NADPH (Cohen et al. 1987). Superoxide is capable of spontaneous dismutation to hydrogen peroxide; however, both cytosol (McCord and Fridovich 1969) and mitochondria (McCord et al. 1977) contain superoxide dismutase, which is capable of increasing the rate of this reaction by a factor of 10^4. Thus its rapid removal would appear to be desirable.

Non Oxidative Killing Mechanisms

In addition to oxidative mechanisms, the role of non-oxidative processes for the killing of micro-organisms is now becoming apparent. These are mediated principally by enzymes present in the

primary granules. Many of the non-oxidative processes are possibly assisted by changes in the pH of the phagolysozome. Immediately following phagocytosis the lysozomal pH rises (Cech and Lehner 1984; Segal et al. 1981), and then falls to below 6. This alkalization and acidification may damage some micro-organisms, but it is more likely to provide optimal pH for the various microbicidal enzymes.

The non-oxidative mechanisms that are probably utilised first are the cationic proteins, since they are effective at neutral pH. They appear to impede bacterial replication but have little effect on the structural integrity of microbes. Odeberg and Olsson (1975; 1976) showed that cationic proteins inhibited synthesis of DNA and RNA in Staphylococcus areus. Lysozyme is also a cationic protein which acts by breaking down the peptidoglycans in bacterial cell walls. Its main role may be in the digestion of killed micro-organisms (Root and Cohen 1981). There are also a range of proteases present in the neutrophil granules having a basic, neutral or acidic pH optima. Lactoferrin present in the secondary granules has also been associated with microbicidal activity; this will be discussed in greater detail in a later section, however.

The importance of these non-oxidative killing mechanisms is illustrated by the neutrophil's ability to kill certain organisms under anaerobic conditions (Mandell 1974). However, certain organisms including Staph. areus and E. coli were not killed under these conditions, suggesting that some organisms are killed preferentially by oxidative processes and others by non-oxidative processes.

1.1.2. Monocyte/Macrophages

The macrophage is a relatively large, phagocytic cell of myeloid origin. Unlike most cells, macrophages have many functions. They are involved in the killing of intra-cellular parasites and tumour cells, and scavenge for foreign material and extra-cellular debris in addition to acting as regulators of certain immune functions. Unlike the neutrophil, macrophages undergo differentiation in response to environmental changes which result in alterations in their morphology, physiology and function. In addition to these direct aspects of the role of macrophages, they are involved in indirect interactions with lymphocytes. Both positive and negative interactions occur via monokines and lymphokines. These will be discussed in greater detail later.

Development

Monocytes develop from the same stem cells as neutrophils. Under the influence of the appropriate factors, this stem cell differentiates into a promonocyte which possesses the characteristics of mature macrophages, namely the presence of immunoglobulin receptors, phagocytic ability and adhesiveness to glass. Promonocytes undergo division and further differentiation into monocytes, which then leave the bone marrow and enter the circulation. The cells then enter peripheral tissues where they develop into discrete macrophage types. The type of cell is typical of individual tissues, for example, Kupffer cells in the liver, alveolar macrophages and connective tissue histiocytes. Together, these cells make up the reticulo-endothelial system. A

similar process of monocyte circulation and migration to the tissues occurs during inflammation, but at a much accelerated rate.

Like PMN, recognition of foreign material by mononuclear phagocytes is dependent on exogenous opsonins such as antibody and complement. Macrophages express distinct Fc receptors, for IgG2a and IgG2b, known as FcR1 and FcR2 respectively. In addition, they possess at least two distinct receptors for fragments of the third complement component (C3), and express a lectin-like receptor for mannosyl or fucosyl terminated ligands known as the mannosyl fucosyl receptor (MFR). They also express receptors for fibronectin and laminin. The function of Fc and C3 receptors on the macrophage is similar to those on neutrophils. Thus C3R are involved in adhesion of the opsonised particle to the cell, and ligation of FcR results in phagocytosis and stimulation of oxygen radical production (Wright and Silverstein 1983). Fibronectin coated ligands and laminin bind to distinct receptors on macrophages and promote Fc and C3 mediated phagocytosis, possibly by increasing contact between the cell and the phagocytic target (Bevilacqua et al. 1981; Bohnsack et al. 1985).

The MFR appears to function in the absence of exogenous opsonins. Under serum free conditions macrophages ingest zymosan, which is rich in manose, via their MFR. (Berton and Gordon 1983). However, this function has been questioned, since macrophages are able to synthesise all the complement components required for local opsonisation (Ezekowitz et al. 1985).

Structure

The morphology of monocytes and macrophages is consistent with that of active cells producing and secreting proteins constantly. The abundant cytoplasm is rich in rough and smooth endoplasmic reticulum, has a well developed golgi apparatus and many lysozomal granules. As monocytes differentiate into macrophages, they enlarge their cytoplasm with an increase in lysozomal granules and endoplasmic reticulum.

Metabolically active macrophages possess lymphocyte receptors, and it has been suggested that the binding of thymocytes to macrophages induces maturation in the former; this underlies the importance of co-operation between macrophages and lymphocytes during inflammation and immunity.

Function

In addition to its phagocytic functions, the macrophage is essentially a secretory cell, and it is the variety of different molecules produced that explains its multi-potency. Unlike neutrophils, the majority of secretions from macrophages are synthesized de novo following stimulation. The exceptions to this are a number of preformed hydrolytic enzymes contained within the lysozomes. These are primarily involved in intracellular degradative functions. A major product of macrophages is lysozyme, accounting for 0.5 to 2.5 % of the cell's total protein. Also, macrophages produce elastase and collagenase which may be involved in wound healing, and plasminogen activator which appears to have a broad spectrum of activities.

The cell stimulatory agents produced by macrophages are now subject to a vast amount of research. These include IL- 1 and TNF, which will be discussed in greater detail later. Also, the macrophage is the source of complement components C2, C3, C4 and C5 (Littman and Ruddy 1977). Thus, local production of complement proteins by macrophages may provide one of the initial responses to tissue injury or microbial invasion. In addition, the complement components could affect local vascular permeability and induce chemotaxis of further leucocytes to the inflammatory locus.

Upon stimulation, macrophages undergo a rapid oxidative burst with the production of reactive oxygen species similar to that occuring in neutrophils. It has been shown that these are involved in the tumouricidal and microbicidal actions of macrophages. Work has indicated that these oxygen intermediates are also responsible for some of the immunosuppresive activity of stimulated macrophages.

In the absence of any inflammatory stimulation, the newly arrived blood monocyte undergoes some morphological and biochemical modifications, but remains as a quiescent cell, known as the resident macrophage. If however, an inflammatory stimulus is present, either monocytes or resident macrophages undergo a process of enlargement and metabolic stimulation that results in a cell referred to as an inflammatory or stimulated macrophage. This process is characterised by a synthesis of 5` nucleotidase, increased ability to phagocytose particles via the C3b receptor and increased hexose monophosphate shunt activity resulting in O_2^- production (however no increase in H_2O_2 secretion occurs).

The final stage in macrophage maturation is the production of an activated macrophage. This cell is usually defined as possessing specific effector functions, such as the ability to kill intracellular organisms or tumour cells. Development into a true activated macrophage only occurs in response to factors produced by activated lymphocytes (Mackaness 1970). Because of this requirement for lymphocyte products, activated macrophages only occur late on in an inflammatory reaction and more usually in conditions of chronic inflammation. The lymphokine responsible for the activation of macrophages is referred to as Macrophage Activating Factor (MAF), and is produced by activated T cells. MAF has recently been identified as Interferon gamma (IFN gamma). The activated macrophage is characterised by the production of large quantities of H_2O_2, which is a potent tumouricidal and bactericidal agent.

The macrophage is now widely accepted as an accessory cell in inflammation and immunity. Mosier (1967) noted that removal of macrophages from spleen cell cultures prevented production of anti-sheep red blood cell antibodies. Oppenheim et al.(1968) showed that lymphocyte proliferation in response to antigens was also macrophage dependent. Since then, macrophages have been shown to be essential for the activation of T cells in response to antigens, mitogens and allogeneic cells. The requirement of macrophages for B cell activation has also been demonstrated. The mechanisms by which macrophages enhance immune responses can be divided into two categories. The first involves antigen uptake, processing and presentation. The second involves production of monokines and at

this level can be considered as non-specific (specificity being maintained at the level of monokine receptor expression as described later).

Nossal et al. (1967) were first to show that labelled antigens were localised and persisted for long periods in macrophages. A report by Schwartz (1987) has demonstrated that peptides are first ingested by the macrophage, then degraded and re-expressed on the macrophage's surface as a complex with HLA-DR antigens. The antigen is then presented to the T cell by the latter's antigen receptor, provided this is complementary with the HLA-DR on the macrophage. The combination of processed antigen and complementary HLA-DR antigens is, however, insufficient for optimal lymphocyte stimulation. In addition, the monokines are also required.

From this information, the macrophage can be considered to be involved in four major functional activities. First, they interact with and degrade infectious agents, immune aggregates and endogenous biological substances. Second, they initiate and enhance immunological activation of lymphocytes. Third, they are effector cells capable of destroying tumour cells and circulating micro-organisms and fourth, they have a suppressive function in immunity. The first function may be seen as a filtering process involving the cell's phagocytic capability and utilising its lysozomal hydrolytic enzymes. If the foreign material persists, then the immune system is recruited. This involves the macrophage presenting the antigen to lymphocytes in an enhanced immunogenic form and increasing the

latter cells' response by production of IL-1. The effector function of macrophages could then be initiated by positive feedback from lymphocytes by, for example, INF gamma . This stage of activation would again require persistence of antigen to maintain the stimulation of the lymphocytes. Finally, a less well defined macrophage suppressor function would come into play and reduce the reaction.

1.1.3. Lymphocytes

The definition of two major sub-populations of lymphocytes was derived from studies in chickens by Cooper et al. (1966). They demonstrated that the bursa of Fabricius and thymus are central lymphoid organisms for the development of immunity in that species. Considerable developments in the understanding of the role T and B lymphocytes in inflammation and immunity has since occurred. Although the lymphocytes are essentially immunocytes, their importance in inflammation is clear. The T cells have essentially two effector functions: the cytotoxic killing of target cells and the release of lymphokines which regulate the function of other inflammatory cells. The principal role of B lymphocytes is secretion of immunoglobulin (by the fully differentiated plasma cell) and the maintenance of immunological memory. Antibody/antigen complexes potentiate inflammation by their direct opsonic effects and by activating the complement cascade which produces further opsonins. Complement components are also directly cytotoxic, and hence the humoral immune effects of antibodies are particularly effective against the extra-cellular phases of bacterial and viral infections.

There is a population of lymphoid cells which behave neither like T cells nor B cells and are designated null cells. Among these cells are natural killer cells (NK) which, unlike other lymphocytes, are capable of lysing a variety of transformed cells in culture without prior sensitisation. NK cells may play a role in the defence against viral infections and tumour cells. The classification of lymphocytes into two major sub-populations began with the work of Raff (1971). He demonstrated that anti-immunoglobulin antibodies and antibody to the THY-1 antigen could be used to identify and separate functionally distinct lymphocytes. A vast amount of data has since emerged on the expression of surface phenotype on both lymphoid and myeloid cells; and this was collated by the Third International Workshop on Human Differentiation Antigens in 1986. The conference produced a list of 50 cluster determinant (CD) groups, which is now the accepted nomenclature for the human leucocyte differentiation antigens.

1.1.3a. T Lymphocytes

The T Lymphocyte plays a critical role in the regulation of the immune response and appears to be the central regulatory cell responsible for focussing inflammatory responses on specific targets, either by direct cytotoxic effects or by enhancing B cell and monocytes/macrophage responses.

T Lymphocyte Development

T lymphocyte precursors are found in the yolk sac and liver of the foetus and in the bone marrow and spleen of the adult. The immature cells then migrate to the thymus, where they differentiate

and acquire the phenotypic characteristics and immunological reactivity of mature T cells (Stutman 1978). Once seeded by T cell precursors, proliferation of T lymphocytes in the thymus occurs at an enormous rate, such that around 99% of thymocytes are destined to die without maturing. This rapid turnover (combined with results of experiments showing that irradiation of murine bone marrow does not affect intra-thymic populations or the mature T cell pool in peripheral tissues), has led to the suggestion that T cell numbers are maintained by an intra-thymic self-renewing precursor pool (Adkins et al. 1987). The importance of the thymus in T cell development is highlighted by the inability of T lymphocyte precursors to exhibit any of the effector functions of mature cells. The T cell functions mentioned above are at least partially dependent upon the expression of a variety of cell surface antigens. These include the T cell receptor, growth factor receptors, the recognition antigens which participate in interactions with class 1 and 2 MHC structures, homing receptors involved in the homing of emergent thymocytes to the peripheral lymphoid organs, and an abundance of molecules which have been characterised but have yet to be assigned a function. Thymocytes express to a greater or lesser degree most of these markers and the relative expression is considered as an indicator of maturation (Adkins et al. 1987).

Using the CD classification, mature T cells are divided into the $CD4^+$ (helper) phenotype and the $CD8^+$ (suppressor/cytotoxic) phenotype. The $CD4^+$ sub-population functions by production of soluble mediators such as IL-2 and B-Cell factors which amplify

immune and inflammatory responses. The $CD8^+$ lymphocytes function as effectors which recognise and eliminate cells bearing infectious agents and tumour antigens. Unlike B cells, which mediate their antigen specific functions via secreted or membrane bound immunoglobulins that bind soluble ligands, T cells are predominantly responsive to cell surface antigens. Specifically, T lymphocyte membrane receptors recognise antigen on antigen presenting cells (APCs), which express autologous class 1 or class 2 major histocompatability complex proteins on their surface. This interaction between T cells and APCs results in the activation of $CD4^+$ and $CD8^+$ T cells and leads to proliferation, lymphokine production and target cell lysis.

T Lymphocyte Receptors

Mature T cells possess a number of cell surface receptors which have functions consistent with the activities of T cells. The T cell receptor (TCR), by which T cells recognise antigen has stimulated a considerable amount of interest over recent years (reviewed by Oettgen and Terhorst 1987). The TCR has been shown to be an 80-90 KDa glycoprotein containing two disulphide-linked chains, alpha and beta. The presence of constant and variable regions of the primary structure has been demonstrated; the rearrangement of the genes encoding the receptor has been observed. Thus the TCR is in many ways similar to antigen specific surface immunoglobulins on B cells. In addition, the TCR is physically associated with a group of proteins termed the T-3 complex (CD3) which consists of three monomorphic molecules (alpha, delta and epsilon). The T3 complex is thought to be involved in

transmembrane signalling.

In addition to the TCR, human T cells bear two other receptors involved in activation, the sheep erythrocyte-binding receptor (E receptor) and the IL-2 receptor. Expression of the latter can be induced by antigen binding to the TCR or by antibodies to the E receptor. Stimulation of IL-2 receptor expression by one of these mechanisms makes the cell unresponsive to the other. Therefore, simultaneous ligation of both TCR and E receptors will inhibit IL-2 receptor expression and prevent proliferation of the T cell. Herbert and Watson (1986) have used this phenomenon to develop a hypothesis to explain autologous T cell tolerance during ontogeny. They postulate that Ts produce a cell surface molecule (suppressor lymphocyte associated molecule, SAM) which is the ligand for the E receptor. Thus, Ts would inhibit proliferation of T cells activated by autologous antigen/TCR interaction by simultaneously ligating the responding cells' E receptor by SAM. During T cell development in the thymus Ts would, therefore, be able to inhibit proliferation of self-reactive T cells whilst enhancing the growth of non-self reactive thymocytes by stimulating expression of IL-2 receptors via E receptor/SAM interaction only.

In addition, T cells express surface adhesion molecules of the Mac 1 family which have been described under the section on neutrophils.

T Lymphocyte Function

T cells mediate the effector arm of cellular immunity via cytolytic effects (Tc) used to combat intracellular infections, tumour cells

and foreign tissues. In addition, T cells perform a regulatory role in either promoting or suppressing the development of immune functions. Antigen specific regulatory T cells can be either helper/inducer (Th/Ti, $CD4^+$) or suppressor (Ts, $CD8^+$) types as previously discussed. Since the Th functions are mediated largely through secretion of cytokines (which are discussed in great detail later), only Ts and Tc functions will be discussed here.

Suppressor T Lymphocytes

The antigen specific Ts functions remain poorly understood; however, they are thought to act by inhibiting Th functions. The work of Damle (1986) suggests that $CD8^+$ Ts recognise antigen specific structures on the surface of antigen reactive $CD4^+$ cells and that this interaction initiates maturation and clonal expansion of the Ts cells.

A better characterised mechanism of suppression by Ts is the production of soluble immune response suppressive factor (SIRS), which non-specifically inhibits in vitro proliferation and antibody responses to thymic dependent and thymic independent antigens (Pierce and Tadakuma 1977). SIRS is not cytotoxic and does not inhibit T cell functions such as induction of cytolytic T cells and the mixed lymphocyte reaction. SIRS does not act directly on B cells, but depends upon the presence of macrophages which are induced to become suppressive. The mechanism by which macrophages mediate this suppression of B cells is unclear; candidates could however be prostaglandins, thymidine analogue and oxygen intermediates, all of which are known to be produced by

macrophages. Another mechanism requires direct cell contact. Ptak et al. (1977) demonstrated that macrophage membranes will block immune responses in vitro. They postulated that these membranes bind necessary growth factors, thereby preventing lymphocyte activation.

T Lymphocyte Cytotoxicity

The majority of the work conducted into cytotoxic T lymphocytes (CTLs) has involved the use of allo-immune T cells produced in vivo by injection of normal or neoplastic allogeneic cells, or in vitro in the mixed lymphocyte reaction (MLR) against irradiated or mytomycin C treated lymphocytes. This work revealed the importance of MHC molecules in CTL responses; however, apart from its obvious importance to transplantations, it is difficult to envisage a true physiological role for the CTL phenomenon in this context. Curiously, in the one physiological condition in which allo grafting occurs, i.e. in pregnancy, allo immune CTL do not appear to be involved in causing abortion. Of greater in vivo significance is the MHC restricted cytotoxic response, whereby immune effector cells and the antigen bearing cells are MHC compatible. This occurs against virus infected cells, with tumour associated antigens and cells that have been modified by chemicals such as trinitrophenol.

The classification of cytotoxic lymphoid cells has remained controversial despite many years of research. Classically, any lymphoid cell capable of non MHC restricted cytotoxicity has been considered a natural killer cell, whereas cells with antigen specificity have been identified as CTL. Lasier and Phillips (1986)

have attempted to rectify this situation by proposing a classification for three types of human cytotoxic lymphocytes. These are:

1) antigen specific, MHC restricted CTL that recognise targets via the T cell receptor complex.

2) CTL which recognise targets independent of MHC restriction but with the T cell receptor.

3) non-MHC restricted NK cells which do not express the T cell receptor. They suggest that these are distinct from T cells.

The Mechanism of Target Cell Lysis

The way in which effector cells mediate the lysis of target cells has yet to be fully elucidated. However, the importance of cell contact is clear for all the cell types involved. As previously discussed, both neutrophils and macrophages are involved in the cytotoxic process, as are eosinophils. The requirement for antibodies and direct contact has resulted in this process being termed antibody dependent cell mediated cytotoxicity (ADCC). In the case of CTLs the contact is mediated via the MHC molecules and the T cell receptor as discussed.

The mechanism by which T cells induce lysis in target cells has been reviewed by Young and Cohn (1986). Following contact of CTL to target, the permeability of the target cell membrane increases. This has been associated with pore formation in the membrane. Unlike PMNs and macrophages, lymphocytes do not utilise oxygen species for their cytolytic activities. However, cytoplasmic granules have been implicated in lytic functions of CTLs and NK

cells. Isolated granules have been shown to fuse with the target cell membrane and to induce pore formation in a calcium dependent manner similar to that seen with intact CTL. Young and Cohn have isolated and partially characterised a pore forming protein (PFP, also termed perforin or cytolysin) from CTL granules and have demonstrated that this molecule is remarkably conserved over a wide range of species. This PFP was shown to share immunological cross reactivity with the ninth complement component (C9). PFP is not, however, the only lymphocyte granule constituent involved in cytolytic reactions. Serine esterases, proteoglycans and lymphotoxin (TNF-beta) have also been implicated. Examination of CTL mediated target cell lysis revealed that release of cytoplasm was paralleled by release of DNA fragments. Since TNF beta appears to mediate nuclear damage, it was suggested that TNF beta is injected through the pore made by PFP, thus causing irreparable cell damage.

1.1.3b. B Lymphocytes

The B lymphocyte is characterised by its expression of surface Ig (sIg) which acts as the cell's antigen receptor. Cross linkage of the sIg by specific antigen molecules results in triggering of the B lymphocyte; however, as will be discussed below, this by itself is insufficient to promote antibody production.

B Lymphocyte Development

B lymphocytes bearing sIg can be detected in the foetal liver of man at approximately 9–12 weeks gestation. The cells are derived from a pluripotent stem cell common to T lymphocytes,

megakaryocytes and erythrocytes. The hepatic B lymphocytes are preceded by pre-B cells characterised by their lack of sIg and the presence of small amounts of cytoplasmic IgM (Raff 1977). However, as foetal development proceeds, the number of hepatic pre-B cells diminishes whilst the number of pre-B cells in the bone marrow and spleen increases. After birth and in the adult, pre-B cells are only found in the bone marrow. The mechanisms which control B cell development are still poorly understood. Recent work using long term bone marrow cultures has indicated a close association between bone marrow stromal cells and lymphopoiesis. This is thought to involve both direct contact between the immature cells and the stroma and also factors produced by the stromal cells. These stromal cell derived pre-B cell factors are thought to represent novel molecules, and are not related to any of the Interleukins so far described (Dorshkind 1987).

A considerable amount of work has been conducted into the perplexing diversity of antigens to which B cell clones are reactive. The way in which B cells become tolerant to self antigens has also been extensively investigated. However, these aspects of B cell function are beyond the scope of this review and therefore only work relating to B cell activation by soluble mediators will be discussed.

B Lymphocyte Activation

The activation of mature B cells to Ig secreting plasma cells is a complex process involving sequential events that are regulated by distinct and specific signals. Briefly, antigen activates a resting B cell to proliferate; the resultant daughter cells then

differentiate to become antibody secreting cells. The importance of the role of T cells in this process is becoming increasingly apparent, particularly in man (reviewed Jelineck and Lipsky 1987b). Current understanding suggests that antigen activates resting B cells to become responsive to T cell derived helper factors which will be discussed in detail below.

The importance of B cell proliferation was first recognised by Burnett (1959) in his clonal expansion theory. This allows for a small number of antigen specific B cells to expand, permitting the generation of an effective antibody response. This phenomenon of low precursor frequency is responsible for the difficulty in studying antigen specific B cell responses and has led to the majority of the work being conducted with polyclonal B cell mitogens such as LPS. Subsequent work using specific inhibitors of cellular DNA synthesis (mytomycin C or hydroxyurea) has demonstrated that proliferation is necessary for the differentiation of B cells. These findings indicate that B cell proliferation plays a required role in the differentiation of antibody secreting cells beyond the requirement to expand the number of antigen reactive cells. It has also been demonstrated that Ig synthesising B cells continue to proliferate whilst producing antibody. Furthermore, the continued proliferation of Ig secreting cells may be important in promoting antibody heavy chain isotype switching and providing the opportunity for somatic mutations that increase the affinity of secreted antibody

B cell responses have classically been defined as T cell

independent (TI) and T cell dependent (TD). TI antigens are distinguished from TD antigens by a small number of common properties. For example, TI antigens are poorly metabolised high molecular weight polymers with simple repeating structures, and it has been postulated that these act by cross linking surface receptors, thus providing a maximal activation signal. Examples of TI antigens in the mouse are LPS, polymerised flagellin and pneumococcal polysaccharide. Few agents have convincingly been shown to induce differentiation of Ig secreting cells in man without T cell influences, and the concept of pure T cell independence in the mouse is also now being questioned.

T cell dependent responses include those induced by both antigen and mitogen and can be further divided into two sub-groups. These are those in which direct physical contact between T and B cells is required, and those which only involve T cell derived lymphokines. Classically, the role of TD antigens was demonstrated with trinitrophenol (TNP), which is unable to evoke an antibody response by itself. Antibodies to TNP could only be raised when it was coupled to a carrier protein such as albumin, and it was postulated that the TNP-albumin conjugate must sequentially interact with macrophages, these then present the antigens to carrier-specific T cells which then activate hapten-specific B cells. More recent studies have demonstrated the ability of B cells to serve as antigen presenting cells. The B cell could therefore be considered to take up the hapten carrier conjugate, degrade the antigen and present the relevant immunogenic moieties to T cells in the context of self MHC molecules, thus illustrating the requirement of

direct T-B contact in B cell activation.

Work investigating the activation of B cells by soluble factors has demonstrated a complex sequential interaction of cytokines, principally of T cell origin but also from monocytes and autocrine signals from B cells. This area of research has been further complicated by recent realisation that B cell factors, as with many other cytokines so far investigated, are pleotropic in their actions, and by the finding that each factor mediates multiple effects on a single target. The sequencing of these factors and their pleotropic nature has made them candidates for Interleukin classification.

The B cell factors were originally described, as with other cytokines, on a functional basis. Thus, the B cell growth factors (BCGF1 and BCGF2) are involved in B cell proliferation, whilst the two B cell differentiation factors, (BCDF1 and BCDF2) are responsible for the maturation of activated B cells into Ig secreting cells. The cloning of the cDNAs for these factors has revealed a considerable overlap in their functions (reviewed by O'Gara et al. 1988). IL-4 was first postulated to equate with BCGF1 because it could co-stimulate with anti-Ig and cause a proliferation of resting B cells. IL-4 has since been shown to induce expression of murine MHC molecules, and low affinity receptors for the Fc portion of IgE (CD23) on resting B cells. IL-4 can also increase IgE antibody production by activated murine B cells. These functions have a number of important implications for the immune response. The increase in MHC expression could enhance B cell antigen presentation to T cells and thus increase the

sensitivity to small amounts of antigen. The induction of proliferation would increase clonal expansion of antigen specific B cells and the regulation of immunoglobulin isotype production may modulate humoral responses to different antigenic stimuli.

BCGF2, which causes DNA synthesis and IgG and IgM secretion by preactivated B cells, and T cell replacing factor 1 (TRF-1), which increases antigen specific antibody responses by B cells from antigen primed mice, are now known to be identical and have been classified as IL-5. Human IL-5 has been demonstrated to induce IgM secretion in activated peripheral blood B cells and splenic cells, to induce the formation of eosinophil colonies from human cord blood and cause the differentiation of thymocytes into cytolytic T cells.

The factor which is now termed IL-6 was originally described as BCDF, which induced antibody secretion by preactivated normal and Epstein Bar Virus transformed human B cells. Although IL-6, as with IL-4 and 5, is a T cell product, the cDNA sequence of IL-6 has been shown to be identical to that of human fibroblast product termed 26 KDa protein. This factor was found to possess anti-viral activity and is co-synthesized with Interferon beta 1; it was therefore also termed Interferon beta 2.

Other lymphokines (IL-1, IL-2, IL-3 and IFN gamma), originally thought to influence cells other than B cells, are now known to modulate B cell function. IL-1 has been shown to enhance B cell proliferation and differentiation to pokeweed mitogen (PWM) stimulated partially T cell depleted cultures. This occurred

when IL-1 was added at the initiation of the culture, but did not occur when added 24 hours into the culture period (Lipsky 1985). This suggested that IL-1 exerted its major effect on B cells before the T cell factors were required. IL-1 has little effect on activated B cells but is able to enhance proliferation and differentiation in the presence of T cell lymphokines (Falkoff et al. 1984).

The role of IL-2 in B cell responses has been controversial, however, activated B cells have been shown to express the Tac antigen and to proliferate following exposure to IL-2. The lymphokine has been shown to induce differentiation of Ig secreting cells. This effect may also be enhanced by IFN gamma.

The importance of IFN gamma in supporting B cell growth and differentiation into Ig secreting cells remains unresolved. In many cases, this lymphokine synergises with IL-1 and IL-2. IFN gamma exerts both stimulatory and inhibitory effects on polyclonal and antigen specific responses. IgG2a production is significantly enhanced by IFN gamma treatment of LPS stimulated murine B cells, whilst IFN gamma preferentially suppresses the IL-4 mediated enhancement of IgG1 and IgE by LPS stimulated murine B cells.

There is also a body of data which suggests an autocrine function in B cell development. B cells have been shown to produce IL-1, (Gordon et al. 1986) which could then feedback and act in the manner described above. Furthermore, the receptor for the Fc portion of IgE (CD23), has been shown to have an autocrine stimulatory function (reviewed by Gordon and Guy 1987). This

functions in concert with a 12 KDa T cell derived BCGF, which induces the cleavage and processing of the B cell bound CD23 to its extracellular auto-stimulatory form. Thus, a potent amplification cascade can be envisaged.

The response of B cells to antigen is therefore a highly complex process involving the sequential interaction of a number of factors which either prime or direct the cell to its next stage of development.

1.2. Cytokines Involved in Inflammation

1.2.1. Interleukin-1

The first reported actions of IL-1 can be traced back to 1972, when Gery et al. reported that cultured human peripheral blood adherent cells produced a factor capable of stimulating proliferation of murine thymocytes in vitro. The early studies on this monokine resulted in considerable confusion, which in the light of later work can be attributed to the pleotropic nature of IL-1. The spectrum of activity of IL-1 initially led to a variety of factors being described; these were subsequently grouped, on the basis of their biological and biochemical similarities, under the heading of IL-1. Thus lymphocyte activating factor (LAF) described the stimulation of thymocytes by IL-1. The term endogenous pyrogen (EP) was used for the effect of IL-1 on the thermoregulatory centre of the hypothalamous which induced fever. Leucocyte endogenous mediator (LEM) described the induction of the acute phase response by IL-1. Later, catabolin, a factor that promoted degradation of cartilage matrix, was found to be equivalent to IL-1. A variety

of effects of IL-1 in inflammation were described, but many of these have recently been attributed to TNF which was found to be contaminating IL-1 preparations.

The initial biochemical investigations only served to confuse matters further. Molecular weights for this factor were reported to be between 2 and 75 KDa and with a range of isoelectric points from 4.0 to 8.0. With the cloning of murine (Lomedico et al. 1984) and Human IL-1 (Auron et al. 1984) a better understanding of this monokine has emerged.

Auron showed that human IL-1 from peripheral blood monocytes is synthesized as a 269 amino acid precursor of molecular weight 32 KDa. This molecule is then cleaved to produce an 18 KDa extracellular form of pI 7.0. A second IL-1 gene was discovered by March et al. (1985), the product of which had an identical molecular weight to Auron's protein, but had a pI of 5.0 (equivalent to that of murine IL-1). The pI 7.0 is referred to as IL-1 beta and the pI 5.0 as IL-1 alpha, the former being the predominant species in man. There is a one hundred fold increase in mRNA for IL-1 beta over IL-1 alpha in the cytoplasm of stimulated monocytes (March et al. 1985).

Although the monocyte has been most extensively studied as a producer of IL-1, a wide variety of cells have been reported to release IL-1 activities. These include B cells, endothelial cells, epithelial cells, fibroblasts and many others. Most normal cell types, however, only produce IL-1 in response to stimulation. Studies on the kinetics of IL-1 production by human monocytes

revealed no intracellular or extracellular IL-1 production by unstimulated cells. Following stimulation by LPS, intracellular IL-1 appeared within 30 minutes and extracellular IL-1 activity could be detected within 60 minutes (Matsushima et al. 1986), high extracellular levels being attained by 3 hours.

An unusual feature of IL-1 revealed by its cDNA sequence is the absence of a signal peptide which is characteristic of secreted proteins. This has raised questions as to whether IL-1 is truly a secretory protein. Gery and Lepe-Zuniga (1983) have suggested that perforation of the plasma membrane provides one mechanism for the release of intracellular IL-1. There is an expanding body of evidence suggesting that IL-1 bound to the monocyte plasma membrane is important in the stimulation of lymphocytes. Kurt-Jones et al. (1985) have suggested that membrane bound IL-1 in association with Ia antigens is involved in the activation of T cells during antigen presentation.

Conlon et al. (1987) used monoclonal antibodies raised against IL-1 alpha and beta to detect and localise the two forms of IL-1 within monokine producing cells. Using flow cytometery they found a dramatic increase in the amount of surface bound IL-1 alpha on LPS stimulated monocytes above that of unstimulated monocytes. They could not, however, detect any membrane bound IL-1 beta, although acetone permeabilisation of the membrane revealed the presence of cytoplasmic IL-1 beta. In the light of March's data on cytoplasmic mRNA for IL-1 beta discussed earlier, Conlon suggested that IL-1 alpha may preferentially be associated with the surface of cells, particularly those involved in antigen presentation, and that IL-1

beta may be active as a secretory factor.

The membrane associated IL-1 may have different functions *in vivo* from secreted IL-1. Membrane bound IL-1 could serve as a local stimulant of immune lymphocytes at the site of infection or injury. Secreted IL-1, which may follow the membrane IL-1 due to positive feedback by IFN gamma released from T cells stimulated locally, may act predominantly as a mediator of the systemic response discussed in detail below.

IL-1 secretion *in vitro* is widely induced by LPS and endotoxin is considered to be the principal stimulus *in vivo*. Okusawa *et al.* (1987) have demonstrated that C5a is able to induce IL-1 production. They showed that C5a is a more potent stimulator than C5a des arg by a factor of 5, and that a potent synergy occurred with endotoxin or IFN gamma. Consistent with Conlon's data they found that the secreted IL-1 was IL-1 beta. This and the other work discussed above illustrates the close co-operation between the various endogenous mediators involved in inflammation. Bacterial and fungal infections, trauma and severe immune complex diseases are associated with complement activation and IL-1 production, which must act synergistically to result in the acute phase response which follows.

Recent work into the effect of IL-1 *in vivo* is further revealing the co-operation that exists between cytokines. Neta *et al.* (1987) administered IL-1 to mice and demonstrated that this treatment

protected the animals from lethal radiation. The phenomenon of radio protection of animals by injection of endotoxin has been known for many years. Neta suggested that this occurs by IL-1 production, which acts on bone marrow progenitor cells to increase their responsiveness to granulocyte macrophage colony stimulating factor (GM-CSF). Broudy et al. (1987) demonstrated that IL-1 stimulates human endothelial cells to produce GM-CSF and granulocyte CSF (G-CSF). Their data, combined with that of Neta et al., suggests a mechanism by which IL-1 could modulate granulocyte numbers during the course of an inflammatory response.

Both positive and negative feedback loops appear to operate in the production of IL-1. Dinarello et al. (1987) showed that IL-1 alpha was able to induce IL-1 beta production in vivo in rabbits and from human mononuclear cells in vitro. The in vitro data showed that maximal IL-1 beta production (the majority of which remained intracellular) occurred in response to 10 ng ml^{-1} IL-1 alpha; increasing the concentration to 100 ng ml^{-1} caused a reduction in IL-1 beta production. They showed that this was due to production of prostaglandin E_2 (PGE_2), which has been shown by others to inhibit IL-1 production (Knudsen et al. 1986; Kunkel et al. 1986). Therefore, it appears that IL-1 can directly increase its own production up to optimal concentrations, whereupon it induces a negative feedback mechanism through PGE_2.

Warner et al. (1987) showed that IL-1 can also induce IL-1 production by endothelial cells and also demonstrated a similar negative feedback mechanism by PGE_2. They suggested that IL-1 may play an important role in altering endothelial function during

inflammatory responses. IL-1 increases the expression of leucocyte adhesion molecules on the endothelial cell surface (Bevilacqua et al. 1985) and thus may be important in initiating the migration of leucocytes from the circulation to the inflammatory locus.

IL-1 has been shown to have other positive feedback effects by causing T cells to produce IFN gamma; this stimulates HLA-DR expression on a range of cell types (Pober et al. 1986). In addition, Haq et al. (1985) showed that IFN gamma is required for the maintenance of IL-1 production in vitro. They reported that human monocytes lose their ability to secrete IL-1 after 24 hours of culture with LPS, although they were able to detect intracellular IL-1 for up to 72 hours in culture. They showed that pre-treatment of the monocytes with IFN gamma enabled the cells to secrete IL-1 for up to 9 days. Their work suggests that IFN gamma, produced by T cells, plays a role in the maintenance of IL-1 production and illustrates a potential positive feedback mechanism to enhance inflammation.

Initial studies on the receptor for IL-1 were carried out in the murine T lymphoma line LBRM-33-1A5 (Dower et al. 1985). This work revealed a surprisingly low receptor number of around 500 per cell. The number of IL-1 receptors found on peripheral T cells was extremely low, around 50 per cell (Dower et al. 1985; Matsushima 1985). A survey of primary cell populations and of cell lines revealed that the expression of IL-1 receptors was consistent with the pattern of biological responsiveness. The receptor has since been shown to bind IL-1 alpha and beta and it appears that the same

receptor is present on all the different cell types tested. Following binding, the receptor-ligand complex is internalised and the IL-1 can be found within the nucleolus. It has been suggested that IL-1 can subsequently regulate gene transcription directly (Perlmutter et al. 1986).

The exact role of IL-1 in T cell activation remains unclear; however, an understanding is emerging. IL-1 induces IL-2 production in lectin activated T cells (Smith et al. 1980; Larsson et al. 1980) of the helper subset. In addition, IL-1 can induce IL-2 receptor expression on T cells (Kaye et al. 1984). The IL-2 produced then causes any IL-2 receptor positive cell to enter S phase. Specificity is maintained by the interaction of the T helper cell and the monocyte. The cross linking of the HLA DR/antigen complex on the monocyte with the antigen receptor on an HLA-DR complementary T cell stimulates the monocyte to produce IL-1 which then activates the T cell. This interaction emphasises a requirement for close contact in the paracrine action of IL-1 (Durum et al. 1984). In addition to its effects on IL-2 production and action, IL-1 induces other T cell effects including chemotaxis and changes in membrane viscosity. Also, T cells produce other lymphokines in response to IL-1: for example, B cell growth factors, IFN gamma and colony stimulating factors.

IL-1 has a number of effects on B cells. The monokine stimulates the maturation of pre-B cells and also induces proliferation in activated mature B cells. In addition, IL-1 has indirect effects on B cells by stimulating T cells to produce the B cell factors described in the previous paragraph.

1.2.2. Tumour Necrosis Factor

The phenomenon of tumour necrosis in humans following challenge with bacterial toxins was first described by Coley in 1893. However, the rational investigation of this did not begin until 1975, when Carswell *et al.* reported the existence of a factor in serum of Bacillus Calmette Guerin (BCG) primed, endotoxin treated animals which was able to induce the necrosis of tumours in recipient animals. Carswell used the term Tumour Necrosis Factor to describe the responsible agent. Further investigations by Green (1977), and Matthews and Watkins (1978) and by others also indicated that TNF was produced by the monocyte. Due to its ability to selectively lyse some tumour cells *in vitro* and *in vivo*, interest in this monokine has been intense. However, the mechanisms by which TNF cause the lysis of tumour cells have yet to be fully elucidated.

Whilst these investigations were taking place, other groups were working on the wasting associated with many chronic disease states. It was shown that this cachexia resulting from LPS administration was due to the production of a serum factor termed cachectin (Kawakami and Cerami 1981). The elucidation of the primary structure of TNF and cachectin by DNA sequence analysis revealed that both activities were produced by the same protein (Pennica *et al.* 1985).

Purified human TNF is a non-glycosylated protein of 157 amino acids with a molecular weight of approximately 17 KDa and an isoelectric point of 5.3 (Aggarwal 1985b). Unlike most secreted proteins but

similar to IL-1, TNF lacks a typical 20-30 amino acid signal peptide sequence.

A compound closely related to TNF is released from activated T cells. This was first described as lymphotoxin by Williams and Granger (1968) and by Ruddle and Waksman (1968). It has been suggested that lymphotoxin be termed TNF beta and the original TNF be designated TNF alpha (Nedwin *et al.* 1985).

Macrophages produce large quantities of TNF, which may amount to 1% of the total secretory products of the cell. Once produced *in vivo* it enters the circulation and causes discrete metabolic effects at distant sites. In rabbits, the rise in plasma TNF occurs within minutes of intraveneous administration of LPS (Beutler *et al.* 1985). Beutler also showed that the circulating half life was around 6 minutes and that the major tissue targets were the liver, skin, kidneys, lungs and gastrointestinal tract.

Several effects previously ascribed to IL-1 are now known to be mediated by TNF. It is a potent pyrogen, both directly by acting on the hypothalmic thermoregulatory centres, and indirectly by inducing IL-1 synthesis which itself acts on the hypothalmus (Dinarello *et al.* 1986). Other functions also ascribed to IL-1 are bone resorption and induction of PGE_2 production (Dayer *et al.* 1985).

Comparative studies on the sensitivity of cell lines to TNF have revealed that some tumour cells are sensitive to the action of TNF, whilst all normal cells tested were resistant (Sugarman *et al.* 1985). This work also revealed that resistance to TNF was not due

to lack of receptors on the target cells or of a low binding affinity for the ligand. A number of studies are beginning to elucidate the action of TNF on sensitive cells. Inhibitors of protein synthesis (for example cycloheximide) greatly enhance susceptibility to the cytotoxic action of TNF (Hahn et al. 1985). This would suggest that TNF does not act by activation of a cellular gene product. Exposure of TNF resistant cells to protein synthesis inhibitors can cause the cells to become sensitive to TNF toxicity. Thus it appears that resistant cells possess repair mechanisms which counteract damage caused by TNF.

As previously discussed, monocyte/macrophages have the capacity to lyse tumour cells. Recent studies have implicated TNF as a mediator of monocyte cytotoxicity. Feinman et al. (1987) showed that the sensitivity of tumour cell lines to monocyte cytotoxicity closely correlated with their sensitivity to TNF. They and others showed that polyclonal and monoclonal antibodies to TNF were able to inhibit monocyte mediated cytotoxicity for tumour cells. It is not yet clear, however, whether target cell lysis is due to TNF released by the monocyte in the extracellular space, or whether TNF bound to the monocyte membrane is responsible. Other factors may be involved in this aspect of TNF function. It has been shown that IFN gamma is capable of sensitising target cells to monocyte killing (Feinman et al. 1986; Feinman et al. 1987). This may be due to upregulation of TNF receptors on the target cell (Aggarwal et al. 1985a) or to IFN gamma stimulating TNF synthesis by monocytes (Nedwin et al. 1985).

TNF also plays an important role in the activation of neutrophils.

Many of these functions were originally ascribed to IL-1. However, highly purified and recombinant IL-1 have been shown to have no activity on neutrophils (Georgilis *et al.* 1987; Yoshimura *et al.* 1987). Yoshimura suggested that previous reports of IL-1 mediated chemotaxis were due, at least in part, to the presence of TNF in the IL1 preparations. Neutrophil emigration from the intravascular space to extra-vascular sites of infection largely depends upon adherence of the neutrophil to endothelial cells. TNF has been shown to enhance this adhesion by stimulating the expression of cell surface molecules on both neutrophils and endothelium (Gamble *et al.* 1985). Berger *et al.* (1988) have reported similar findings showing that TNF increased surface expression of receptors for C3b and C3bi (CR1 and CR3 respectively) on peripheral blood neutrophils. They observed that TNF containing monocyte conditioned medium was able to increase CR1 and CR3 by 300 to 400%. In addition, they measured the release of the granule marker lysozyme and B_{12} binding protein and showed that only 15% of the B_{12} binding protein and 10% of the PMN lysozyme were released. This suggested that the supernatant induced receptor expression may not require fusion of granule membranes with the plasma membrane and that the majority of the granule contents could be retained in cells expressing maximal numbers of receptors on their surface. It has been suggested that TNF, released early in an inflammatory reaction, can increase expression of these receptors on circulating PMN and induce their margination and egress into the tissues. Since this can occur without appreciable degranulation, the neutrophil can be directed to the site of infection with its anti-

microbial systems intact.

TNF is also able to stimulate phagocytosis of latex beads and to increase PMN-mediated antibody dependent cellular cytotoxicity (Shalaby et al. 1985). The majority of this work was done with TNF beta and the effects revealed a potent synergy between TNF beta and IFN gamma.

Despite the initial report that TNF caused the necrosis of tumours in vivo, a recent publication has suggested that TNF functions to enhance the immune response in vivo. Ghiara et al. (1987) showed that simultaneous injection of TNF alpha and antigen into rats dramatically enhanced antibody production to the antigen. This only occurred, however, when a T cell dependent antigen was used; no enhancement could be induced when a T independent antigen was injected. In their report they point to an increase in IL-2 receptors and of IL-2 production by T cells in response to TNF (Scheurich et al. 1986), and suggest that their observations may be due to an amplification of the antigen specific helper T cell population. Further support for the direct effect of TNF on T cells is given by Yokota et al. (1988). Using immobilised monoclonal antibodies to the CD3 complex (an accessory cell independent stimulus) to stimulate purified $CD4^+$ and $CD8^+$ lymphocytes, they observed an enhancement of proliferation by TNF alpha of up to 600%. Their data also suggests that the T cells themselves are able to produce TNF alpha.

A direct action of TNF alpha on B cells was reported by Jelinek and Lipsky (1987a). Although they could not stimulate B cells to

become immunoglobulin secreting cells (ISC) by either recombinant IL-1 or recombinant TNF in the absence of T cells and macrophages, the cytokines did enhance the effect of pokeweed mitogen in mixed cell cultures. To illustrate the direct effect of TNF and IL-1 on B cells, they used Staph. aureus Cowans strain 1 as a stimulus. This is able to stimulate purified B cells in the presence of IL-2. Both TNF and IL-1 were able to support the differentiation of B cells to ISC only when IL-2 was present; they were shown to act in a similar manner during every aspect of their investigations apart from the timing of the cytokines' activity. IL-1 was only active when added at the initiation of B cell cultures, whilst TNF possessed a late acting activity. Their work and that of others suggests that IL-1 delivers a signal that is important in preparing B cells to respond to T cell derived lymphokines. TNF, however, exerts its primary effect following activation.

The work of Jelinek and Lipsky is a good example of the similar roles played by IL-1 and TNF alpha. Although their respective receptors have been shown to be distinct (Matsushima et al. 1986) and they are products of different genes (Nathan 1987), both are now recognised as possessing pleotropic activities.

Autocrine modulation of monocyte function by TNF has been suggested by the discovery of receptors for TNF on monocytes (Imamura et al. 1987). Their work demonstrated the presence of a low number (230) of high affinity and a high number (2879) of low affinity receptors per monocyte. They also demonstrated that TNF, once bound, is rapidly internalised and degraded by the monocyte.

1.2.3. Interleukin 2

The discovery of Interleukin-2 (IL-2), then called T cell growth factor (TCGF), was made in 1976 by Morgan et al. who showed that the supernatants of activated T cell cultures were capable of promoting and maintaining the growth of human T cells. The cytokine is produced by T cells following stimulation by antigen or mitogen and functions by providing an essential signal for activated T cells. It has since been shown that IL-2 is able to modulate many other functions on cells including CTL, B cells, lymphokine activated killer cells (LAK) and NK cells.

As with most cytokines, which are characteristically produced naturally in very small amounts, biological studies were initially hampered by insufficient quantities of purified material. Human IL-2 has now been purified to homogenity, the cDNA isolated and recombinant IL-2 produced in a variety of cell types. This has revealed a protein of molecular weight 15 KDa, although a range of molecular weights have been reported due to different levels of glycosylation. Taniguchi et al. (1983) demonstrated that the protein is synthesised as a 153 amino acid precursor peptide. The 20 amino acid signal peptide is then cleaved and the protein is secreted (Robb et al. 1983). Taniguchi also determined the primary structure of the molecule by sequence analysis of cDNA clones; this was found to be identical to that of the protein secreted by the leukaemic T cell line Jurkat (Robb et al. 1984). Subsequent genetic analysis located the gene for human IL-2 on chromosome 4g band 26-28 (Siegel et al. 1984).

Resting T cells do not produce or respond to IL-2; however, upon contact with a range of stimuli they begin to produce IL-2 and express its receptor. All signals which induce T cell proliferation do so via this mechanism. It is now clear that limited T cell proliferation occurs in the absence of monocytes, and that this phenomenon is due to the requirement of two signals for optimal IL-2 production (Larsson et al. 1980). One signal is provided by a mitogenic lectin or antibody binding to the T cell receptor/T3 complex. A second signal is provided by monocyte production of IL-1.

After these signals have been received, lectin or antigen is no longer required, and the proliferation of activated T cells becomes dependent upon the presence of IL-2 (Ruscetti and Gallo 1981). In the absence of IL-2 the cells cease to proliferate and ultimately die. The IL-2 receptor, required for the acquisition of the IL-2 responsive state, has been the subject of intensive investigations and will be discussed in detail below. Since IL-2 will stimulate any IL-2 receptor positive cell, it is thought that the expression of this receptor (previously known as the Tac antigen) provides specificity in an IL-2 driven system. Ruscetti and Gallo proposed a model for IL-2 mediated proliferation. Antigen or mitogenic lectin are first processed by histocompatible macrophages which then present the antigen to compatible T cells via the latter's antigen receptors. This, in conjunction with IL-1 from the macrophage, results in the production of IL-2 by helper T cells. A similar process of antigen presentation to effector T cells results in the expression of IL-2 receptors. Ligation of the receptor by

IL-2 from the helper cells results in the proliferation and, in the case of NK cells, the activation of effector functions.

A possible role for the control of this process was provided by the work of Cantrell and Smith (1983), who showed a rapid increase in IL-2 receptor expression on peripheral blood T cells following stimulation by antigen. Maximal levels were detectable between 3 and 7 days in culture and were absent by 10 days. However, no proliferation occurred after 48 hours of the cultures, even in the presence of saturating concentrations of IL-2. Addition of phytohemaggutinin (PHA) after 10 days reinduced the Tac antigen, and the cells began to proliferate again. This was followed by a decline over a period of 7 days. They were able to reproduce this pattern of response over and over again in the same culture. It appears, therefore, that antigen recognition ultimately determines the magnitude of the T cell response by regulating the expression of IL-2 receptors.

Binding studies with radio labelled IL-2 have revealed the presence of two receptors on T cells of high and low affinity. The initial investigations into the structure of the IL-2 receptor resulted in a paradox (reviewed by Smith 1987). The antibody, anti-Tac, was found to compete with IL-2 for binding to IL-2 receptors. However, a large discrepancy was apparent between the number of receptors per cell as revealed by radio labelled anti-Tac and the numbers disclosed by radio labelled IL-2, with the former always outnumbering the latter.

Another paradox became apparent during metabolic studies which

revealed a turnover of high affinity receptors 5-6 times faster than the turnover of the Tac antigen. The discovery of a low affinity receptor which also carried the Tac epitope provided the answer to the discrepancy between the number of high affinity receptors and the number of Tac epitopes. In subsequent investigations, the cDNA for the IL-2 receptor was isolated and cloned. When this was transfected into recipient cells, it was found that only the low affinity receptor was expressed. Ligation of the receptor by IL-2 failed to produce a response in the recipient cell. The answer to the whole paradox was provided by the discovery of a novel IL-2 binding protein by Teshigawa et al. (1987). This protein bound IL-2 with intermediate affinity between the high and low affinity receptors and was not recognised by anti-Tac. Further evidence showed that clones expressing the Tac antigen by itself produced only low affinity receptors, and clones expressing high affinity receptors displayed both IL-2 binding proteins. The implication of this was that both IL-2 binding proteins are involved in the formation of a fully functional, high affinity receptor. Teshigawa et al. therefore proposed that the two units of the high affinity receptor be referred to as the alpha chain for the 75 KDa novel peptide and the beta chain for the 55 KDa Tac antigen positive unit. Smith concluded by suggesting that the alpha and beta chains bind to different amino acid residues on the surface of the IL-2 molecule and that the two chains are products of different genes.

An impressive report by Ju et al. (1987) could confirm Smith's suggestion about the binding sites on IL-2. They produced

recombinant IL-2 with defined amino acid substitutions and deletions. These were assessed for the competitive binding of native IL-2 and for their ability to stimulate the proliferation of T cell lines. Using this approach, they were able to identify three regions on the IL-2 molecule that were required for full biological activity. They suggested that two of these regions, the NH_3-terminal 20 amino acids and the COOH-terminal 13 amino acids, participate directly in forming contact points with the IL-2 receptor. The third region, they suggest, maintains an essential disulphide bond required for the biological activity of the protein.

As mentioned above, when the Tac antigen (beta chain) of the receptor is expressed alone no proliferation occurs in response to IL-2. A recent publication by Tanaka et al. (1988) has suggested that it is the alpha chain which is responsible for signal transduction of IL-2, and that the beta chain mediates the internalisation of IL-2.

Although the spectrum of activity of IL-2 is limited in comparison to that of IL-1 and TNF, its actions go beyond those of promoting T cell proliferation. IL-2 has been implicated in the activation of NK functions, which is partially due to induction of IFN gamma production. IL-2 in high concentrations can also activate LAK cells to lyse solid tumours (Lotze et al. 1981). This phenomenon recently triggered great excitement due to its therapeutic potential in the treatment of cancers (Rosenberg et al. 1985). Although the preliminary trials were encouraging, they have been

associated with extreme toxicity. The distinction between LAK and NK cells has been questioned and it has been proposed that the majority of LAK activity in human peripheral blood is mediated by IL-2 activated NK cells (Lotzova and Herberman 1987).

IL-2 is able to affect B cells both directly and indirectly. It has been shown to stimulate the proliferation and differentiation of activated B cells (Nakagawa et al. 1985), and it appears to do this by binding to the IL-2 receptor (Tac antigen) on activated B cells (Tsudo et al. 1984). Ralph et al. (1984) showed that high concentrations of IL-2 (greater than 100 U ml^{-1}) were able to stimulate Ig secretion by human B lymphocytes. At variance with Tsudo's report, they suggested that the IL-2 was acting through a receptor distinct from Tac. However, they did not measure Tac expression on peripheral human B cells, only on the SKW 6.4 cell line, which may explain the discrepancy. Indirect activity of IL-2 on B cells has been reported by IL-2 stimulated T cell production of B cell growth factor 1 (IL-4) (Howard et al. 1983).

More recently, a role for IL-2 in the activation of monocytes has been reported. Holter et al. (1987) demonstrated the presence of large numbers of the Tac antigen on human peripheral blood monocytes stimulated with LPS or IFN gamma. In addition, they showed that recombinant IL-2 increased H_2O_2 production by activating monocytes. In support of these finding, Malkovsky et al. (1987) showed that recombinant IL-2 is able to augment the cytotoxicity of human monocytes to a human urinary bladder carcinoma cell line T24. These findings would suggest that IL-2 may have a role in enhancing monocyte effector functions.

1.2.4. Interferon Gamma

There are three major classes of interferon: leucocyte or interferon alpha, fibroblast or interferon beta and immune or interferon gamma. Alpha and beta IFN have been the subject of intensive research over recent years because of their potential antiviral and antitumour effects. The work on IFN gamma has, however, been conducted more recently, and it appears that the molecule has close functional links with other cytokines involved in inflammation. Therefore only IFN gamma will be discussed here.

The gene for human IFN gamma which is located on the long arm of chromosome 12 has been isolated, sequenced and cloned (Gray and Goeddel 1982). This encodes for a protein of 146 amino acids with a molecular weight of 17 KDa.

IFN gamma is produced by T lymphocytes of both the $CD4^+$ and the $CD8^+$ phenotype in response to allo antigens or mitogens; NK cells can be also induced to become potent producers. The production of IFN gamma by T lymphocytes is dependent upon the secretion of IL-1 by monocytes and on IL-2 from T helper cells. Resting T cells can not be activated to produce IFN gamma by IL-2 alone; however, NK cells respond to IL-2 by producing large quantities of IFN gamma. It has been suggested that this distinction is due to the absence of IL-2 receptors on resting T lymphocytes and the presence of these receptors on NK cells. This may represent an important amplification mechanism in vivo, whereby the IL-2 produced by T cells in response to antigenic stimulation may recruit NK cells (which represent around 15% of circulating lymphocytes, and are

therefore much more numerous than antigen specific T cells) into the IFN gamma producing population (Trinchieri and Perussia 1985).

Similar to the IFN alpha and beta, IFN gamma was originally reported to possess cytotoxic effects against normal and neoplastic cells. However, later reports have indicated that these results were produced with IFN gamma preparations contaminated with lymphotoxin (TNF beta, the cytotoxic effect of which is potentiated by IFN gamma). Many of the functions of IFN gamma are amplificatory; for example, the binding of IFN gamma to its receptor on macrophages enhances antigen presentation, increases the respiratory burst and induces the expression of a wide variety of macrophage proteins. One of the most extensively studied effects of IFN gamma is the induction of class 2 MHC antigens (Steeg et al. 1982). This occurs in a large number of cell types, including myelomonocytic cells, lymphoid cells, melanocytes and melanoma cells, and on a large series of tumour derived cell lines.

IFN gamma also induces the expression of FcR1 on both neutrophils and monocytes. The cytokine can also induce bone marrow cells from normal individuals and from patients with chronic myeloid leukaemia (CML) to differentiate along the monocytic pathway (Perussia et al. 1983). Studies on the effect of IFN gamma on mature PMNs have revealed that it activates the oxidative burst and augments the cell's ability to release proteolytic enzymes. IFN gamma is also involved in B cell activation, where it has a number of synergistic effects with B cell factors; this, however, is discussed in the section on B cell activation.

The synergistic effects of IFN gamma with macrophage products, namely IL-1 and TNF, has been extensively studied. Tsujimoto et al. (1986a) demonstrated that IFN gamma increased the expression of TNF on a number of human tumour cell lines. They suggest that this effect may contribute to the potent synergistic cytotoxic action of TNF and IFN gamma seen in some tumour lines. The importance of such a synergism was suggested recently by Billiau (1988) who postulated a role for IFN gamma in the Shwartzman reaction. Briefly, the Shwartzman reaction is a potentially lethal inflammatory response to bacterial LPS. It is induced by a preparatory intradermal injection of LPS followed by a provocative intravenous injection 24 hours later. This results in an accumulation of PMN at the site of the preparative injection which is associated with intravascular coagulation, vascular occlusion and haemorrhage. This leads to necrosis of the blocked vessels. If the preparative injection is given intravenously the generalised Shwartzman reaction occurs, which results in leucocyte and platelet accumulation in the small vessels of various organs including skin and lungs, which again leads to haemorrhage and necrosis. Many of the effects of the Shwartzman reaction can be initiated by IL-1 and TNF, and IL-1 has been used to replace LPS as either the preparative or the provocative injection for the local reaction in rabbits. However, IL-1 and TNF cannot completely induce the Shwartzman reaction alone. Billiau reported that mice pre-treated with anti-IFN gamma antibody were partially protected from the localised Shwartzman reaction and dramatically protected from the generalised response.

As mentioned above, macrophages treated with IFN gamma develop many characteristics which make them more sensitive to environmental stimulation. A positive feedback loop occurs between IL-1 and IFN gamma, whereby IL-1 production is enhanced by IFN gamma and production of the latter is enhanced by the former. This could, therefore, induce a dramatic increase in inflammation, and blockage of IFN gamma by antibodies would therefore remove one arm of the feedback loop resulting in an inhibition of the Shwartzman reaction.

It appears, therefore, that IFN gamma plays a central role in inflammation and immunity by potentiating the majority of the central events in these processes.

1.2.5. Colony Stimulating Factors

The colony stimulating factors (CSFs) are a group of polypeptides first described by their ability to induce proliferation and differentiation of haemopoietic stem cells in vitro. Functional studies, and more recently sequence analysis, has revealed four distinct CSFs acting on the myeloid line of development. The names are self-explanatory, granulocyte CSF (G-CSF), macrophage CSF (M-CSF), granulocyte macrophage (GM-CSF) and multi-CSF (Interleukin-3). There is considerable overlap in the activities of these factors. Multi-CSF and GM-CSF stimulate all compartments of differentiation, although GM-CSF has weaker and incomplete actions on multi-potential and stem cells. G-CSF is predominantly restricted to a subset of granulocytic progenitor cells, but also allows survival of more primitive cells. M-CSF is restricted to

restricted to a subset of granulocytic progenitor cells, but also allows survival of more primitive cells. M-CSF is restricted to macrophage committed cells.

Although the CSFs are an interesting group of compounds, only GM-CSF, which has a number of functions relating to inflammation, will be discussed in any detail here. GM-CSF is a glycoprotein of molecular weight 23 KDa which is released by activated T cells. The complete amino acid sequence of the protein is known from sequence analysis of cDNA clones (Wong et al. 1985). The concentration of GM-CSF in vitro affects the outcome of the colonies produced, with lower concentrations resulting in macrophage colonies (Metcalf 1985). It has also been shown to stimulate some functional activities in mature granulocytes and macrophages. For example, Gasson et al. (1984) reported that GM-CSF inhibits neutrophil migration. Reports have also shown that GM-CSF is able to stimulate neutrophil phagocytosis (Metcalf 1986) and enhance macrophage tumoricidal activity (Grabstein et al. 1986). Thus, production of GM-CSF at the site of inflammation would promote local retention of granulocytes and enhance their activity.

Similar to TNF alpha and IL-1, GM-CSF is a pleotropic effector molecule, although more limited in its range than the monokines. GM-CSF appears to exhibit its pleotropic effects by interaction with the specific high affinity receptor present on early myeloid progenitor cells and on differentiated cells of the myeloid series (Gasson et al. 1986). There is now a body of data emerging which suggests that GM-CSF (and possibly G-CSF) act synergistically with

TNF and IL-1 in a number of functions. Cannistra et al. (1987) showed that rGM-CSF is inhibitory to the human macrophage like cell line U937. This inhibition was partially due to rGM-CSF stimulating the production of biologically active TNF by U937. They also showed that rGM-CSF primed human peripheral blood monocytes to release elevated levels of TNF following stimulation with either endotoxin or phorbol myristate acetate (PMA). A positive feedback loop has been demonstrated whereby TNF induces secretion of GM-CSF from fibroblasts and endothelial cells (Munker et al. 1986).

1.3. Lactoferrin and Inflammation

So far, this review has focused on the positive events controlling inflammation. However, following the eradication of the injurious agent it is highly likely that negative feedback mechanisms come into play to inhibit the reaction which, if allowed to continue, would be harmful to the host. A role for the iron binding proteins in the control of inflammation was first suggested by DeSousa (1978). Work in this area has remained controversial to date due to inconsistency of data from different laboratories. The majority of this work has been conducted on lactoferrin (Lf) by Broxmeyer and his colleagues, who have shown that Lf inhibits granulopoeisis in vitro by inhibiting production of GM-CSF.

Lf was first isolated from milk whey by Sorensen and Sorensen in 1939. They described its characteristic salmon pink colour which is now known to be due to the binding of iron to one or both of its two iron binding sites. The protein has since been extensively characterised. It is an 80 KDa single polypeptide chain to which

two oligosaccharide moieties are attached (Matsumoto et al. 1982). There are great similarities between Lf and the plasma iron binding protein transferrin; however, the two are immunologically distinct (Mazurier et al. 1983). Both proteins bind two ferric ions per molecule and incorporate one mole of bicarbonate per mole of iron bound.

Lf is found in many secretions including pancreatic juice, bile, tears and seminal plasma (Masson et al. 1966). Its probable function in these secretions is to bind free iron and thus make it unavailable for the proliferation of micro-organisms (Bortner et al. 1986). Lf is also found in plasma where its sole source is the neutrophil secondary granule. There is a close correlation between plasma Lf levels and the number of circulating PMN (Baynes et al. 1986).

Work by Parran et al. (1969) first suggested that neutrophils release a factor capable of inhibiting the production of granulocyte progenitor cells in vitro. This finding was subsequently supported by Baker et al. (1975) and Herman et al. (1978) and at least one of these inhibitory factors was identified as Lf (Broxmeyer et al. 1978). Broxmeyer also demonstrated the importance of iron binding to the molecule for the inhibition to occur. However, the work was viewed with scepticism due to the extraordinarily low concentrations at which Lf was claimed to be active, down to 10^{-17}M. Broxmeyer's data was supported by Bagby et al. (1981), but Winton et al. (1981), using murine Lf on murine cells failed to show any effect of Lf. Stryckmans et al. (1984), in an attempt to answer Winton's criticisms, repeated Broxmeyer's

work, but they also failed to show an effect with Lf. However, more recently Fletcher and Willars (1986) have produced evidence to support Broxmeyer's claims.

The work of Broxmeyer (1979) and Broxmeyer and Platza (1984) has suggested that Lf inhibits granulopoiesis by directly affecting production of GM-CSF. The work of Bagby et al. (1983) has implicated an effect on an intermediary molecule produced by monocytes.

In addition to these effects, the role for Lf in the regulation of many other events involved in inflammation has been suggested. Kijlstra and Jeurissen (1982) have shown that Lf is able to inhibit the complement system. The molecule has been shown to enhance neutrophil adherence and aggregation (Oseas et al. 1981), enhance the production of leucocyte migration inhibitory factor (Kijlstra and Boersma, 1984), suppress the primary antibody response (Duncan and McArthur 1981), modulate natural killer cell activity (Nishiya and Horwitz 1982) and to enhance the production of the hydroxyl radicals (Gutteridge et al. 1981).

The preceding review has highlighted the many close interactions between cell types and the cytokines produced by these cells in the regulation of inflammation. It is possible that some of the functions ascribed to Lf outlined in the previous paragraph, namely the effects on antibody production, NK activation and CSF production, could be mediated by Lf affecting a central function in these processes. This project was therefore undertaken to provide further evidence either in support of or contrary to Broxmeyer's

initial observations about Lf. By investigation of the effects of both purified Lf and Lf derived from phagocytosing neutrophils on mononuclear cells stimulated in a variety of ways, it was anticipated that an insight into the mechanism of action of Lf could be gained, which could explain the variety of effects of this enigmatic protein on many cell types.

Since cytokines are also involved in GM-CSF production, it was anticipated that determination of the effects Lf on secretion of a molecule of central importance to this process could unify the many described effects of Lf on inflammation, immunity and haemopoiesis.

CHAPTER TWO

GENERAL METHODS

Supliers of all the materials used in this and subsequent chapters are listed in appendix 2.

2.1. Blood

Venous blood was obtained from healthy normal donors amongst the laboratory staff. This was taken into dipotassium ethelene diaminetetra acetic acid (EDTA) to give a final concentration of 0.3 mM. The blood was always used immediately after venesection.

2.2. Neutrophils

PMNs were prepared by density gradient centrifugation of whole blood over Mono-Poly Resolving Medium (MPRM, Ficoll Isopaque) following the manufacturer's instructions. Briefly, 5 ml of blood was layered aseptically over 3 ml of MPRM in a sterile 10 ml centrifuge tube. This was then centrifuged at 400 x g for 45 minutes at 20°C. Occasionally, depending on the donor or on the batch of MPRM, the time needed to be extended up to 60 minutes to obtain a PMN band free of red blood cells. This could be done without affecting subsequent PMN responses. Following centrifugation the erythrocytes were pelleted, there was a lower leucocyte band comprising the PMNs and an upper band of mononuclear cells (MNCs), while the plasma remained above the MPRM. The plasma was aspirated and the MNCs harvested, these were utilised if required. The PMN band was collected and the cells suspended in cold phosphate buffered saline (PBS). They were then washed once by centrifugation at 400 x g for 10 minutes at 4°C. The PMN were

resuspended in a small volume of fresh PBS and placed on ice until required. The cells were usually used immediately, but for the chemiluminescence work it was necessary to leave them for up to 1 hour. This did not significantly affect the PMN responses.

A sample of the suspension was diluted 1:10 in PBS containing 0.1% w/v trypan blue for determination of cell numbers using an improved Neubauer Haemocytometer. This separation procedure routinely produced cells of greater than 98% viability, as judged by dye exclusion. No loss of viability occurred after 1 hour on ice. Separate examination of stained (Diff Quick) cytospin preparations showed the suspensions to be approximately 90% PMNs with the majority of the contaminating cells being erythrocytes and about 1% MNCs. Removal of the erythrocytes by lysis with 0.8% w/v ammonium chloride in distilled water for 10 minutes at room temperature reduced the PMNs' responses; this procedure was therefore not employed. Immediately prior to use, the neutrophils were made up to the required concentration in PBS plus calcium, magnesium and bovine serum albumin (PBS-A, see appendix 1).

2.3. Mononuclear Cells

Mononuclear cells were obtained either from the MPRM preparation described above or prepared by centrifugation over Lymphocyte Separation Medium (LSM). The LSM separation was achieved by diluting whole blood 1:2 in RPMI 1640. 20 ml of diluted blood was layered aseptically over 6ml of LSM in a sterile 30 ml plastic universal container. This was then centrifuged for 20 minutes at 400 x g at 20°C. The MNC band which formed between the plasma and

the LSM was harvested and the cells washed twice in RPMI 1640. The final pellet was resuspended in a small volume of fresh RPMI and the cells counted as described for PMNs. This process routinely produced an MNC fraction of greater than 98% viability with a purity of around 97%. The MNCs contained on average 30% monocytes as determined from Diff Quick stained cytospin preparations.

2.4.1. Mononuclear Cell Culture

MNCs obtained by the procedures outlined above were suspended in complete culture medium containing 10% foetal calf serum (FCS),2mM L-Glutamine, penicillin 100 IU ml^{-1}, and streptomycin 100 ug ml^{-1} in RPMI (cRPMI). The pellet was diluted to obtain the appropriate cell concentration for the stimulus that was to be used.

2.4.2. Three Way Mixed Lymphocyte Cultures

MNCs from 3 donors were suspended at 1.1 x 10^6 ml^{-1} in cRPMI containing 10^{-6} M indomethacin. Equal volumes of each suspension were mixed together and triplicate 180 ul samples added to the wells of 96 well tissue culture plates. The geometry of the wells was found to be crucial to the experiments conducted on mixed lymphocyte cultures (MLCs), and therefore plates possessing either round bottomed wells (RBWs) or flat bottomed wells (FBWs) were used for particular investigations. The MLCs were then incubated at 37°C, 100% relative humidity and 5% carbon dioxide in air (standard incubation conditions) for various time periods, with the standard time being 72 hours.

At the end of the culture period, cell proliferation was determined by measuring incorporation of tritiated uridine (^{3}H-UdR) or

tritiated thymidine (^{3}H-TdR). 0.2 uCi in 20 ul of PBS were added to the wells for the last 6 hours of the culture period.

After this incorporation period, the cells were harvested on to glass fibre filter mats using a Dynatech Automash 2 cell harvester. The filter discs, representing individual wells, were transferred to 7 ml scintillation vials containing 200 ul of 0.2 M KOH to degrade the RNA or DNA and obtain an even distribution of nucleotide throughout the scintillation cocktail. 4 ml of scintillant was added and the vials mixed vigorously. The samples were then counted for three minutes each in a Packard Tri Carb Series 4000 Liquid Scintillation Counter with window settings 2-19 meV. The means of the triplicates were determined and taken to represent the proliferation for a particular sample.

2.4.3. One Way MLCs

To investigate an individual's response to allogeneic stimulation and the effects of Lf, one-way MLCs were prepared. MNCs were suspended at 2.2 x 10^{6} ml^{-1} in complete medium containing 10^{-6}M indomethacin. 90 ul of the cell suspension were added to the wells of 96 well tissue culture plates and an equal volume of mitomycin C treated stimulator MNCs at 1.1 x 10^{6} ml^{-1} were added. The ratio of 2:1, responders:stimulators was found to be optimal from preliminary experiments investigating a range of ratios from 1:1 to 5:1.

The stimulator cells were prepared by incubation with 40 ug ml^{-1} mitomycin C for 45 minutes at 37°C. They were then washed four times in RPMI at 4°C. This process produced cells of approximately

90% viability as determined by trypan blue exclusion. The one-way MLCs were incubated under standard conditions for between 3 and 7 days and the proliferation determined as described above. Controls, containing mitomycin C treated stimulators only, showed no proliferation after the culture period. In an attempt to standardise the one-way MLC, a large batch of stimulator cells was prepared from a unit of blood obtained by routine venesection from a pseudopolycythaemic patient. The MNCs were prepared and mitomycin C treated as described. They were then resuspended at 1 x 10^8 ml^{-1} in complete medium containing 10% dimethylsulphoxide (DMSO) and frozen at a rate of 1^oC $minute^{-1}$ down to -70^oC in a Planar Biomed Programmable Freezer in 1ml aliquots. These were transferred to liquid nitrogen, where they were stored until required. Thawing was carried out by immersion of a vial into a water bath at 37^oC. The cells were washed once and resuspended at $1.1x10^6$ ml^{-1}. After this procedure, viability was routinely reduced to around 50%. The stimulators were then used as described.

2.5. Preparation of Zymosan Activated Serum and Serum Treated Zymosan

Zymosan

Zymosan A from Saccharomyces cerevisiae was boiled for 15 minutes in PBS. Afer cooling, it was pelleted by centrifugation at 1000 x g for 10 minutes and was washed twice in fresh PBS.

Zymosan Activated Serum

30 ml of blood was obtained from six healthy individuals. This was

transferred into 10 ml glass tubes and allowed to clot for approximately 1 hour at room temperature (longer times were occasionally necessary). The tubes were then centrifuged at 400 x g for 10 minutes, the serum was aspirated and pooled. Zymosan was next added to the serum to give a final concentration of 2mg ml^{-1}. This was then incubated at 37°C for 1 hour with end over end rotation. The zymosan was removed by centrifugation at 1000 x g for 10 minutes. The ZAS was collected and passed through a 0.2 um filter to remove smaller zymosan particles which could act as a phagocytic stimulus. The ZAS was then dispensed into 500 ul aliquots and frozen at -70°C until required.

Serum Treated Zymosan

The zymosan used for the preparation of ZAS was washed three times by resuspending the pellet in fresh PBS and centrifuging at 1000 x g for 10 minutes. The STZ was suspended at 20 mg ml^{-1}, aliquoted as above and frozen at -70°C until required.

2.6. Assay of Lactoferrin

Lactoferrin concentrations were measured by an enzyme linked immunosorbent assay (ELISA). The assay utilised polyclonal anti-Lf for both the antigen capture antibody and the detection antibody. The sensitivity of the ELISA was increased by conjugating biotin to the detection antibody and then using an avidin-peroxidase conjugate to amplify the signal.

Upon receipt of anti-Lf, half was stored according to the manufacturer's instructions (4°C in the presence of preservative), while the remaining half was biotinylated. The biotinylation was

achieved by combining 1 ml of antibody at 1 mg ml^{-1} in 0.1M bicarbonate buffer pH 9.0 with 120 ul of 1mg ml^{-1} biotinyl-N-hydroxysuccinimide and incubating for four hours at room temperature. This was then dialysed for 24 hours against PBS at $4^{o}C$. The composition of the buffers is given in appendix 1.

The assay procedure was as follows:

1. 96 well flat bottomed micro ELISA plates were coated with antibody by adding 100 ul of a 1:1000 dilution of antibody in coating buffer pH 9.6 to the wells and incubating at $4^{o}C$ overnight.

2. Wells were washed twice with PBS (see below for washing procedure).

3. Any unbound binding sites on the plate were blocked by adding 200 ul of 0.5% w/v gelatin in PBS and incubating at room temperature for 30 minutes.

4. Wells were washed 4 times in PBS wash buffer.

5. 50 ul of standard Lf (Calbiochem Lf standard) or samples diluted in 0.1% gelatin/PBS were added to duplicate wells and incubated for 2 hours at room temperature.

6. Wells washed 4 times with PBS wash buffer.

7. 50 ul of a 1:1000 dilution of biotinylated anti Lf in 0.1% gelatin/PBS were added to the wells and incubated for 1.5 hours at room temperature.

8. Wells were washed 4 times with PBS wash buffer.

9. 50 ul of avidin-peroxidase conjugate diluted 1:1000 in borate saline were added to the wells and incubated for 1 hour.

10. Wells were washed 4 times with borate wash buffer.

11. 100 ul of substrate were added and the plates incubated for 30 minutes at room temperature. The substrate was 5 amino salycylic acid, this was diluted to 1 mg ml^{-1} in PBS. Excessive colour was removed by the addition of activated charcoal at 0.01 mg ml^{-1} followed by passage through a Whatman no.1 filter. Immediately prior to use 0.2 ml of 1% H_2O_2 were added per 20 ml of substrate.

12. 50 ul of 3 M NaOH were added to each well to stop the reaction.

13. The absorbance in the wells was read at 455.5 nm in an automated micro-plate reader (Kontron).

For each plate, no sample or standard was added to two wells for determination of non-specific binding. The non-specific binding was automatically subtracted from the readings by the microplate reader.

The washing procedure consisted of emptying the wells by rapid inversion and then filling with wash buffer. For the first wash the wells were emptied immediately; for subsequent washes the plate was allowed to stand for four minutes before replacing with fresh buffer.

2.7. Assay of Interleukin-2

IL-2 was assayed using the IL-2 dependent cell line, CTLL-2. This cell proliferates rapidly in the presence of the lymphokine and dies in its absence. The degree of proliferation, as assessed by ^{3}H-TdR incorporation, can be related to the amount of IL-2 in the test supernatant.

Maintenance of CTLL-2

CTLL-2 were maintained in culture and harvested as required for the assay. Since the cells use IL-2 rapidly, after which they quickly die, the cultures required considerable attention to ensure that cell numbers remained low.

The cultures were maintained in RPMI 1640 supplemented with 10% FCS, penicillin 100 IU ml^{-1}, streptomycin 100 ug ml^{-1}, L-Glutamine 2 mM, 2.5 x 10^{-5} M 2-mercaptoethanol and 20 U ml^{-1} IL-2 at 37°C with 5% CO_2 in air and 100% relative humidity. Because of the difficulties described above, they were routinely passaged every 2 days by centrifuging the cultures at 400 x g for 10 minutes and resuspending the cells between 5 x 10^{3} and 1 x 10^{4} ml^{-1} in fresh medium. Using this approach, CTLL-2 could usually be maintained for up to one month. They were never kept for longer than this, since they would either die or become unresponsive to IL-2. For this reason, a large stock of cryopreserved CTLL-2 was required.

Cryopreservation of CTLL-2

Freezing of CTLL-2 was conducted at regular intervals when, assay results and the general appearance and behaviour of the culture indicated a healthy IL-2 responsive population. The cells were

suspended at 10^6 ml^{-1} in the supplemented media described above with the addition of 10% DMSO. 1 ml aliquots of the suspension were transferred to cryotubes. These were then frozen by placing the vials into polystyrene and leaving at -70°C overnight prior to transferring to liquid nitrogen.

New cultures were initiated by thawing a vial of frozen cells rapidly in a water bath at 37°C. The cells were then rapidly transferred to 20 ml of supplemented medium which had been prewarmed to 37°C. The culture was incubated under standard conditions for 24 hours, after which the cells were passaged as described. Using this approach, rapidly growing cells were usually obtained within 5-7 days. In accordance with the reputation of this cell line, however, periodic failures occurred and it was necessary to return to the frozen stocks.

Assay Procedure

CTLL-2 were passaged the day before a planned assay to obtain healthy cells in log phase growth. For the assay, the cells were washed once in RPMI, counted and resuspended at 10^5 ml^{-1} in supplemented medium containing no IL-2. The suspensions were incubated for 2 hours at 37°C to allow internalisation of receptor bound IL-2 and to reduce background counts (Michalski and McCombs 1985). Meanwhile, 100 ul of samples or standard were added in triplicate to the wells of 96 well flat bottomed tissue culture plates. The standard used was the interim international standard from the Biological Response Modifiers Program (a kind gift from Dr. Rossi). Serial dilutions of sample and standard were produced in situ using a multi-channel pipette. A negative control of

supplemented medium alone was also included. 100 ul of CTLL-2 suspension were then added and the plates incubated under standard conditions for 24 hours. For the last 4 hours of incubation, 0.5 uCi ^{3}H-TdR in 20 ul were added to each well. The cells were harvested as previously described and their proliferation determined by liquid scintillation counting. The means of each triplicate were used to determine the IL-2 concentration by probit analysis. This was achieved using a program for an Apple IIe microcomputer published by Schmitt and Ballet (1983).

2.8. Assay of Interleukin 1

IL-1 was measured using the method of Gearing et al. (1987), which relies upon IL-2 production from the cell line EL-4.NOB-1 in response to the monokine. The IL-2 is then assayed by its effect on CTLL-2 as described in section 2.7.

Maintenance of EL-4.NOB-1

EL-4.NOB-1 were cultured in RPMI 1640 supplemented with 5% FCS, 100 IU ml^{-1} penicillin, 100 ug ml^{-1} streptomycin and 2 mM L-Glutamine. The cell density was maintained at between 1 and 5 x 10^{5} ml^{-1} by subculturing every three days with a 1:10 dilution. It was found necessary not to allow the cells to achieve too high a density, since their sensitivity diminished due to very high background counts. At high concentrations, the phenotype changes to a spontaneous IL-2 producer (Gearing, personal communication) which could explain this observation.

A frozen stock of EL-4.NOB-1 was maintained by resuspending the cells at 10^{7} ml^{-1} in 50% FCS, 10% DMSO and 40% RPMI 1640 and

freezing as described for the CTLL-2 line.

Assay Procedure

The EL-4.NOB-1 were harvested and washed twice in RPMI 1640, the cells were then resuspended at 2 x 10^6 ml^{-1}. Serial dilution of sample and standard (National Institute of Biological Standards interim standard IL-1 beta, a kind gift from Dr. Gearing) were prepared in triplicate in 96 well microtitre plates, leaving 100 ul in each well. 100 ul of the cell suspension were then added and the plates incubated under standard conditions for two hours. The cells were then washed in situ by spinningthe plate at 200 x g for 10 minutes. The supernatants were carefully aspirated using a multi-channel pipette and 200 ul of fresh medium replaced. The washing procedure was repeated twice before 200 ul of fresh complete medium was added and the cells incubated for 24 hours. The plates were again centrifuged to remove all cells in suspension and the top 50 ul of medium transferred to a fresh plate. 50 ul of CTLL-2 at 1 x 10^5 ml^{-1} were then added to all the wells of this duplicate plate, which was then incubated for a further 24 hours. The proliferation of the CTLL-2s was assessed following a pulse of 0.5 uCi ^{3}H-TdR in 20 ul of PBS as previously described.

2.9. Statistics

All statistical analyses were by Students T test.

CHAPTER THREE

DETECTION AND IDENTIFICATION OF A MONONUCLEAR CELL INHIBITOR RELEASED FROM PHAGOCYTOSING NEUTROPHILS

Previous studies have indicated that phagocytosing neutrophils release an inhibitor of myelopoiesis *in vitro* (Philip *et al.* 1982). The following experiments were designed to investigate whether phagocytosing neutrophil conditioned medium (PNCM) could inhibit mononuclear cell proliferation in response to various stimuli.

3.1. Preparation of PNCM

PNCM was prepared using the conditions providing maximum inhibitor production as previously determined (Philip *et al.* 1982). Opsonised *Candida guilliermondii* were used to stimulate PMNs for the production of PNCM. This organism has the advantage of not producing hyphae and of not killing neutrophils. The opsonins were provided by a pool of normal human serum, prepared as described in section 2.5. The yeast was grown overnight in glucose broth (nutrient broth supplemented with 2 g l^{-1} glucose) at 37°C in a shaking water bath. The Candida were then heat killed by immersion of the flask in boiling water for 15 minutes, after which they were washed three times in PBS by centrifugation at 600 x g for 10 minutes. The organisms were counted using a haemocytometer and subsequently opsonised by incubation in pooled normal serum at 1 x 10^8 ml^{-1} for 1 hour at 37°C with end over end rotation. They were then harvested by centrifugation at 600 x g for 10 minutes, after which the cells were washed three times in PBS and counted after

the final wash. Prior to use, the Candida were resuspended in PBS-A at 1 x 10^7 ml^{-1} and equilibrated to 37°C. Neutrophils prepared as described in Chapter 2, were suspended at 2 x 10^6 ml^{-1} in PBS-A and raised to 37°C. Equal volumes of PMN and Candida were mixed together giving a ratio of 1 PMN to 5 Candida. These were then incubated at 37°C for 30 minutes with end over end rotation to allow phagocytosis to occur. This was checked by examination of stained (Diff Quick) cytospin preparations. The cells were removed by centrifugation at 600 x g for 10 minutes and the PNCM collected. This was then sterilised by passage through a 0.2 um micropore filter and aliquoted into 1 ml polypropylene vials. The PNCM was stored at -20°C until required.

3.2. Determination of the Effect of PNCM on Mononuclear Cell Proliferation

The effect of PNCM on mononuclear cell proliferation in response to a range of stimuli was then investigated.

The mitogens used were PHA and Con A. Mononuclear cell suspensions were prepared in cRPMI at 1.25 x 10^6 ml^{-1}. 160 ul of cell suspension were added to the wells of flat bottomed 96 well tissue culture plates. 20 ul of PNCM were added to half of the wells with 20 ul of PBS added to the remaining half to act as controls. 20 ul of mitogen were then added to triplicate wells to produce a range of stimulus concentrations. The plates were incubated for 72 hours, after which the cell proliferation was assessed by measuring ^{3}H-TdR incorporation.

The resultant dose response curves for PHA and Con A and the effect of PNCM on these can be seen in Figures 1 and 2. PNCM was found to be inhibitory at every stimulus concentration, producing a greater effect against PHA than Con A.

Each experiment was conducted using a different donor for both the mononuclear cells and to produce the PNCM. To investigate the reproducibility of the inhibition by PNCM, two batches of PNCM were randomly selected and tested against different donor MNCs stimulated by 4 ug ml^{-1} PHA. Table 1 shows the results of these experiments expressed as percentage inhibition of control values. Percentage inhibition was calculated as follows:

$$\% \text{ Inhibition} = 100 - \left(\frac{100}{\text{control cpm x test cpm}} \right)$$

PNCMa gave 40.7% inhibition and PNCMb 32.5% This compares very favourably with the data in Figure 1 at 4 ug ml^{-1} PHA, with PNCM producing a 35.6% inhibition below control values.

Initial experiments with MLCs were disappointing, with little proliferation occuring that was not affected by PNCM (Figure 3). However, a report by Teodorescu (1983) demonstrated the importance of close cell contact to stimulate protein synthesis by lymphoctes.

Therefore, the experiments were repeated using ^{3}H-UdR to measure RNA synthesis. The effect of cell contact was also investigated by culturing the cells in round bottomed and in flat bottomed wells for 72 hours. Microscopic examination of the spread cultures revealed that little contact occurred between the cells, other than

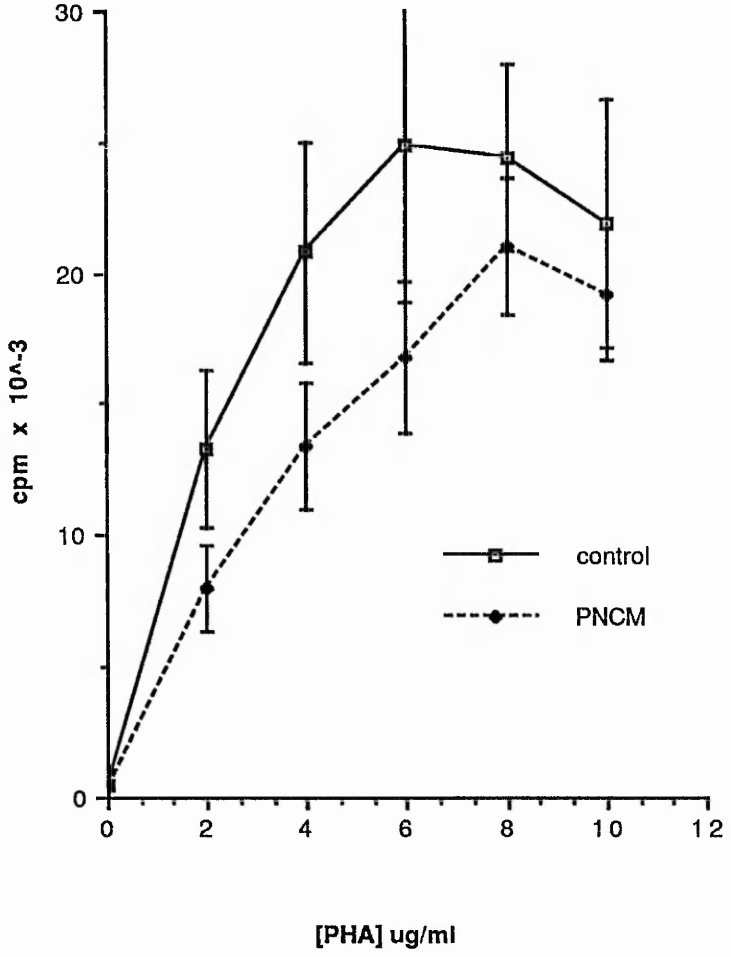

Figure 1. The effect of 10% PNCM on mononuclear cell proliferation in response to PHA. Data represents ^{3}H-TdR incorporation after 72 hours incubation for six individual MNC donors and six PMN donors for the PNCM.

Results represent means +/- SEM.

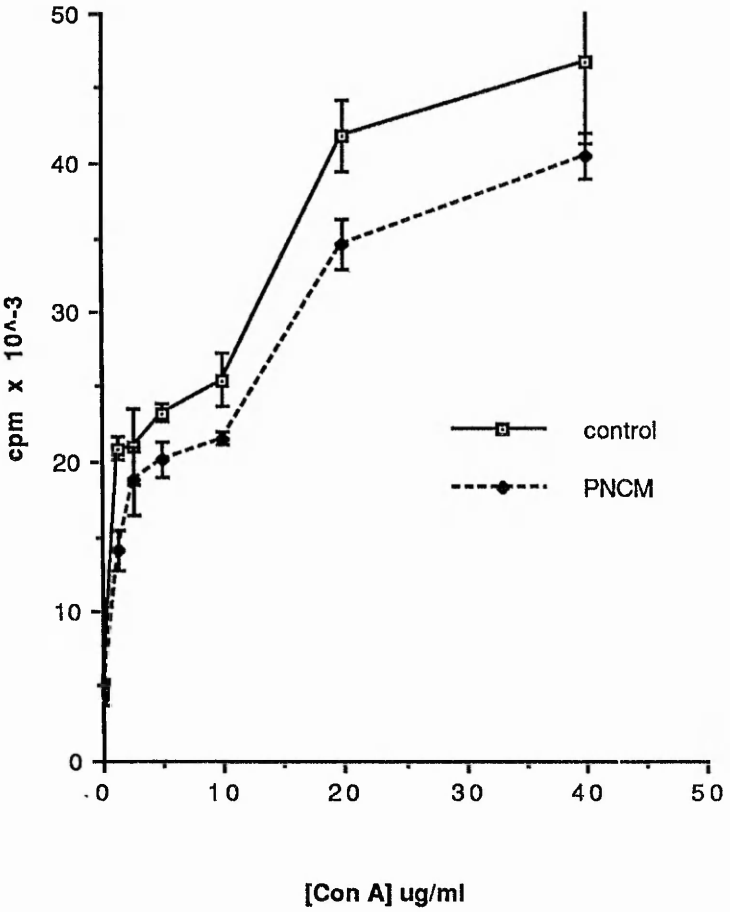

Figure 2. The effect of 10% PNCM on mononuclear cell proliferation in response to Con A. Data represents ^{3}H-Tdr incorporation after 72 hours incubation for 3 individual MNC donors and 3 PMN donors for the PNCM.

Results represent means +/- SEM.

Donor	% Inhibition PNCMa	PNCMb
1	35	17
2	68	31
3	58	49
4	27	28
5	29	38
6	27	32
Mean	40.7	32.5
SEM	7.2	4.3

Table 1

Comparison between the inhibition of proliferation by six MNC donors stimulated with 4 ug ml^{-1} PHA in response to two different batches of PNCM.

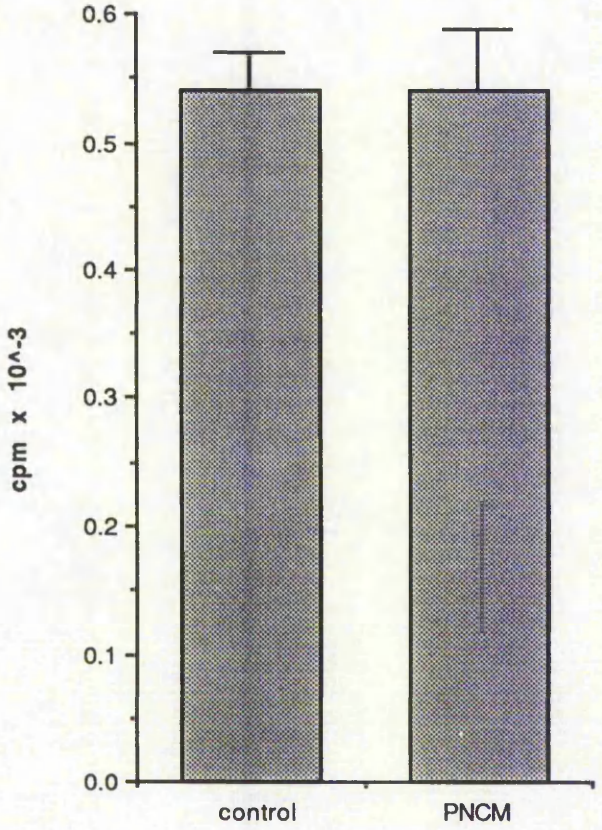

Figure 3. The effect of 10% PNCM on ^{3}H-TdR incorporation into 3-way MLCs after 72 hours incubation in FBWs.

Results represent means of six separate experiments +/- SEM with a different PNCM donor for each.

around the edge of the wells. The cultures in RBWs formed a very clear, tightly packed pellet in the base of the well. The results of these experiments were in accordance with Teodorescus' work, with considerably more uridine incorporation occuring in the crowded cultures than in the spread (Figure 4). In addition, the enhanced transcriptional activity occuring in response to cell crowding was inhibited by PNCM, whereas no effect was observed in the spread cultures.

To determine whether PNCM was only affecting RNA synthesis or whether proliferation was also inhibited, crowded MLCs were prepared and the effect of PNCM on both ^{3}H-UdR and ^{3}H-TdR incorporation measured. To investigate the time course of the response, nucleotide uptake was assessed over a seven day period. Figure 5 shows that although higher counts were obtained with TdR, the pattern of response was very similar. Figure 6, which represents the same data expressed as percentage inhibition of control values shows that both UdR and TdR incorporation were affected identically by PNCM.

The effect of PNCM on a single donor's MNCs stimulated allogeneically in a one-way MLC was next investigated. The conditions initially used were those found to be optimal for the 3-way MLC, namely 72 hours in crowded conditions, with proliferation being determined by ^{3}H-UdR incorporation. However, as illustrated in Table 2, only one of three donors responded to PNCM under these conditions. Since one-way MLCs are generally incubated for 7 days (Shou et al. 1980) experiments were repeated using the longer incubation time. In an attempt to standardise the response, a

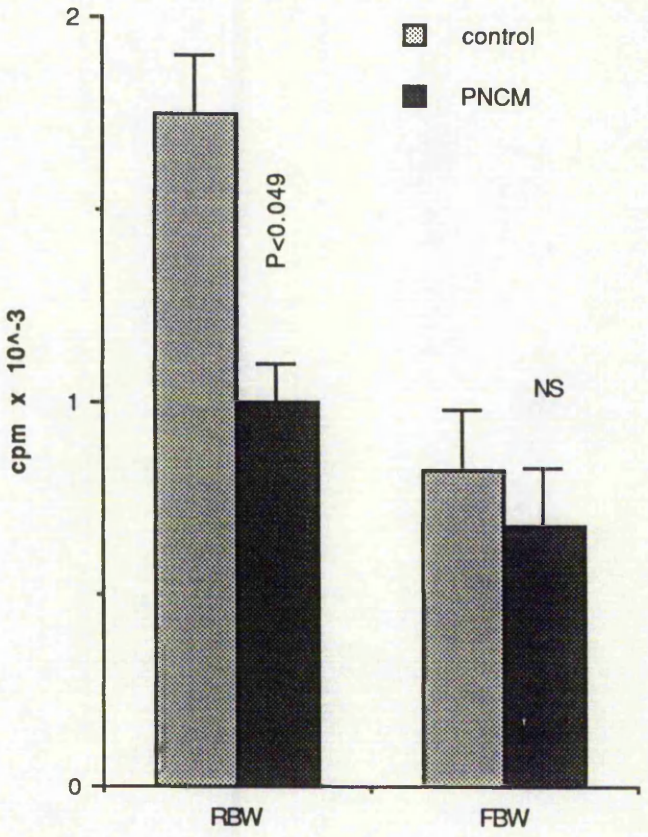

Figure 4. The effect of cell crowding and PNCM on ^{3}H-UdR incorporation into 3-way MLCs incubated for 72 hours.

Results represent means of six separate experiments +/- SEM with a different PNCM donor for each.

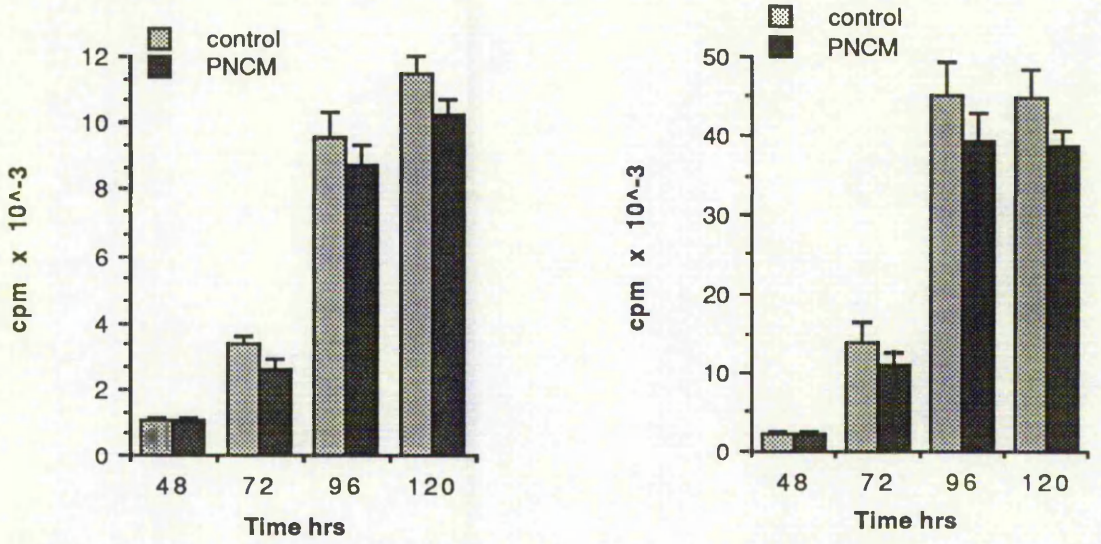

Figure 5. The effect of time and 10% PNCM on ^{3}H-UdR (left) and ^{3}H-TdR (right) incorporation into 3-way MLCs cultured in RBWs.

Results represent means of six separate experiments +/- SEM.

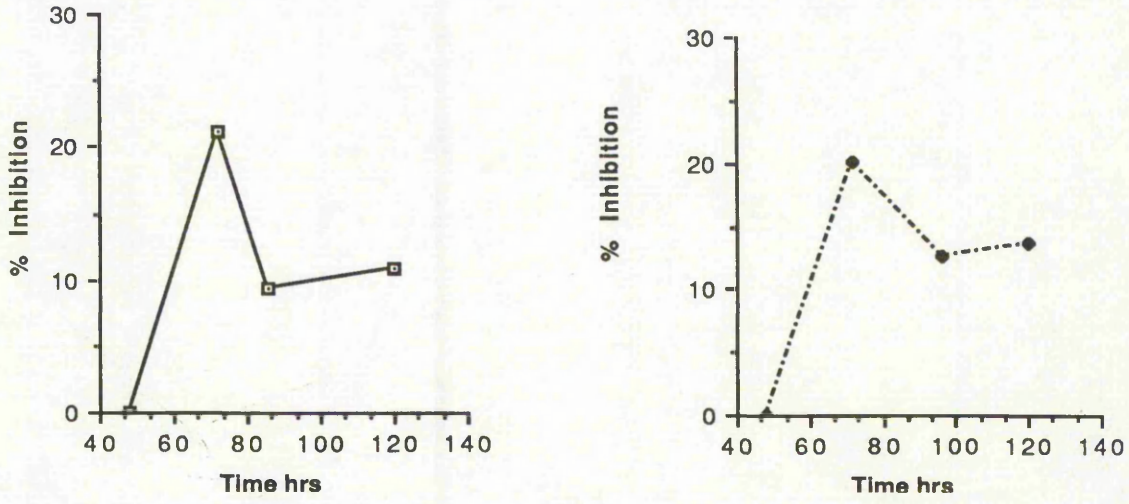

Figure 6. The data illustrated in Figure 5 expressed as % inhibition induced by PNCM below control values obtained without PNCM.

	CPM	
MNC Donor	Control	10% PNCM
1	3641	3542
2	4583	4738
3	4185	3652

Table 2

The effect of 10% PNCM on ^{3}H-UdR incorporation into 72 hour one-way MLCs cultured in RBWs.

batch of stimulator cells was prepared and frozen from a unit of blood obtained from a pseudopolycythaemic patient undergoing routine venesection. In addition, the MNCs were incubated in the absence of stimulator cells. The results of these experiments are shown in Table 3. It can be seen that a considerable amount of variation occurred between donors both in response to stimulator cells and to PNCM, with some individuals being inhibited by the neutrophil exudate whilst others were either unaffected or stimulated. The one-way MLC was therefore considered unsuitable for continuing the investigations.

To determine whether the inhibition produced by PNCM was neutrophil derived and that phagocytosis was required for its release, control conditioned media were prepared alongside PNCM. These were the Candida only at $5x10^6$ ml^{-1} and the PMN at 10^6 ml^{-1} and were treated exactly as the combination of the two for production of PNCM. Addition of either control at 10% to crowded MLCs had no effect on the proliferation of the cultures, as shown in Figure 7. Thus, it was concluded that the inhibitory factor was released by neutrophils in response to phagocytosis.

3.3. Characterisation of the Inhibitor

Work by Broxmeyer *et al.* (1978) demonstrated that neutrophil derived Lf is able to inhibit production of GM-CSF *in vitro*. Since Lf is known to be released under the conditions used to prepare PNCM, this was considered to be the most likely factor responsible for the observed inhibition. PNCM was therefore depleted of Lf by affinity chromatography.

CPM

Donor	R		R + S		(R+S) - R	
	-PNCM	+PNCM	-PNCM	+PNCM	-PNCM	+PNCM
1	9356	6180	16897	22820	7041	16640
2	4629	6353	11993	17427	7364	11074
3	19324	12135	26462	22275	7138	10140
4	4941	4892	13286	19176	7445	14284
5	5450	4492	6844	6036	1394	1544
6	7622	12989	27308	28542	19688	15553
Mean	8554	7840	16982	19379	8345	11539
SEM	2277	1526	3393	3088	2462	2249

Table 3

The effect of 10% PNCM on ^{3}H-UdR incorporation into one-way MLCs cultured for 7 days in RBWs.

R = Responder cells.

R+S = MLC of responders + Mytomycin C treated stimulators.

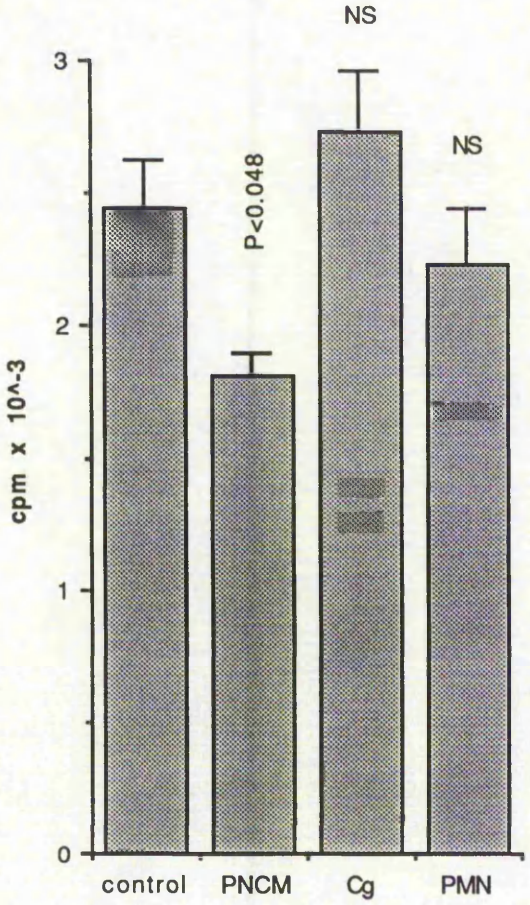

Figure 7. The effect of medium conditioned by Candida alone (Cg), neutrophils alone (PNM) and the combination of the two to produce PNCM on ^{3}H-UdR incorporation into crowded MLCs.

Results represent means of four separate experiments +/- SEM.

3.3.1. Affinity Chromatography

Monoclonal anti-Lf was a generous gift from Dr. J. Brock. This was subsequently bound to cyanogen bromide activated Sepharose 4B, according to the method of Van Eijk and Van Noort(1976) for transferrin affinity chromatography.

Lyophilised Sepharose 4B was obtained from Sigma. 3 grams of the dried powder were swollen for 15 minutes in 1 mM HCl in a 2 cm diameter Econocolumn (Biorad). The HCl was then allowed to drain off and the gel washed by passing 600 ml of the same solution through the column. 15 ml of coupling buffer (0.1 M $NaHCO_3$ containig 0.5 M NaCl, pH 8.3) were then used to wash the gel, after which it was transferred to a 50 ml tube and 20 ml of coupling buffer containing the antibody at 10 mg protein ml^{-1} added. This was maintained at $4^{o}C$ with gentle end over end rotation overnight. The following morning, any active groups remaining on the gel were blocked by replacing the coupling buffer with 0.2 M glycine at pH 8.0 for two hours. The gel was then washed to remove excess uncoupled ligand by alternately passing 100 ml of 0.1 M acetate buffer (0.1 M $NaCl_2H_3O_2$ containing 0.5 M NaCl, pH 4.0) and coupling buffer at pH 8.3 down the column 5 times. The affinity gel was then stored at $4^{o}C$ in 0.1 M Tris/HCl pH 7.2 buffer containing 15 mM sodium azide.

The efficacy of the anti-Lf column was determined using 50% ^{59}Fe-saturated Lf. ^{59}Fe was bound to a solution of purified human Lf in 0.1 M Tris/HCl buffer according to the method of Gutteridge *et al.* (1981) which is detailed section 3.3.3. A stock solution was made to a final concentration of 10^{-5} M. Approximately 2 ml of anti-Lf

Sepharose 4B were added to a 0.5 cm diameter Econocolumn and the column allowed to form by gravity. This was washed with 20 ml of 0.1 M Tris/HCl buffer before 2 ml of the ^{59}Fe-Lf solution was overlaid. The column was allowed to run by gravity, and after the sample had entered the gel, 20 ml of Tris/HCl were added. 2 ml fractions were collected and counted for radioactivity. No activity was detected in any fraction and a clearly defined pink band indicative of iron saturated lactoferrin could be observed approximately halfway down the column. Glycine/HCl buffer at pH 2.8 was used to elute off the bound Lf. The maintenance of the pink colour indicated that this treatment did not cause dissociation of the Fe from the Lf. Subsequent measurement of the absorbance at 460 nm confirmed this observation. Elution occurred very rapidly, and all the radioactivity added to the column was collected in the second 2 ml fraction.

To investigate the cross reactivity of the column, ^{59}Fe labelled transferrin and vitamin B_{12} binding protein were used. Neither were retained by the affinity gel. The B_{12} binding protein was kindly assayed by Mr. K. Sheppard, City Hospital, Nottingham.

Following recovery of the Lf, the gel was regenerated prior to storage at 4^{o}C. The column was washed with 20 ml of 0.1 M Tris/HCl containing 0.5 M NaCl at pH 8.5. This was followed with 20 ml of 0.1 M sodium acetate buffer containing 0.5 M NaCl at pH 4.0 after which the column was re-equillibrated with 0.1 M Tris/HCl at pH 7.2 containing 15 mM sodium azide.

Having verified the efficacy of the anti-Lf column, it was used to

remove Lf from PNCM. The conditions used were identical to those described above, with the exception that the gel was washed initially in PBS rather than Tris/HCl buffer, since PNCM was prepared in PBS. 2 ml of PNCM were added to the column and allowed to enter the gel. This was followed by an excess of PBS. 2 ml fractions were collected and sterilised by passage through a 0.2 um filter and stored at -20°C until required. Glycine/HCl buffer at pH 2.8 was then passed down the column and further 2 ml fractions collected. The fractions were transferred to dialysis tubing and dialysed against an excess of PBS at 4°C for 24 hours. These were sterilised and stored at -20°C until required.

These fractions were then tested against the MLC. It can be seen from Figure 8 that passage of PNCM down the anti-Lf column completely abrogated the inhibition observed with neat PNCM. Removal of Lf from the column resulted in re-expression of the inhibition. This routinely occurred in the second 2 ml fraction in accordance with the results obtained with purified ^{59}Fe saturated Lf.

3.3.2. Neutralisation of Lactoferrin in PNCM

To determine whether antibody to Lf could affect the inhibitory activity of PNCM when added in the liquid phase, polyclonal anti-Lf (Dako Patts) and PNCM were incubated together. The antibody was first dialysed against PBS at 4°C for 24 hours to remove all preservative present. Serial dilutions of antibody were then added to PNCM giving a dilution range from 1:16 to 1:512. These were then incubated at 37°C for 1 hour. The neutralised PNCM was next added to crowded MLCs as described above and the cell proliferation

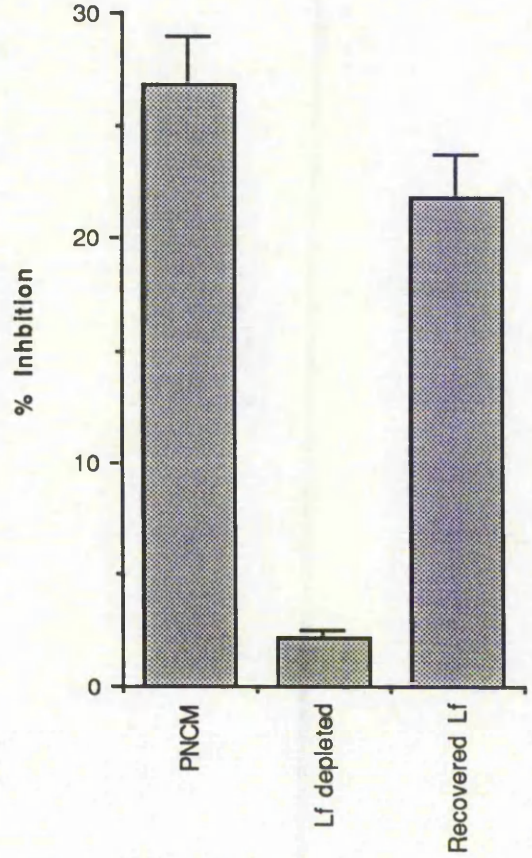

Figure 8. The effect of removing Lf from PNCM by affinity chromatography on the inhibition of ^{3}H-UdR incorporation into 3-way crowded MLCs.

Results represent means of six separate experiments +/- SEM with a different PNCM donor for each.

determined after 72 hours. Figure 9 shows that pretreatment of PNCM with polyclonal anti-Lf completely abrogated its inhibitory activity against the MLC. The minimum inhibitory concentration of antibody was found to be 1:128, which would correspond to a Lf concentration in PNCM of approximately 10^{-9} M.

3.3.3. The Effect of Adding Purified Human Lf to MLC

The work with PNCM strongly suggested that the active factor was Lf. This raised the obvious question: could purified Lf from human breast milk have the same effect? The work of Broxmeyer et al. (1978) has shown that for Lf to inhibit GM-CSF production the protein must be carrying iron. Therefore, both native Lf and Lf to which further iron had been added to obtain 50% saturation were used.

Since Lf is a strongly cationic glycoprotein, it adheres very avidly to glass and plastic. Therefore, to reduce loss of the protein during its preparation and use, all glassware was previously siliconised (Sigmacote). All the tubes used for the storage and dilution of Lf were made of polypropylene, to which the protein does not stick.

Human breast milk Lf (approximately 98% pure) was obtained from Sigma and was diluted in 0.2 M Tris/HCl buffer at pH 7.3. To ensure that the iron saturation of the Lf was indeed 50%, and to prevent non-specific binding, it was important to minimise the addition of extra iron which is ubiquitous; therefore, the buffer was prepared immediately before use with freshly distilled water collected into acid washed glass. Addition of iron to Lf was

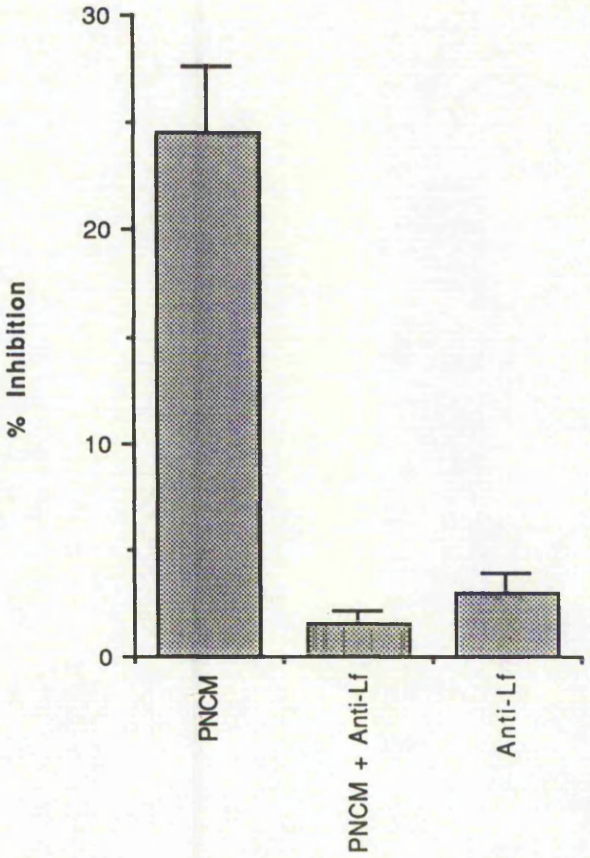

Figure 9. The effect of neutralisation of Lf in PNCM with a 1:128 dilution of polyclonal anti-Lf on its inhibitory activity against 3-way crowded MLCs.

Results represent means of six separate experiments +/- SEM with a different PNCM donor for each.

conducted according to the method of Gutteridge et al. (1981). The Lf was diluted in half the volume of 0.2 M Tris/HCl buffer needed to give the required concentration. The buffer contained 10 mM $NaHCO_3$ since this is required for iron binding. $Fe(NH_4)_2(SO_4)_2.6H_2O$ was prepared in freshly distilled water collected into glass. Based on the calculation that 1.4 ug Fe gives 100% saturation of 1 mg Lf, 49 ug of ferrous ammonium sulphate per 10 mg Lf was required to give 50% saturation. Equal volumes of the two solutions were mixed and incubated together for 30 minutes at 37°C. At a Lf concentration of around 10^{-4} M, the iron saturation could be seen to occur virtually instantaneously by the rapid development of the characteristic salmon pink colour. The protein was then dialysed agianst Tris/HCl for 24 hours at 4°C. To control for loss of Lf by it's binding to the dialysis membrane, the native Lf was also dialysed. The iron saturated and iron poor Lf samples ($5x10^{-4}$ M) were scanned spectrophotometrically between 350 and 550 nm. It can be seen from figure 10 that a peak at 465 nm corresponding to iron saturation occurred in the former but not in the latter sample, thus confirming successful iron saturation. The native and 50% iron saturated Lf (Fe-Lf) were diluted down to 10^{-6} M, in 0.1M Tris/HCl pH 7.3, aliquotted into polypropylene tubes and stored at -20°C until required.

The effect of native and Fe-Lf on the crowded MLC was then investigated. Dilutions of Lf were made in Tris/HCl buffer ranging from 10^{-7} to 10^{-15} M. These were added to the crowded MLC, with a further dilution of 1:10, thus giving a final concentration range of 10^{-8} to 10^{-16} M. 0.1 M Tris/HCl buffer at 10% of the final

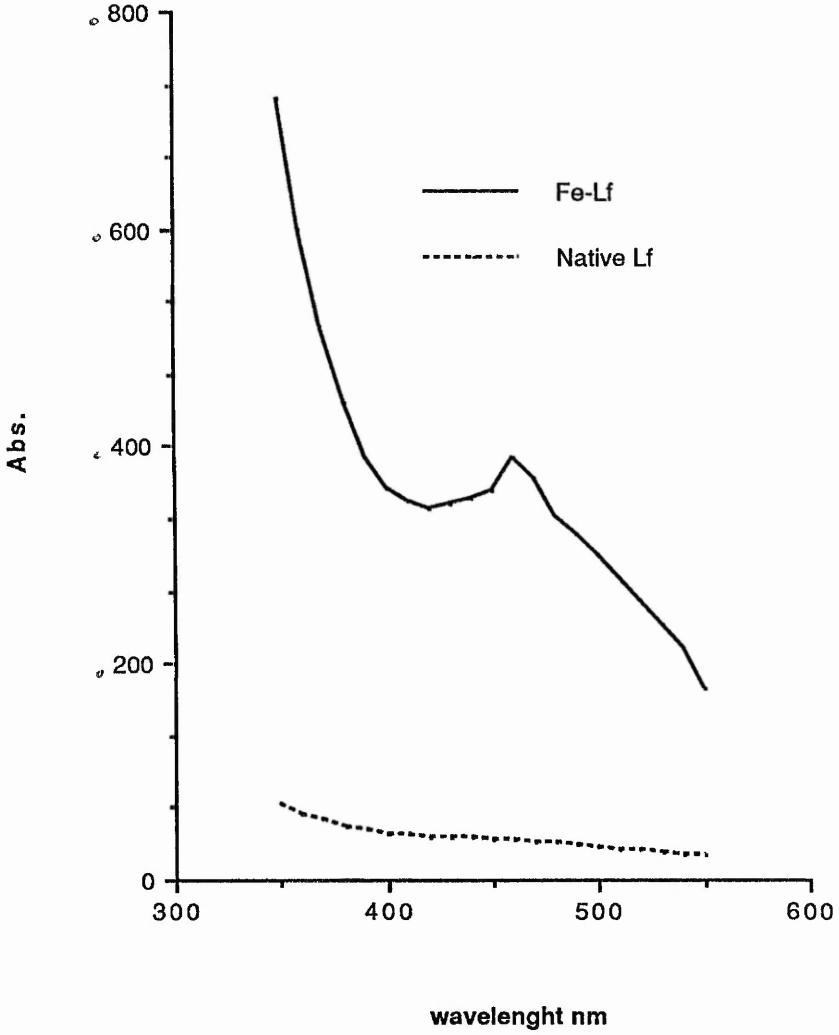

Figure10. Spectrophotometeric scan of native Lf and Lf to which Fe has been added to obtain 50% saturation.

culture volume was used as a control. Following incubation for 72 hours the proliferation was determined by measuring ^{3}H-UdR incorporation.

Figure 11 shows that Fe-Lf was inhibitory to the MLC between 10^{-8} and 10^{-12} M, all inhibition being lost below 10^{-14} M. No inhibition by native Lf occurred at any concentration.

3.3.4. Measurement of Lf in PNCM

To investigate whether a similar dose response curve to that obtained with purified Lf could be produced with PNCM, it was necessary to know the concentration of Lf released from phagocytosing neutrophils. To this end, an enzyme linked immunosorbent assay (ELISA) was used based on that developed by Dr. John Porter (unpublished); the protocol is detailed section 2.6.

Figure 12 shows a typical standard curve for Lf obtained with this ELISA. An internal standard of pooled human serum was incorporated into every assay and from this an inter-assay variation of 11.7% was determined. The detection limit can be seen to be 0.1 nM.

Initially, this assay was used to follow the release of lactoferrin from neutrophils during phagocytosis of Candida. The results of these experiments are shown in Figure 13. It can be seen from this figure that maximal release of lactoferrin had occurred by 30 minutes. This is therefore consistent with the work of Maallem *et al.* (1982) on the release of vitamin B_{12} binding protein by phagocytosing neutrophils.

Measurement of Lf in PNCM produced from six individual donors gave

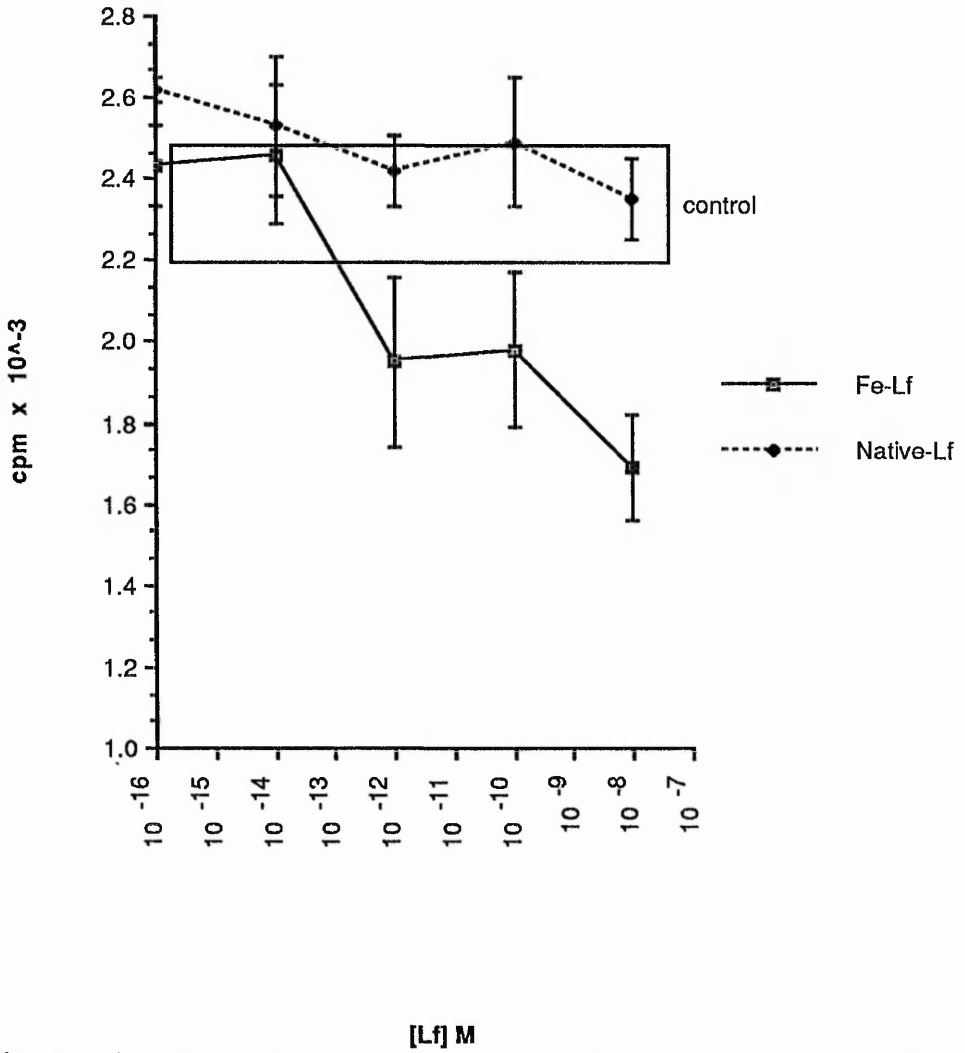

Figure 11. Dose response curve for 50% Fe saturated Lf and native Lf against crowded 3-way MLCs.

Results represent means of six separate experiments +/- SEM.

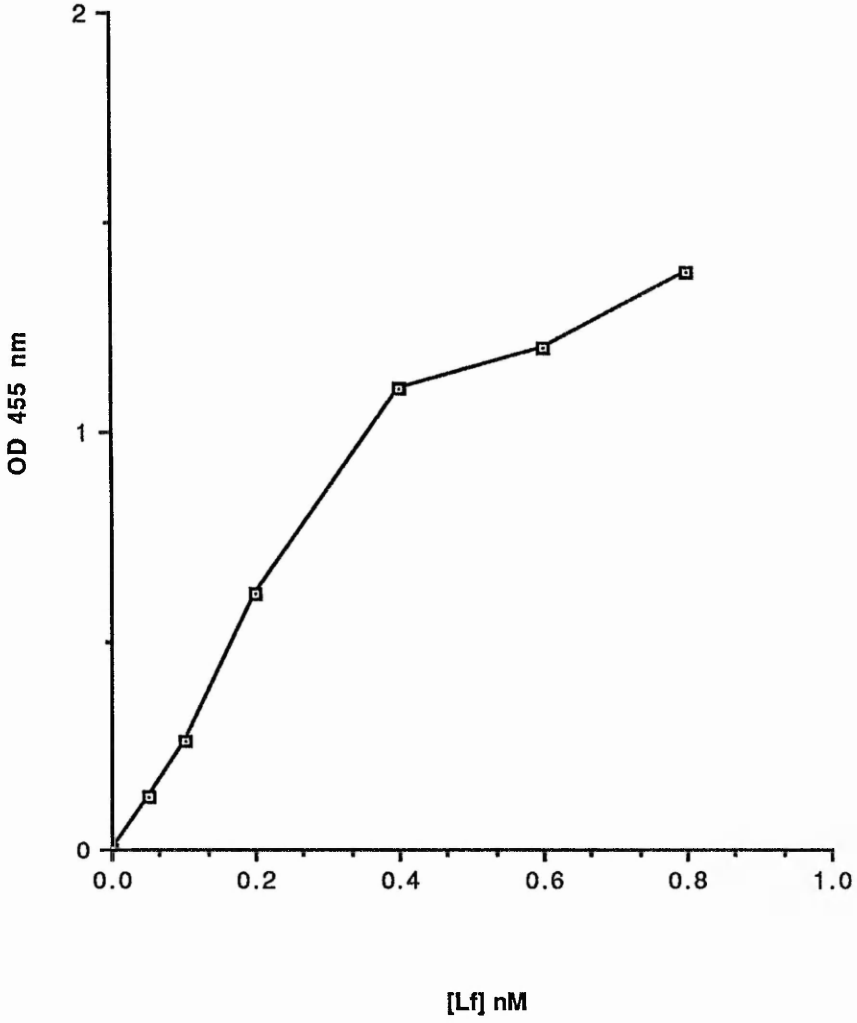

Figure 12. Typical standard curve for Lf as determined by ELISA.

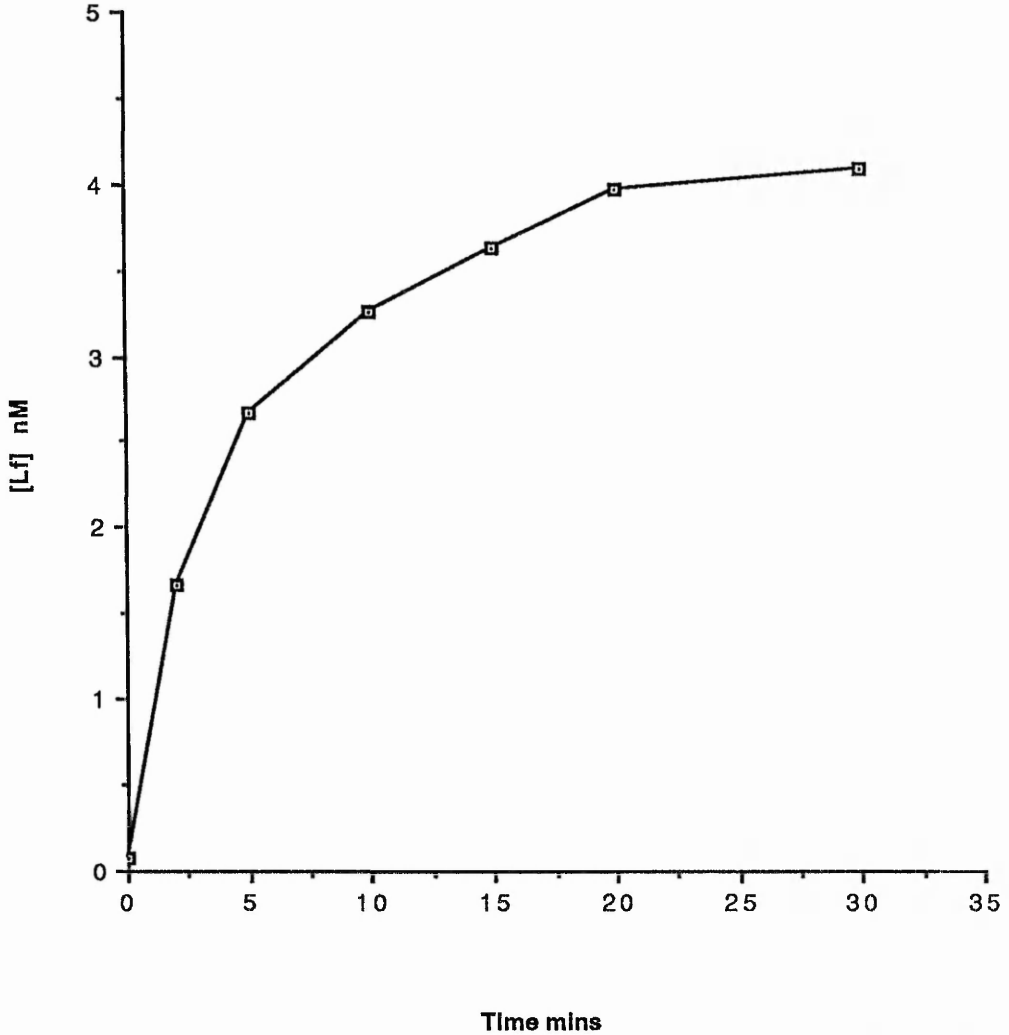

Figure 13. Typical time course for the release of Lf from neutrophils phagocytosis Candida guilliermondii.

a mean Lf concentration of 3.27 nM with a range between 2.01 and 4.38 nM, this is consistent with the concentrations determined by neutralisation of Lf in PNCM (section 3.3.2.). These PNCM samples were diluted appropriately in PBS to give a final concentration range of between 10^{-10} M and 10^{-13} M and were tested against the MLC. The results were then compared with the purified Fe-Lf dose response curve and are shown in Figure 14. It can be clearly seen that when PNCM is expressed in terms of its Lf concentration, the dose response curve produced is almost identical to that produced by purified Fe-Lf.

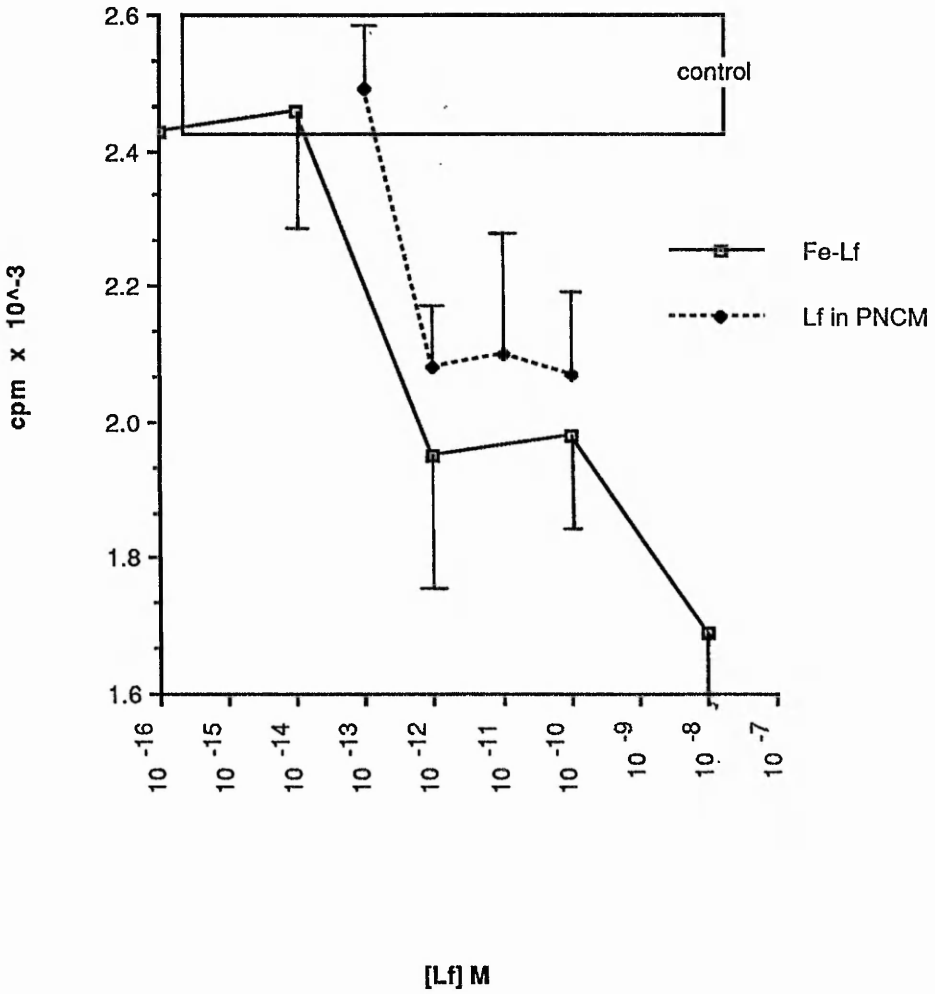

Figure 14. Comparison between the inhibition of crowded MLCs by Fe-Lf and of PNCM expressed in terms of its Lf content.

Results represent means of six separate experiments +/- SEM.

Discussion

The work presented in this chapter has demonstrated that phagocytosing neutrophils release a factor capable of inhibiting mononuclear cell proliferation in response to a range of stimuli. However, in no experiment was the inhibition by PNCM complete. The response to both mitogens and the three way MLC was usually inhibited by approximately 30% by PNCM. When stimulated by PHA or Con A, every donor's MNCs were inhibited by PNCM, and the effect of a batch of PNCM was quite consistent when tested against different individuals' MNCs stimulated by PHA. The results obtained with one way allogeneic MLCs were considered to be too variable to produce valuable data; and this stimulus was therefore abandoned.

Although consistency of response in the three way MLCs was not as good as with mitogens (about one in five three way MLCs did not respond to PNCM or Lf), the requirement for crowding the cells to obtain an effect with PNCM was considered to offer an insight into the action of the inhibitor. Also, since the MLC provides a particulate stimulus, growth factor production could be examined in the absence of soluble stimuli such as mitogens. The three way MLC was therefore used to identify the inhibitory factors present in PNCM.

A series of experiments involving affinity chromatography, neutralisation by antibodies and the use of purified protein has identified the inhibitory factor as Lf. This finding was supported by the observation that purified 50% iron saturated Lf produced a parallel dose response curve to PNCM when the latter was expressed

in terms of its Lf content. Work with purified Lf demonstrated a requirement for iron saturation above that of native Lf for the inhibition to occur. The observation that phagocytosing neutrophils release Lf which possesses inhibitory activity would therefore suggest that this Lf is also carrying iron. Although the concentration of Lf in PNCM was too low to obtain a spectrophotometric measurement of iron saturation, removal of Lf from PNCM by affinity chromatography provided evidence for this. The passage of PNCM down the affinity column occasionally produced a distinctive pink band which was eluted off the column by glycine/HCl buffer. Thus, there is circumstantial evidence that Lf released from phagocytosing neutrophils is carrying iron.

The release of Lf from neutrophils has generally been considered to occur in the iron poor (native) state. This was based on the finding that iron free Lf is bacteriostatic by binding and removing available iron for bacterial growth (Masson et al. 1969). Further investigations were carried out by Van Snick et al. in 1974, who demonstrated that rabbit heterophils release Lf when phagocytosing Staph. albus. However, their measurements of the iron saturation of heterophil Lf (8%) were made on cell extracts obtained by repeated freeze thawing. Similarly, Broxmeyer et al. (1978) suggested that Lf was released from neutrophils in an iron free form; however, his work was also conducted using freeze thawed extracts. This would suggest that granule associated Lf contains little iron. Nevertheless, the evidence presented here would indicate that when released by phagocytosis, Lf does carry iron. This poses the question: what is the source of the iron? Two

possibilities exist. Either the neutrophil mobilises iron from its own ferritin stores, or the iron is acquired from killing and digestion of the phagocytosed micro-organism. Further experiments are required to answer this question. One approach would be to allow neutrophils to phagocytose ^{59}Fe labelled micro-organisms. Detection of ^{59}Fe on the Lf released would implicate the microbes as the source of the iron. The hypothesis could be tested further by allowing neutrophils to phagocytose inert, iron free latex beads and determining the inhibitory capacity of the PNCM produced. Demonstration of inhibition would imply that the neutrophil was providing iron.

The fact that Lf requires iron to inhibit mononuclear cell responses suggests that Lf released passively by cell death would be iron free and consequently inactive. However, Lf released by phagocytosis would acquire iron, and with it inhibitory activity.

The majority of work conducted into the function of Lf has focussed on the inhibition of granulopoiesis *in vitro*. However, following many years of research, there still remains little agreement over its precise role. The field is split between workers who believe that lactoferrin has a regulatory role and those who do not. Broxmeyer *et al.* (1978) demonstrated that Lf purified from human breast milk and fully saturated with iron was capable of inhibiting CSA production *in vitro* down to 10^{-17} M. The degree of iron saturation of Lf affected the protein's inhibitory capacity, with native Lf being inhibitory down to 10^{-14} M, and apo-Lf was shown to be active only at concentrations greater than 10^{-7} M. This data was viewed with some scepticism, since the

concentration of Lf in FCS used to culture the cells would be higher than the minimum inhibitory concentration observed by Broxmeyer (Burgess and Metcalf 1980). However, the work was repeated by Bagby et al. (1982) who depleted FCS of Lf by affinity chromatography. They were also able to demonstrate inhibition of CSA production by cultured human mononuclear cells with 85% Fe saturated Lf at concentrations down to 10^{-17} M.

Other workers have attempted to repeat these experiments but were unable to obtain any effect with Lf. Delforge et al. (1985) used purified fully iron saturated Lf from human milk and a sample of Lf obtained from Dr. Broxmeyer. They determined the effect of these Lf samples on production of CSA by normal bone marrow mononuclear cells, and found no inhibition, either by their own preparation or by that of Broxmeyer. They repeated the experiments using different cell separation procedures and culture media, also depleting FCS of Lf. In addition, they used 10^{-6} M indomethacin during incubation, which Broxmeyer and Platzer (1984) had shown to be necessary to demonstrate inhibition by Lf (due to the inhibitory effect of Lf on PGE_2 production which itself inhibits GM-CSF synthesis, and hence masks the effect of Lf). Even with these stringent conditions, they still failed to demonstrate any inhibition. They also used Broxmeyer's FCS but still obtained no effect.

The work presented here on the inhibition of the MLC is in partial agreement with Broxmeyer and Bagby on the effect of Lf on CSA production. However, the inhibitory effect of Lf against the three

way MLC was found to be lost at a concentration of approximately three orders of magnitude greater than that claimed by Broxmeyer or Bagby. In agreement with the former but not with the latter, Lf was found to be inhibitory to MLC at concentrations greater than 10^{-10} M. Bagby provided evidence that the loss of activity by Lf above 10^{-10} M was due to calcium dependent polymerisation of the protein and that the polymeric form failed to inhibit granulopoiesis.

Although the three authors attempted to standardise their conditions in order to investigate the phenomenon of inhibition of CSA, they each used different concentrations of FCS in their experiments. The highest FCS concentration was used by Delforge (20%) with Bagby employing 15% and Broxmeyer 10%. This may provide an explanation for the differences in their results. Bagby's observation that Lf polymerised and lost inhibitory activity at concentrations greater than 10^{-10} M could give some insight into the discrepancies. If the difference between Bagby's dose response curve and that of Broxmeyer is due to the additional 5% of FCS used by the former, it is possible that the absence of inhibition observed by Delforge over the entire concentration range could be due to the use of 20% FCS. Winton et al. (1981) also used an FCS concentration of 20%, and were unable to demonstrate an effect by Lf; thus, supporting the hypothesis that Lf is ineffective as an inhibitor at high FCS concentrations. The inhibition of the MLC by Lf at concentrations greater than 10^{-10} M accords with this explanation, since the FCS concentration used was 10% and hence equivalent to that used by Broxmeyer.

If the inhibitory activity of Lf depends on the FCS concentration used, then the criticism of Burgess and Metcalf would not apply to Broxmeyer's work where, according to this hypothesis, the FCS concentration should be low enough to enable inhibition to occur. This hypothesis could also explain how normal cell functions could continue in the circulation, where the Lf concentration is reported to be between 10^{-9} M (Bennet and Mohla 1976) and 10^{-11} M (Broxmeyer et al. 1983). According to the data presented in this thesis such concentrations would inhibit mononuclear cell proliferation. If the inhibitory capacity of Lf is lost at high FCS concentrations it would surely be ineffective in the plasma.

Although the data presented here is in accordance with the work of Broxmeyer at higher concentrations of Lf, inhibition of the MLC is lost at 10^{-14} M, not 10^{-17} M as determined for CSA production. Many workers in the field have found an effective concentration of 10^{-17} M difficult to accept, particularly when the cell numbers used by Broxmeyer are taken into consideration. Addition of 10^{-17} M Lf to 5×10^5 monocytes (as used by Broxmeyer) results in there being one Lf molecule for every 50 monocytes. Broxmeyer's dose response curve for fully iron saturated Lf shows maximal inhibition at 10^{-12} M; this would give 1204 Lf molecules per monocyte. Birgens et al. (1983) found the number of Lf binding sites on human monocytes to be 1.6×10^6 per cell. Applying this to Broxmeyer's data, Lf at 10^{-12} M would occupy 0.07% of the available binding sites. If Birgens' measurements are correct, the production of so many apparently redundant receptors by a cell can be explained in one of two ways: either the binding sites measured by Birgens are

principally involved in some unrelated function, or an anomaly exists in Broxmeyer's data.

If the Lf receptor is involved in a negative feedback mechanism requiring Lf, it would be reasonable to assume that the increase in receptor occupancy above 0.07% would result in an increased response. This can be seen in figure 11. Taking approximate monocyte numbers in the mononuclear cell preparation to be 30%, the addition of Lf at 10^{-8} M would result in there being 18.06 x 10^{6} molecules per monocyte, which is tenfold more than the theoretical number of binding sites calculated by Birgens. If the effect of Lf is proportional to receptor occupancy, then the maximal response would occur at 10^{-9} M. This is not inconsistent with the data shown in Figure 10. The pattern of inhibition of the MLC by Fe-Lf is therefore very similar to the effect on CSA production as shown by Broxmeyer et al. (1978). However, the dose response curve for the former occurs over a concentration range three orders of magnitude greater than that observed by Broxmeyer. It is difficult to find an explanation for this discrepancy, although, active concentrations determined in this work are in closer accordance with reported monocyte Lf receptor expression.

At no concentration did the iron free Lf inhibit the MLC. This was an unexpected observation for a number of reasons. No attempt to deplete the Lf completely of iron was made. This was because all the evidence indicates that Lf in vivo is not completely iron free, and the results which show no apo-Lf activity (Broxmeyer et al. 1978) could be due to denaturation of the protein by the 0.1 M citrate used to liberate bound iron (Ainscough et al. 1980).

However, the Lf used must have been very low in iron, since no absorbance peak of a 5×10^{-4} M solution could be detected around 465 nm (Figure 10). Despite this, if the iron free Lf contained only 1% of iron carrying molecules, the 10^{-8} M sample would contain 10^{-10} M of Fe-Lf, a concentration which is clearly inhibitory to the MLC (Figure 11). This difference could be explained if differential binding of iron to the two sites occurred (as hypothesised for transferrin by Fletcher and Huehns 1968). Thus, an atom of iron would preferentially attach to, for example, site A, resulting in an increase in the affinity of site B. At low concentrations of available iron, the monoferric species would be favoured. If the affinity of site B became greater than that of site A, then with the increasing availability of iron the prevalence of diferric Lf would increase. This hypothesis also requires the diferric species only to be active as an inhibitor. Based on this assumption, native Lf would be entirely monoferric and hence lack activity. The 50% iron saturated Lf would, however, contain diferric molecules and hence be inhibitory.

A number of publications have suggested that differences exist in the binding properties of the two iron sites of Lf (Harrington et al. 1987; Anderson et al. 1987). A large body of evidence now exists showing that the binding of iron to Lf results in a significant conformational change, with the structure becoming markedly more compact (Anderson et al. 1987; Querinjean et al. 1971). It is presumably this alteration in tertiary structure of the protein which facilitates the activation of Lf following iron binding. The degree of iron saturation affects its interaction

with the receptor on monocytes and also its subsequent fate. Van Snick et al. (1977) demonstrated that mouse peritoneal macrophages accumulate twice as much Fe-Lf as apo-Lf over a 25 hour incubation period. Broxmeyer et al. (1980) studied the binding of Lf to human monocytes by immunofluorescence techniques. They found that apo-Lf bound to 41% of the cells, native Lf to 78% and fully iron saturated Lf to 91%. Thus, there is considerable data to support the observation that iron is required to activate Lf to its inhibitory state.

Summary

An inhibitory factor released from phagocytosing neutrophils and effective against mononuclear cell proliferation in response to a range of stimuli has been demonstrated. This factor has been identified as Lf and a requirement for iron was observed to activate the protein to its inhibitory state. The finding that PNCM contains active Lf suggests that following phagocytosis, neutrophils release Lf which carries iron. The work has partially confirmed the controversial results of Broxmeyer and of Bagby, and has presented a possible explanation for the discrepancy between their work and that of Delforge.

CHAPTER FOUR

DETERMINATION OF THE ACTION OF PNCM AND LF ON THE MLC

Having established that Lf released from phagocytosing neutrophils inhibits the MLC, the next question was: how is this inhibition achieved? The finding that considerably more UdR incorporation occurred when the cultures were incubated in round bottomed wells (RBW) than when in flat (FBW), and that only this extra proliferation in the RBWs was inhibited by Lf, suggested the importance of cell contact in this phenomenon.

4.1. Demonstration of a Growth Factor Produced in Response to Cell Crowding

One possible explanation for the increase in proliferation in the crowded cultures could be that the enforced contact between the cells resulted in enhanced production of a growth factor or factors. To test this hypothesis, MLCs were grown in RBWs and FBWs for 72 hours. The contents of the wells were then aspirated, pooled and the supernatants harvested by centrifugation at 400 x g for 10 minutes. 100 ul of crowded cell supernatants (CC-S) were transferred to 100 ul of MLC at 2 x 10^6ml in FBWs (thus the final concentration and degree of crowding was identical to that of all previous spread MLCs). These cultures were then incubated for a further 72 hours before their proliferation was assessed by ^{3}H-UdR incorporation. Figure 15 shows that CC-S were able to increase the proliferation of the secondary spread cultures.

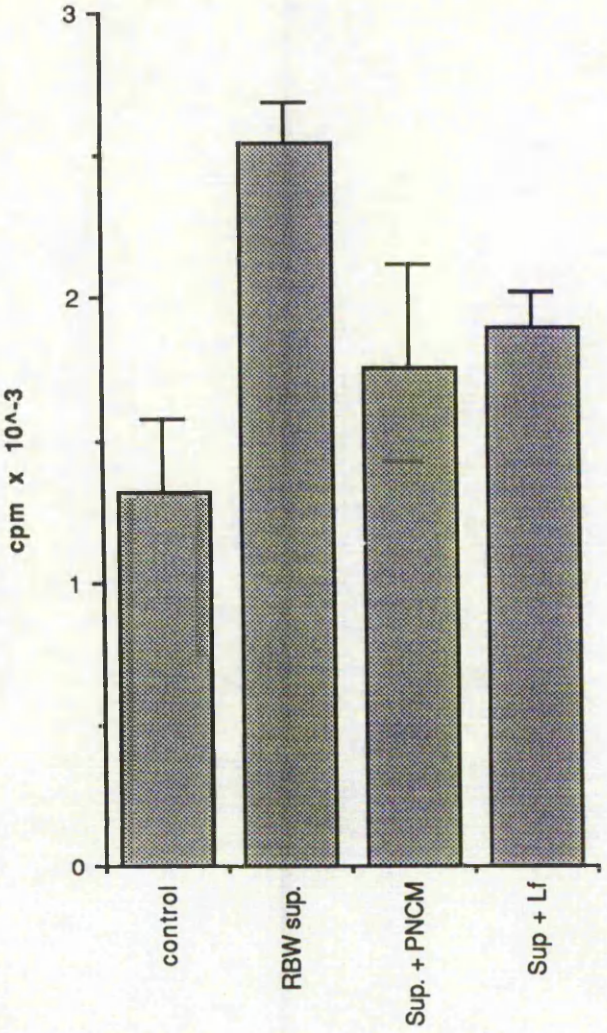

Figure 15. The effect of transferring supernatants from crowded MLCs (RBW Sup.) to those cultured in spread conditions and the effect of adding 10^{-10} M Fe-Lf or PNCM to the crowded cells.

Results represent means +/- SEM from six separate experiments. The stimulation produced by RBW Sup. above the control was significant, $P<0.016$. Inhibition of this by PNCM and Lf was also significant, $P<0.038$ and $P<0.025$ respectively.

This suggested that cultures grown in crowded conditions do produce a growth factor or factors capable of stimulating proliferation in spread cultures. Also, the addition of 10^{-10} M Fe-Lf or PNCM to the crowded cultures used to produce the supernatants caused a reduction in the subsequent proliferation of the cells in FBWs. This would therefore support the hypothesis that Lf was inhibiting the production of a growth factor/s produced in response to cell crowding.

To determine whether this factor/s was genuinely produced in response to enforced cell contact, and was not simply a product of cultured mononuclear cells, the experiments were repeated using supernatants from MLCs produced in FBWs. Figure 16 shows that these supernatants had no effect on subsequent spread cultures, and also illustrates that neither Lf or PNCM had any inhibitory effect.

Thus, crowding did appear to produce a growth factor which was inhibited by Lf. However, the CC-S were diluted by only one in two. This therefore resulted in the potential transfer of 5×10^{-11} M Lf to the secondary spread cultures, a concentration which, from Figure 11, can be seen to be inhibitory to the MLC. These experiments did not therefore, rule out the possibility that Lf was inhibiting the effect of the growth factor rather than its production. To answer this question, control CC-S was produced as previously described. To these, a final concentration of 5×10^{-11} M Fe-Lf was added before transferring the supernatants to secondary spread cultures. The results of these experiments are shown in Figure 17.

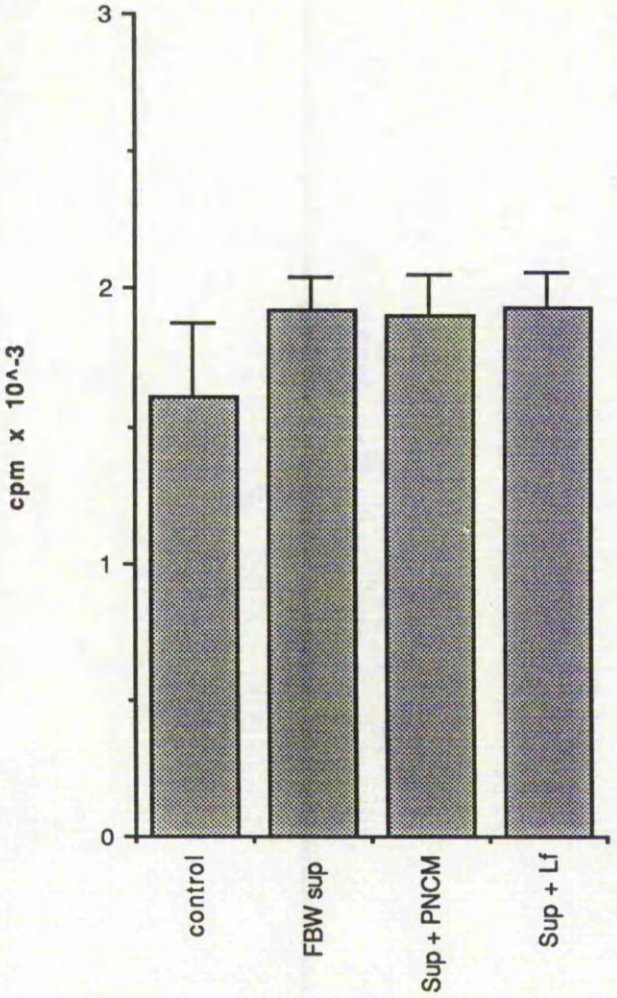

Figure 16. The effect of transferring supernatants from spread MLCs (FWB Sup) to those cultured in spread conditions and the effect of adding 10^{-10} M Fe-Lf or PNCM to the spread cells used to produce the supernatants.

Results represent means +/- SEM from three separate experiments.

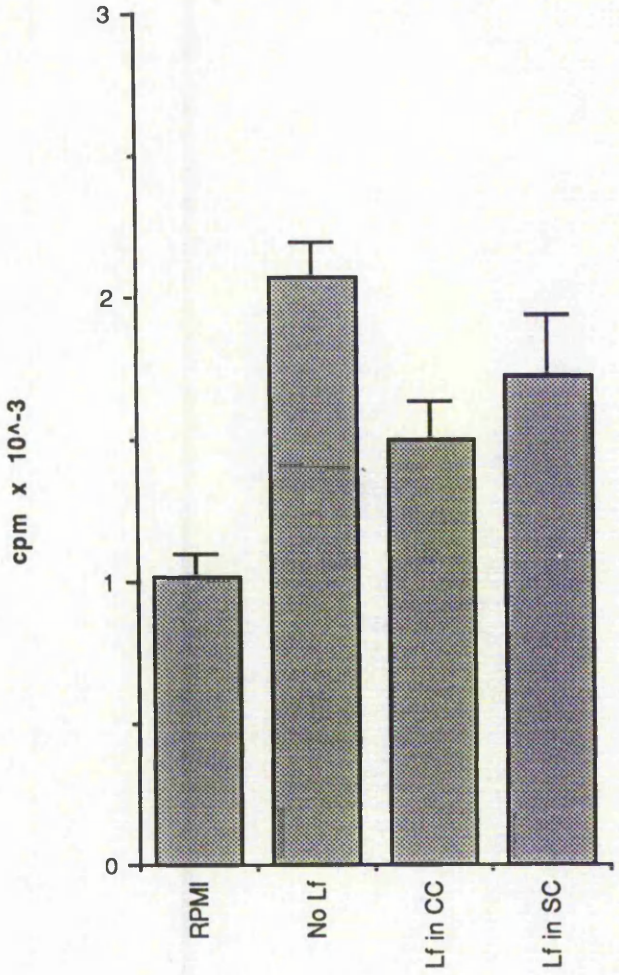

Figure 17. The effect of adding 5 x 10^{-11} M Fe-Lf to crowded cell conditioned medium on the enhancement of spread cell proliferation.

Lf in CC:- Lf added at initiation of crowded cultures.

Lf in SC:- Lf added at initiation of spread cultures containing crowded cell conditioned medium.

Results represent means +/- SEM from five separate experiments.

Addition of Lf to CC-S after the factor had been produced resulted in an intermediate level of stimulation of the secondary spread cultures; between that occuring when no Lf was present and when Lf was added at the initiation of the cultures used to produce supernatants.

Since these results failed to indicate whether Lf was inhibiting the production or effect of the growth factor, further experiments were conducted in which the Lf carried over to spread cultures in crowded culture supernatants was removed by affinity chromatography. Crowded cell supernatants were produced from MLCs to which 10^{-10} M Fe-Lf was added at the initiation of the cultures. These were then passed over the affinity gel which had previously been extensively washed by passage of 50 ml of RPMI down the column. The supernatants were sterilised through a 0.2 um filter before being added to spread MLCs. The effect of these supernatants on secondary spread cultures was then determined. Figure 18 shows that similar results were obtained in the experiments to those illustrated in Figure 17. Thus, it appeared that Lf inhibits both the production and effect of the growth factor.

4.2. Characterisation of the Growth Factor

Preliminary attempts to identify the growth factor focussed on the time course of its production. Crowded MLCs were incubated in the presence and absence of 10^{-10} M Fe-Lf. Supernatants were harvested at 24 hour intervals over a 72 hour period. In addition, the proliferation in replicate cultures was assessed at the time of harvesting. These supernatants were stored at -70°C before being

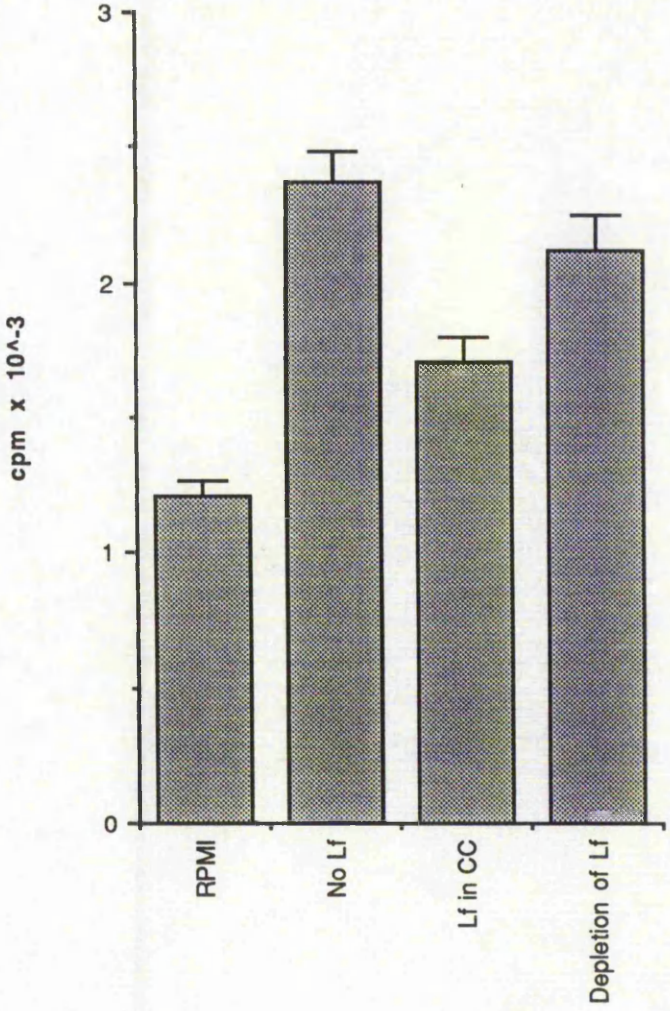

Figure 18. The effect of depleting Lf from crowded cell conditioned medium which was produced in the presence of 10^{-10} M Fe-Lf on the enhancement of spread cell proliferation.

Results represent means +/- SEM from three separate experiments.

tested against secondary spread cultures. Previous investigations had shown that freezing once did not affect the activity of the growth factor. Figure 19 shows the degree of proliferation in the crowded cultures in the upper panel, and the amount of growth factor present is shown in the lower panel. It can be seen that the production of growth factor precedes the proliferation in the crowded cultures with maximal levels being achieved by 72 hours. In addition, the inhibition of growth factor by Lf precedes the inhibition observed in the crowded cultures.

The MLC is considered to be a T cell mediated phenomenon driven by IL-2 (Ilonen and Karttunen 1984). The time course for the production of the growth factor was consistent with that of IL-2. Therefore, two approaches were adopted to investigate the possibility that Lf was functioning by inhibiting IL-2 production from T cells.

To determine whether Lf was affecting T cells, the mononuclear cells used for the MLC were either depleted or enriched for T cells by rosetting with sheep red blood cells (E-rosetting). The MNCs were obtained from 50 ml of peripheral blood, donated by three separate individuals, by centrifugation over LSM. Following washing the cells twice in RPMI they were counted and the concentration adjusted to 10^7 ml^{-1} in RPMI containing 20% FCS. Concurrently, sheep red blood cells (SRBC) were washed three times in RPMI. After the third wash, the medium was removed and the pellet resuspended in 10 times its volume in RPMI containing 20% FCS. 0.5 ml of the 10% SRBC suspension were added to 2 ml of MNCs. The cells were then centrifuged at 200 x g for 10 minutes. The

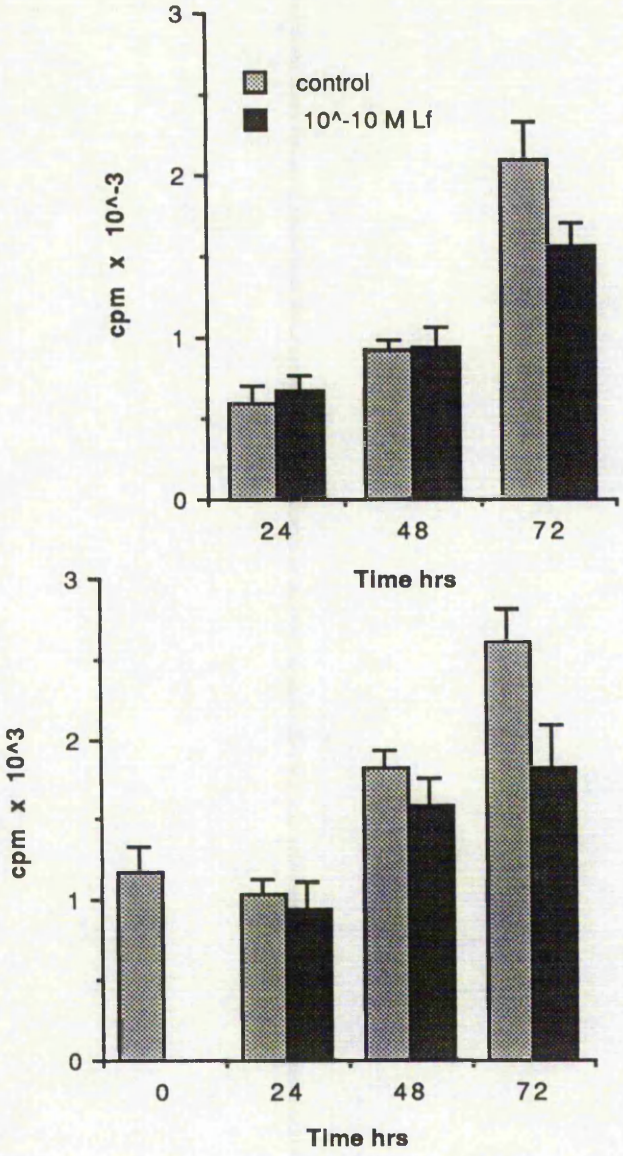

Figure 19. The proliferation of crowded MLCs over a 72 hour period and the effect of Fe-Lf on this (upper diagram). The lower panel shows the production of growth factor in these cultures as assessed by the effect of the crowded culture supernatants on spread cell proliferation.

Results represent means +/- SEM from six separate experiments.

pellet was then maintained at 4°C for 2 hours. The rosetted cells were separated from the non-rosetting cells by centrifugation over LSM at 400 x g for 20 minutes. The rosettes (T enriched, T^+) passed through the centrifugation medium and formed a pellet at the base of the tube; the non-rosetting cells (T depleted, T^-) were maintained above the LSM. The two populations were aspirated and washed twice in RPMI. The T depleted cells were maintained at 4°C until required, while the SRBCs were lysed from the rosette positive cells. This was achieved by resuspending the pellet in 0.8% w/v ammonium chloride and allowing the suspension to stand at room temperature for 10 minutes. The cells were washed twice more in RPMI before both the T depleted and T enriched populations were counted. This procedure routinely resulted in greater than 98% viablity for both T^+ and T^- populations as determined by Trypan blue exclusion. The T^+ and T^- populations from each donor were then adjusted to 1.1×10^6 ml^{-1} in cRPMI. MLCs of the T^+ cells only and the T^- cells only were then prepared by mixing equal volumes of the cell suspensions from each of the three donors. 180 ul of this MLC suspension was then added to the wells of RBW plates. 20 ul of Fe-Lf, giving a final concentration of 10^{-10} M, was then added to the test wells in triplicate, with 20ul of Tris/HCl buffer acting as control, also in triplicate. The T^+ and T^- MLCs were incubated under standard conditions for 72 hours, after which ^3H-UdR incorporation was determined.

Figure 20 shows the results of these experiments. It can be seen that considerably more proliferation occurred in the T^+ than in the T^- cultures. In addition, Lf was able to inhibit the T^+ MLCs but

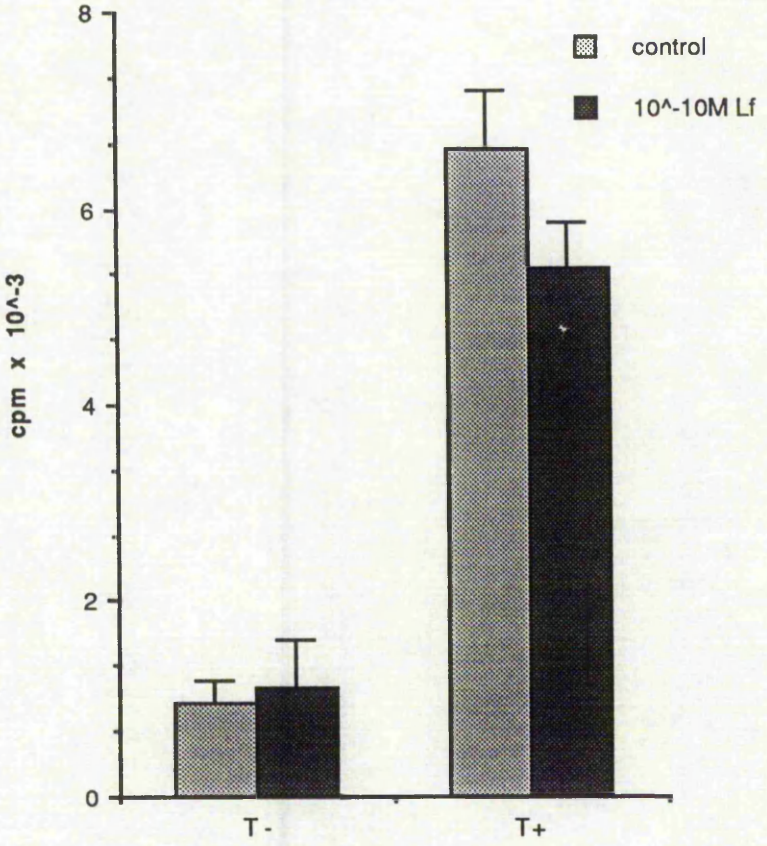

Figure 20. The effect of Fe-Lf on 3-way MLCs derived from T-cell depleted (T^-) and T-cell enriched (T^+) mononuclear cells in crowded conditions.

Results represent means +/- SEM from three separate experiments.

had no effect on the T^- cultures. Thus it appeared that the three-way MLC was indeed a T cell phenomenon, and that it was the T cells which were inhibited by Lf.

It is, however, very unlikely that highly purified T cells would proliferate in the absence of monocytes, which are required for antigen presentation and to produce IL-1. The presence of contaminating monocytes was therefore investigated in the T^+ population by nonspecific esterase staining (kindly performed by Ms.D. Bradbury, Haematology Dept., City Hospital.). These investigations revealed the presence of monocytes in the T^+ population at 5.5% of the total T cell numbers.

This data therefore suggests that growth factor produced in crowded cultures and effective against spread MLCs could be IL-2. The amount of IL-2 in crowded MLC supernatant was therefore determined using the method of Gillis et al. (1978). See section 2.7 for protocol.

Figure 21 shows a typical standard curve for IL-2. The inter-assay variation for this method was found to be 13.8% with a sensitivity down to 0.1 U ml^{-1}.

4.3. The Effects of Lf on IL-2 Production in Crowded MLCs

The IL-2 assay was used to measure IL-2 production in the MLC supernatants. Initial experiments investigated the time of production of IL-2 in MLCs. The cultures were grown crowded in RBWs and supernatants harvested at 24, 48 and 72 hours by resuspending the pellet in replicate wells followed by

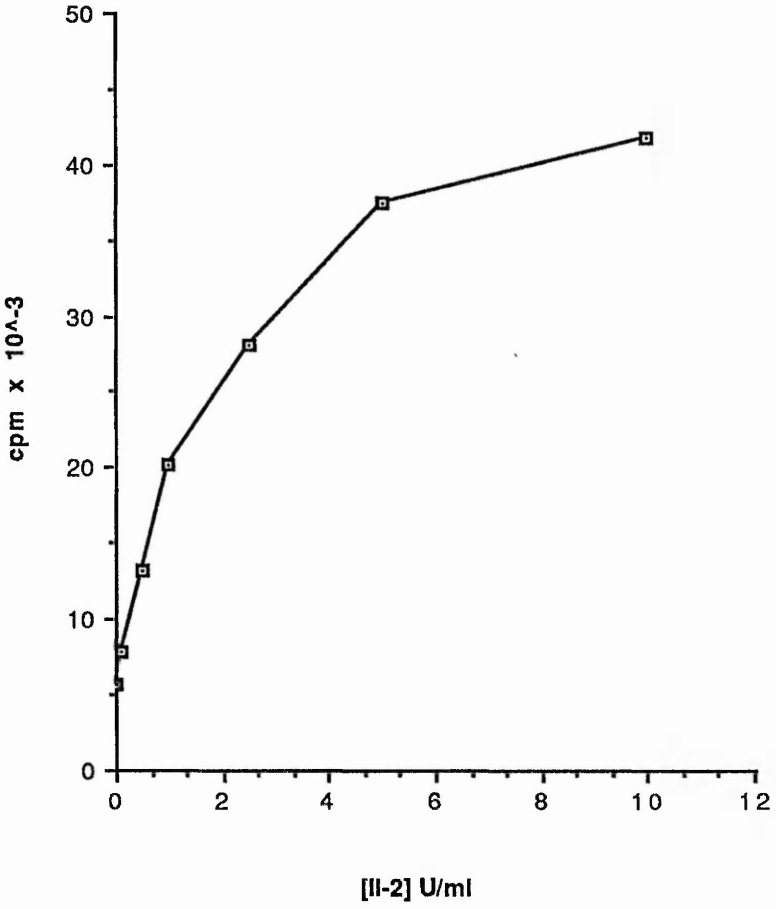

Figure 21. Typical standard curve for the proliferation of CTLL-2 in response to Il-2.

centrifugation at 400 x g for 10 minutes. The MLC supernatants were then frozen at -70°C until required. Figure 22 shows the results of these experiments. It can be seen that under these conditions maximal IL-2 production occurred at 72 hours, which is consistent with the data presented in Figure 19. IL-2 levels were therefore measured in 72 hour supernatants, which had been prepared whilst investigating the dose responses of Lf on the MLC. Figure 23 shows that Lf induced inhibition of proliferation in the MLCs and also suppressed IL-2 production in these cultures. It can be seen that the dose related inhibition of these two parameters was virtually identical.

To provide further evidence that IL-2 was the growth factor produced by crowded cultures and effective against spread cultures, the effect of purified human IL-2 on the latter was investigated. MLCs were prepared in FBWs as previously described. Serial dilutions of purified human IL-2 were then added to triplicate wells. 10^{-10} M Fe-Lf was also added to a second series of triplicate wells. The plates were incubated for 72 hours and the proliferation of the MLC determined by measuring ^{3}H-UdR incorporation. The results of these experiments are illustrated in Figure 24. Increasing concentrations of IL-2 stimulated the growth of cultures in a dose dependent manner, with maximum proliferation occuring at 10 U ml^{-1}. This concentration produced a five fold increase above controls containing no IL-2. It is noteworthy that Lf did not inhibit the effect of IL-2 on the spread MLCs.

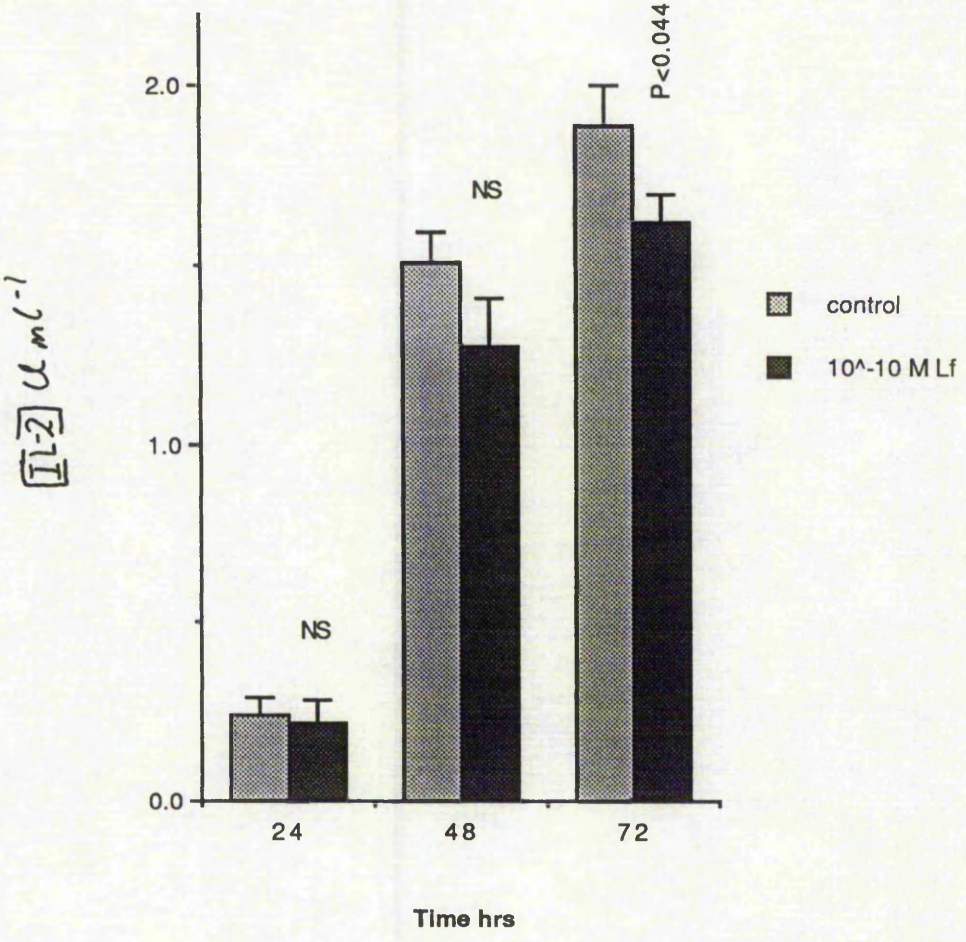

Figure 22. The production of Il-2 by crowded MLCs over a 72 hour period and the effect of Fe-Lf on this.

Results represent means +/- SEM from four separate experiments.

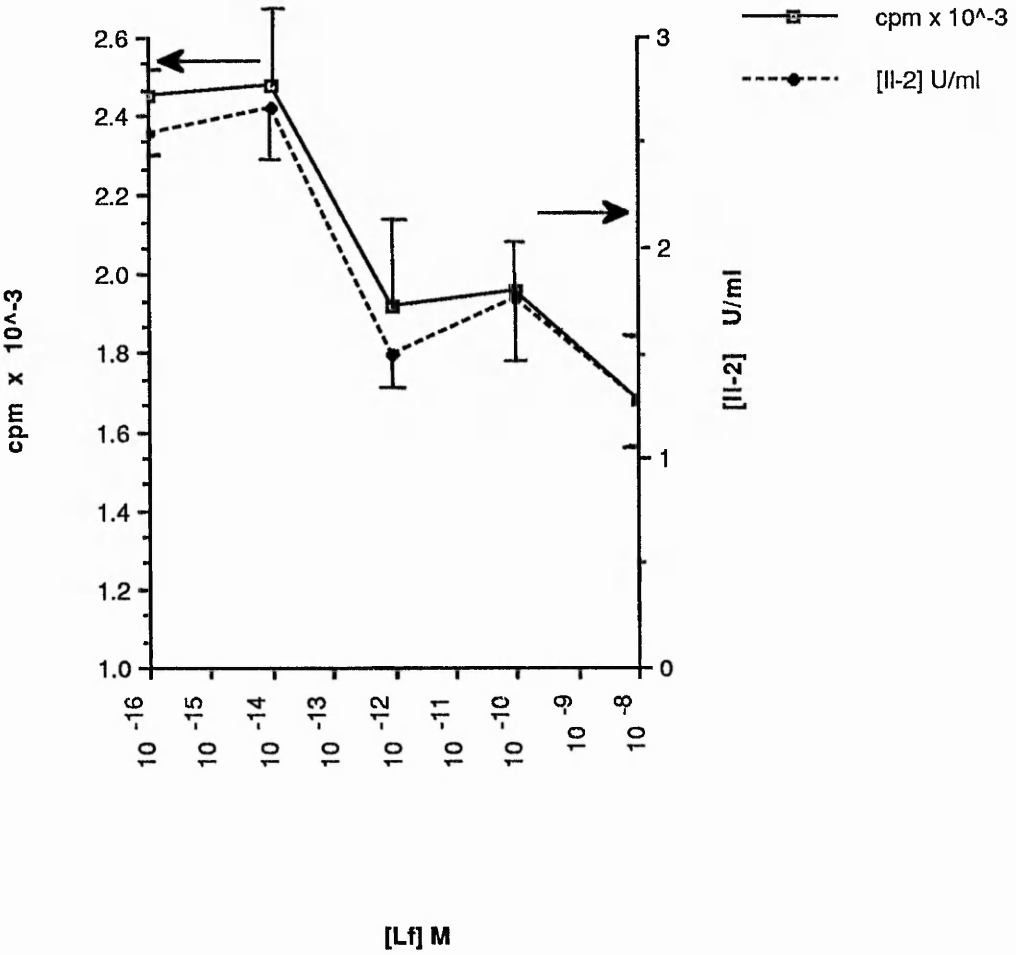

Figure 23. Comparison between the dose dependent inhibition of crowded MLCs and of Il-2 production in these cultures by Fe-Lf. The arrows indicate control cpm and Il-2 production in the absence of Lf.

Results represent means +/- SEM from six separate experiments.

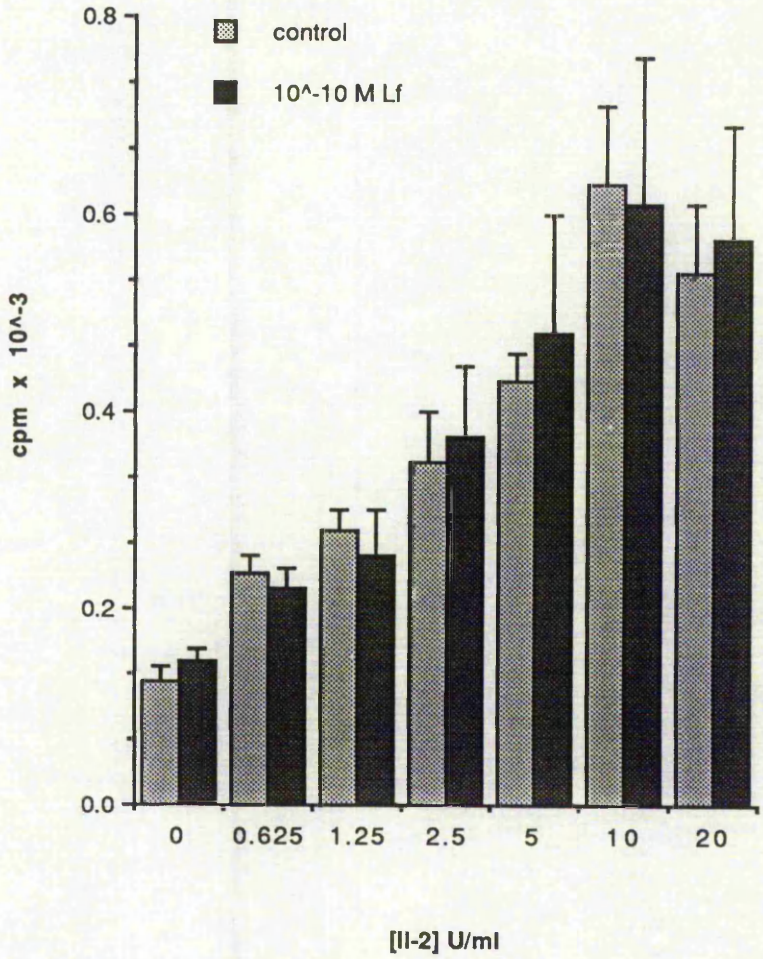

Figure 24. The effect of adding purified Il-2 and 10^{-10} Fe-Lf to spread MLCs.

Results represent means +/- SEM from three separate experiments.

4.4.1. The Effect of Lf on IL-1 Production in MLCs

Production of IL-2 by T cells is known to be dependent on the presence of IL-1 from monocytes (Durum et al. 1984). Therefore, the effect of Lf on IL-2 could be secondary to its affect on monocyte functions leading to production of IL-1. Indeed, work by Bagby et al. (1981) has indicated that the effect of Lf on GM-CSF production is secondary to its effect on a monokine. The amount of IL-1 in MLC supernatants produced in the presence and absence of Lf was therefore determined. This was technically difficult, however, since most assays for IL-1 are also sensitive to IL-2 which was shown to be present in MLC supernatants. The method of Gearing et al. (1987) provided a solution to the problem. By using a sub-clone of the cell line EL-4 called EL-4.NOB-1, it is possible to measure IL-1 in supernatants containing IL-2. This is achieved by preincubating the sample with EL4.NOB-1 for two hours, during which time the IL-1 binds to its receptor on the cell. Cells can then be washed in situ in the microtitre plate before incubation, thus removing any IL-2 that may be present in the sample. During the incubation period, the EL4.NOB-1 responds to IL-1 by producing IL-2, which can be measured using CTLL-2 as previously described.

Figure 25 shows a typical standard curve for the IL-1 assay. The sensitivity of the assay was found to be 0.1 pg ml^{-1} IL-1 beta. Addition of Fe-Lf at a final concentration of 10^{-10} M to the standards had no effect on the resultant standard curve. However, no IL-1 activity could be detected in any of the MLC supernatants harvested at 24, 48 and 72 hours.

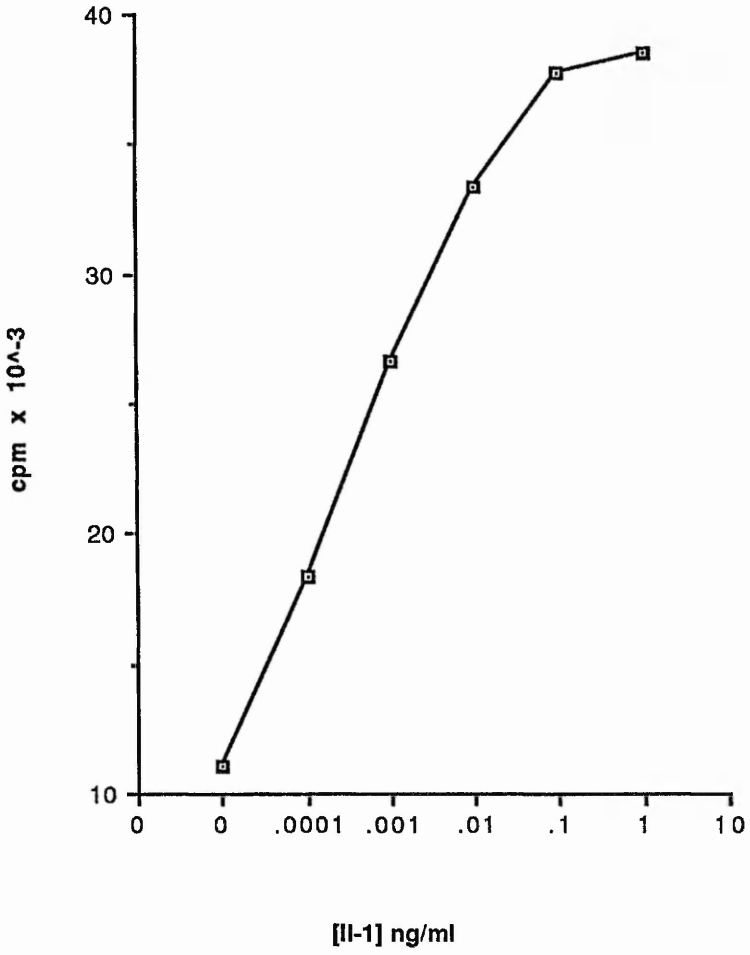

Figure 25. Typical standard curve for Il-1 as measured using EL-4.NOB-1 and CTLL-2.

4.4.2. The Effect of Lf on Production of IL-1 in Response to LPS

Stimulation of mononuclear cells by LPS is widely used to induce IL-1 secretion (Gearing *et al.* 1985). Optimal conditions require adhesion of the monocytes to plastic followed by stimulation with approximately 10 ug ml^{-1} LPS for 18 hours. These conditions were employed to investigate the effect of Lf on IL-1 secretion.

Peripheral blood mononuclear cells were isolated as previously described and suspended in cRPMI containing 10^{-6} M indomethacin at 1.25 x 10^{6} ml^{-1}. 800 ul of the suspension were transferred to the wells of 12 well tissue culture clusters. 100 ul of a 10^{-9} M solution of Fe-Lf were then added to half of the wells, the remaining half receiving 100 ul Tris/HCl buffer as a control. To all the wells 100 ul of 100 ug ml^{-1} LPS (*E. coli* strain 055:B4) were added. Thus, the final conditions were 10 ug ml^{-1} LPS stimulating mononuclear cells at 10^{6} ml^{-1} +/- 10^{-10} M Fe-Lf. The cells were incubated under standard conditions for 18 hours, after which the supernatants were harvested aseptically by centrifugation at 400 x g for 10 minutes. These were transferred to 1 ml cryotubes and stored at -70°C until required.

The supernatants were then assayed for IL-1 activity using EL-4.NOB-1 as described above. Figure 26 shows that IL-1 was secreted under these conditions and that its production was inhibited by 10^{-10} M Fe-Lf.

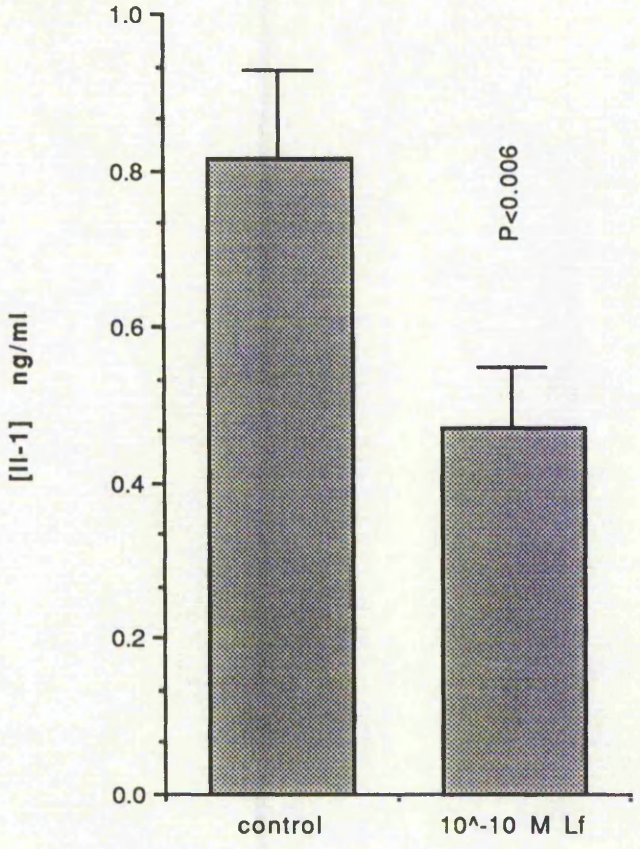

Figure 26. The effect of 10^{-10} M Fe-Lf on Il-1 secretion from single donor mononuclear cells stimulated by 10 ug ml^{-1} LPS.

Results represent means +/- SEM from three separate MNC donors.

Discussion

The requirement for close cell contact between the cells of the MLC to demonstrate inhibition by Lf has been clearly established. The work discussed in this chapter aimed to investigate the mechanisms involved in this phenomenon. Transfer of supernatants produced by crowded MLCs to those in spread conditions has revealed that a growth factor was produced by the former and was effective against the latter. In other experiments, whereby supernatants produced in FBWs were transferred to fresh MLCs also in spread conditions, no growth factor could be detected. Thus, it appeared that the additional proliferation of crowded MLCs was indeed due to production of a growth factor in response to close cell contact. The addition of Lf to crowded cultures used to produce the supernatants demonstrated that the inhibitory activity of the protein occurred via inhibition of this growth factor.

The obvious question arising from these data is: what interactions are occuring in the crowded cultures which lead to the production of this factor, and how is this process modulated by Lf? The enhanced stimulation in response to crowding must initially result from enforced contact between the cells. This does not occur in the flat bottomed wells due to the negative charge on the cells, causing them to repel each other.

The data illustrated in Figure 20 clearly implicated the T cells as being principally involved in this phenomenon. This is consistent with a large body of data demonstrating the central role of T cells to alloantigens in MLCs. However, it is now widely accepted that T cell proliferation does not occur in the absence of accessory cells

such as monocytes or B cells (Durum et al. 1984). The interaction with monocytes involves three signals: presentation of processed antigen, autologous MHC molecules and the production of IL-1. Thus, mononuclear cell responses to antigenic stimulation are MHC restricted. However, Durum showed that this requirement for MHC compatibility could be overcome by preincubating the T cells in an allogeneic MLC.

The direct interaction between the T cell receptor and MHC molecules on monocytes suggests the importance of cell contact in mononuclear cell responses. There are a number of reports which illustrate this. Weaver and Unanue (1986) demonstrated that murine T-helper cell clones were able to induce expression of membrane bound IL-1 on either Ia restricted, antigen specific mouse peritoneal macrophages, or on allogeneic macrophages. This occurred in two ways, either by direct cell contact or by release from the T cell of a novel lymphokine. A similar role for the accessory cell function of B cells has been demonstrated by Arnold et al. (1985). They showed that a B cell line provided an IL-1 like signal to T cells via direct cell contact, which induced IL-2 production in the responding mitogen treated T cells. They also found a similar function of the B cell line in stimulating MLCs.

It therefore appears that cell contact is an important requirement for some lymphocyte functions. This suggestion was recently supported by Poo et al. (1988), who demonstrated receptor directed lymphokine release by a helper T cell line. This polarised release of IL-4, they suggested, was due to localised receptor cross

linkage and functions by focussing the lymphokine on to the activating accessory cell, in this case a B cell.

In the light of these reports, the significance of crowding the MLC to obtain enhanced responses becomes apparent. By bringing the cells together, the number of interactions between HLA-DR molecules on the responding cells is increased; this in turn increases the strength of the stimulus and hence the response. That Lf is inhibitory only to the crowded MLCs becomes of particular interest in this context, when the work of Broxmeyer and Platzer (1984) is taken into consideration. In this report (which investigated the effect of Lf on GM-CSF production from mouse peritoneal macrophages) Broxmeyer demonstrated that Lf inhibits GM-CSF production from a population of Ia^+ cells; no effect was observed against Ia^-macrophages. Thus, it can be suggested that the cells initiating proliferation in the crowded MLCs will be $HLA\text{-}DR^+$, and according to Broxmeyer will respond to Lf. Broxmeyer showed that the inhibitory effect of Lf was lost at high macrophage concentrations, due to a concentration dependent production of PGE_2. This situation would not, however, apply to the crowded cultures since 10^{-6} M indomethacin was used throughout, which was shown by Broxmeyer to abrogate the cell concentration effect.

All the evidence indicates that the increase in stimulus strength by crowding cells resulted in increased production of a growth factor. A number of observations have implicated IL-2 as this growth factor:-

1. The time course for production of IL-2 by crowded MLC (Figure 22) is very similar to that for the production of the growth factor detected in the spread of MLC (Figure 19). In addition, both were inhibited by Lf.
2. Dose response curves for Lf induced inhibition of the MLC and for IL-2 production by these cultures were superimposable (Figure 23).
3. Spread MLCs proliferated in response to crowded MLC supernatants (Figure 15) and to purified human IL-2 (Figure 24).

Thus, it can be concluded that Lf inhibits IL-2 production in the MLC. This conclusion provides further circumstantial evidence that Lf is in some way involved with HLA-DR molecules if the the work of Ilonen and Karttunen (1984) is taken into consideration. They demonstrated that the degree of proliferation and of IL-2 production in human two-way MLCs correlated with the degree of HLA-D locus identity between the two individuals. Thus, the addition of Lf to MLCs may be considered to mask HLA-DR antigens on the accessory cells and hence reduce the proliferative response.

The demonstration that spread MLCs responded to exogenous IL-2 by proliferating indicated that the cells were not entirely quiescent. Resting T cells possess few, if any, IL-2 receptors, but following stimulation they acquire the Tac antigen, and with it IL-2 responsiveness (Robb et al. 1984). The fact that the spread MLCs responded to exogenous IL-2 could be explained by a report from Lakhanpal and Handwerger (1986). They found that normal adult

peripheral blood mononuclear cells, negative for IL-2 receptors, acquire the receptor and become responsive to the lymphokine after incubation in medium containing FCS. Thus the T cells in spread MLCs would resond to exogenous IL-2 due to FCS induced IL-2 receptor expression.

A consistent problem encoutered whilst attempting to investigate the inhibitory activity of Lf was whether the production or the effect of the growth factor was being influenced. Figures 17 and 18 clearly show that Lf inhibits both production of the growth factor in the crowded cultures and its effect on the spread cultures. However, when purified IL-2 was added to MLCs in FBWs, Lf did not influence the subsequent proliferation (Figure 24).

It is quite clear that Lf was inhibiting the production of IL-2 in the crowded MLCs from the data presented in Figure 23. Control experiments had shown that Lf did not affect proliferation of the CTLL-2 cells used to assay IL-2.

These results can be explained in one of two ways. The first explanation is based on the biochemistry of IL-2. It has been widely reported that both the molecular weight and the isoelectric point of purified IL-2 exhibit heterogeneity and this has been shown to be due to different degrees of glycosylation (Robb and Smith 1981). Although the various molecular forms of IL-2 have been shown not to differ in their biological activity (Welte and Mertelsmann 1985), it is possible that different degrees of glycosylation would render the protein susceptible to inactivation by Lf, by the latter binding to the former. Thus it could be

argued that some of the IL-2 molecules produced by the MLC would be sensitive to inactivation by Lf whilst others would be unaffected. By extension, the lack of inhibition of the effect of purified IL-2 could be due to this being glycosylated to a degree which is unaffected by Lf.

The alternative explanation is based upon the production of a second factor by crowded MLCs. In this case, IL-2 production would be inhibited by Lf but its effect would not, as suggested by Figures 23 and 24. Production of the second factor also capable of stimulating the spread cells may not be affected by Lf; however, its effect on MLCs in FBWs could be inhibited. A number of authors have demonstrated the production of a lymphokine distinct from IL-2 and active on cytotoxic T lymphocytes. Reddehase et al. (1982) found that IL-2 alone was insufficient to support the activation of CTL responses of murine splenocytes and that an additional factor produced by allogeneically stimulated Th cells was required. Conlon et al. (1982) showed that immature murine thymocytes required, in addition to IL-2, a factor produced by allogeneically stimulated splenocytes to develop cytotoxicity. Similarly, Wagner et al. (1982) demonstrated that IL-2 was insufficient to induce differentiation of primary CTL in vitro. They named the required second signal cytotoxic T cell differentiation factor (CTDF). They suggested that IL-2 is required to expand the CTL precursor clone and that CTDF is then involved in the differentiation to the cytolytic effector cells. This, they suggest, is analogous to the proliferation and differentiation signals required for the development of the mature antibody producing B cells.

Although these reports clearly illustrate the involvement of factors other than IL-2 in T cell responses, they are all involved in the differentiation of cytotoxic precursor cells. Is this T cell function being measured in the augmentation of uridine uptake into spread MLCs by crowded cell conditioned medium? The fact that uridine would still be incorporated into differentiating cells due to protein synthesis does not rule out the possibility of the involvement of a differentiation factor. However, the possibility of this factor also inducing proliferation cannot be excluded, since Olabuenaga et al. (1983) found that an NK-like cell line, B10G7, would proliferate in the presence of stimulated murine spleen cell supernatants, but not with purified IL-2 alone. Therefore it is possible that proliferation is also occuring.

The inactivation of a growth factor by Lf binding to its active sites is a strong possibility due to the high cationic nature of the iron binding protein. Indeed, this has been shown by Ashorn et al. (1986) who demonstrated that Con A stimulated lymphocyte proliferation was inhibited by Lf, and that this inhibition was due to Lf binding to and deactivating the lectin. However, as often occurs when considering Lf, data exists to the contrary. Broxmeyer et al. (1980) were unable to demonstrate inhibition of GM-CSF production from Con A stimulated human lymphocytes by Lf. The data in Figure 2 is in agreement with the former but not the latter group. This disparity could be explained by the use of a different source of Con A, since the degree of sialation between lectin preparations is known to vary. Differences in the sialic acid

residues on Con A could affect the binding of Lf, and consequent inactivation of the stimulus in an analogous way to that proposed for the inhibition of IL-2 molecules differing in their degree of glycosylation.

From the available data it is not possible to distinguish between the two proposed explanations discussed above. However, it is likely that both apply. Experiments involving neutralisation of IL-2 in crowded culture supernatants by specific antibodies are required to demonstrate the involvement of a second factor. Also, the possibility of Lf binding to IL-2 could be investigated by measuring the effect of IL-2 preparations on spread MLCs after passage of the IL-2 down a column of immobilised Lf.

The finding that no IL-1 activity could be detected in the MLC supernatants was unexpected, since the central role of IL-1 in IL-2 production has been well documented (Smith et al. 1980; Larsson et al. 1980). However, under conditions reported to induce IL-1 secretion, namely after stimulation with LPS for 18 hours (Gearing et al. 1985), IL-1 activity was detected and it can be seen from Figure 26 that this was partially inhibited by Fe-Lf.

The weight of evidence for the involvement of IL-1 in IL-2 production as observed in the MLC is such that an explanation for the lack of IL-1 in MLC supernatants is required. This has emerged from the work of a number of investigators over recent years. Kurt-Jones et al. (1985) showed that T cell responses to paraformaldehyde fixed murine peritoneal macrophages were independent of IL-1 secretion. They demonstrated that the response

of T cells was due to membrane-bound IL-1. In a similar study on human cells, Goeken and Staggs (1987) demonstrated that fixed human macrophages secreted IL-1 in response to either mumps or tetanus toxoid; however, after washing away the IL-1 the cells were still able to stimulate T cell proliferation. By employing FACs analysis they demonstrated that the T cell response was due to membrane-bound IL-1. Bakouche et al. (1987) demonstrated the presence of membrane IL-1 on LPS stimulated human monocytes in addition to secretion of the monokine. They suggested that the membrane-bound form is involved in direct contact with T cells inducing expression of high affinity IL-2 receptors and that the secreted IL-1 functions as a true monokine. Conlon et al. (1987) suggested that membrane IL-1 was IL-1 alpha and the secreted form was IL-1 beta.

Thus a large body of evidence exists to demonstrate the importance of membrane-bound IL-1 in T cell activation. It is therefore proposed that the inactivation of IL-2 secretion in the MLCs is due to suppression of the membrane IL-1 on the monocyte. This would further explain the requirement of cell crowding to obtain maximum proliferation of the cultures. The observation that Lf inhibited the crowded MLCs only could therefore be due to the iron binding protein inhibiting IL-1 expression which could be detected by the enforced cell contact in the RBWs.

Summary

Proliferation in the three-way MLC was found to be much greater when the cells were cultured in RBWs than in FBWs. The enhanced growth in crowded conditions could be inhibited by Lf; no effect

was observed in spread conditions. This phenomenon was found to be due to the production of a growth factor by the crowded MLCs, and it was this substance that was inhibited by Lf. The growth factor was subsequently identified as IL-2. Secretion of IL-2 is dependent upon the presence of IL-1; however, this monokine could not be detected in MLC supernatants. Stimulation of mononuclear cells with LPS induced IL-1 secretion, and Lf was found to be inhibitory under these conditions. These observations suggest that membrane-bound IL-1 was inducing IL-2 production in the MLCs, which could explain the requirement for crowding to obtain a maximal response. Inhibition of IL-1 expression on the surface of monocytes by Lf could explain the observed reduction in IL-2 production and consequent proliferation as demonstrated.

CHAPTER FIVE

THE EFFECT OF LACTOFERRIN ON A MONONUCLEAR CELL DERIVED NEUTROPHIL PRIMING FACTOR

The demonstration that Lf inhibits lymphocyte proliferation *in vitro* presents a number of problems when attempting to assign an *in vivo* role to this phenomenon. Of course, the involvement of IL-1 in colony stimulating factor production is well documented, and one explanation has already been suggested by Bagby *et al.* (1983) for the inhibition of granulopoiesis by Lf via a monokine, probably IL-1. However, a number of hypotheses arising from the preceding work have implicated a role for Lf in the control of localised inflammation. The suggestion that Lf is ineffective as an inhibitor in plasma (page 111) would rule out such a role for the protein in the circulation. The requirement for crowding the MLC to obtain an effect with Lf and the proposed involvement of MHC molecules in the inhibitory functions of Lf (Broxmeyer and Platzer 1984) indicate a role in inflammatory lesions where both mononuclear and polymorphonuclear leucocytes are packed together. One of the principal initiating signals in inflammation is thought to be endotoxin. This functions as a chemotaxinigen (Colditz and Movat 1984) by stimulating the localised production of inflammatory mediators which ultimately result in the migration of leucocytes to the site of infection. The finding that Lf inhibits IL-1 production in response to LPS further supports the hypothesis that Lf functions as a regulator of localised inflammation.

Cybulsky *et al.* (1987) found that IL-1 was a potent chemotactic

agent for PMN when injected into rabbits, and suggested that IL-1 mediates endotoxin-induced neutrophil emigration. However, Yoshimura et al. (1987) found that both highly purified and recombinant IL-1 was not chemotactic for PMN in vitro. They were able to demonstrate that LPS stimulated mononuclear cells to release a neutrophil chemotactic factor (or factors) which may include TNF. Ming et al. (1987) showed that TNF was chemotactic for PMN in vitro.

TNF has been shown to be involved in the priming of neutrophils by enhancing their response to subsequent stimulation (Shalaby et al. 1985). Could the production of this or other factors produced by macrophages, which are involved in the initiation of inflammation, be influenced by Lf? The inhibition of a PMN chemotactic factor or a substance which enhances neutrophil function by a protein, released from phagocytosing neutrophils at an inflammatory locus, could have significant implications. Thus, as neutrophil numbers accumulate and release Lf, a negative feedback mechanism would be initiated to prevent excessive accumulation and activation of PMNs, which due to their highly toxic nature could become destructive to the host.

This chapter describes experiments which attempted to investigate this hypothesis. Sullivan et al. (1988) have demonstrated that monocytes stimulated by LPS rapidly release a factor which dramatically enhances PMN superoxide production in response to fMLP. This phenomenon was consequently investigated by employing lucigenin enhanced chemiluminescence as an indicator of PMN superoxide production.

5.1. Demonstration of a PMN Priming Factor Produced by LPS Stimulated Mononuclear Cells

MNCs were prepared as described in Chapter 2 using MPRM. The concentration of the cells was adjusted to $2x10^6$ ml^{-1} in cRPMI and 800 ul of the suspension added to 12 well tissue culture clusters. 100 ul of LPS and 100 ul of Lf or TRIS/HCl were added. The cells were then incubated under standard conditions for various times. At the end of the culture period, the supernatants were harvested by aspirating the contents of the wells and removing the cells by centrifugation at 400 x g for 10 minutes. The mononuclear cell conditioned medium (MNC-CM) was transferred to polypropylene vials in 900 ul aliquots and stored at -70^oC until required. This was usually within one month of production. Supernatants were used immediately after thawing and were not refrozen.

These supernatants were then tested for their priming effects against PMNs prepared as described in Chapter 2. The PMNs were adjusted to 10^7 ml^{-1} in RPMI. 100 ul of the suspension were added to 900 ul of MNC-CM which was maintained on ice. As a control, PMN were added to 900 ul of fresh cRPMI on ice. The cells were incubated at 4^oC for 15 minutes, after which they were washed by centrifugation at 400 x g for 5 minutes. The medium was removed and the PMN resuspended in cold PBS containing 1 mg ml^{-1} BSA. The cells were centrifuged a second time, after which the PBS was replaced with RPMI containg 1 mg ml^{-1} BSA (RPMI-A). 400 ul of the PMN suspension were transferred to luminometer cuvettes in duplicate. 50 ul of lucigenin, diluted in PBS to 2.5×10^{-4} M, had previously been added to the cuvettes. These were then

transferred to the carousel of an LKB 1251 automated luminometer which had previously been heated to 37°C. The suspensions were allowed to equilibrate to 37°C for approximately 2 minutes before 50 ul of stimulus were added. The stimulus chosen was zymosan activated serum (ZAS). This was used immediately after thawing and was diluted 1:2 in RPMI-A to give a final concentration of 5% in the cuvette.

Figure 27 shows a typical chemiluminescence curve for PMN stimulated by ZAS following incubation in MNC-CM, and compares this with controls incubated with cRPMI. It can be seen that considerably more chemiluminescence occurred with the primed PMN. The reaction was quantified by measuring the rate of chemiluminescence over the steepest section of the curve. This was routinely over the first 28 minutes following addition of stimulus. Figure 28 shows the results of a number of experiments expressed as the rate of chemiluminescence following priming of the PMN.

Investigations were conducted by culturing the mononuclear cells for 18 hours before harvesting the supernatants. To determine the optimal time for the production of this PMN priming factor (PMN-PF), MNCs were incubated with 10 ug ml^{-1} LPS for various lengths of time. The supernatants were then tested for their ability to enhance PMN chemiluminescence. Figure 29 shows the results of these experiments.

Maximal production occurred between 5 and 6 hours, after which time the PMN-PF begins to decline. A 5 hour incubation was therefore used for all subsequent investigations.

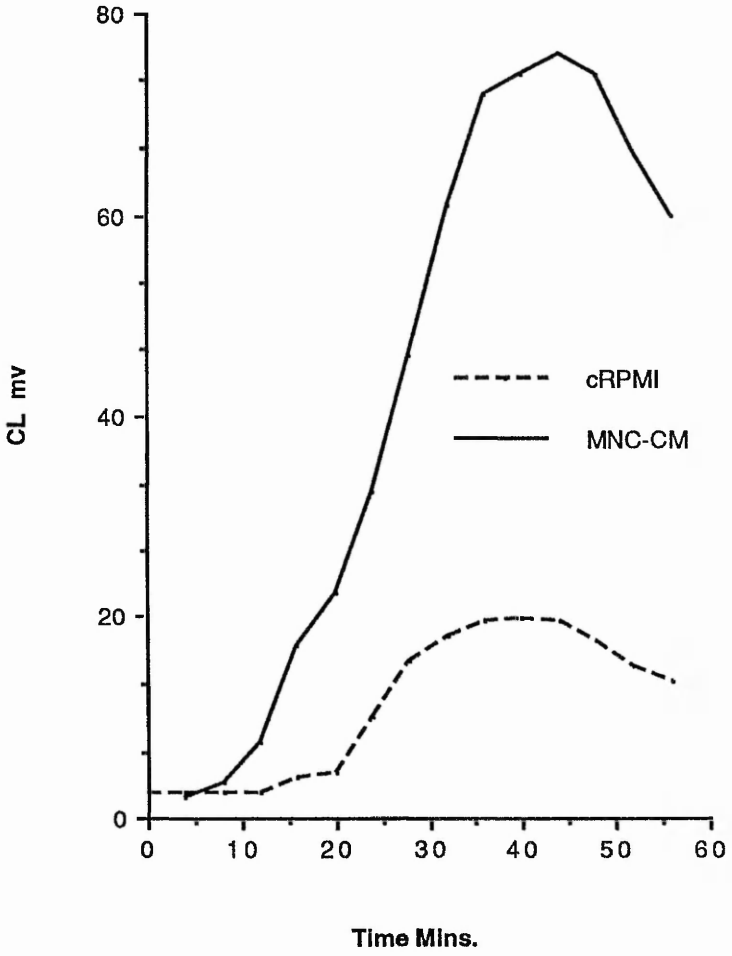

Figure 27. The effect of preincubation of neutrophils in mononuclear cell conditioned medium (MNC-CM) on ZAS stimulated chemiluminescence.

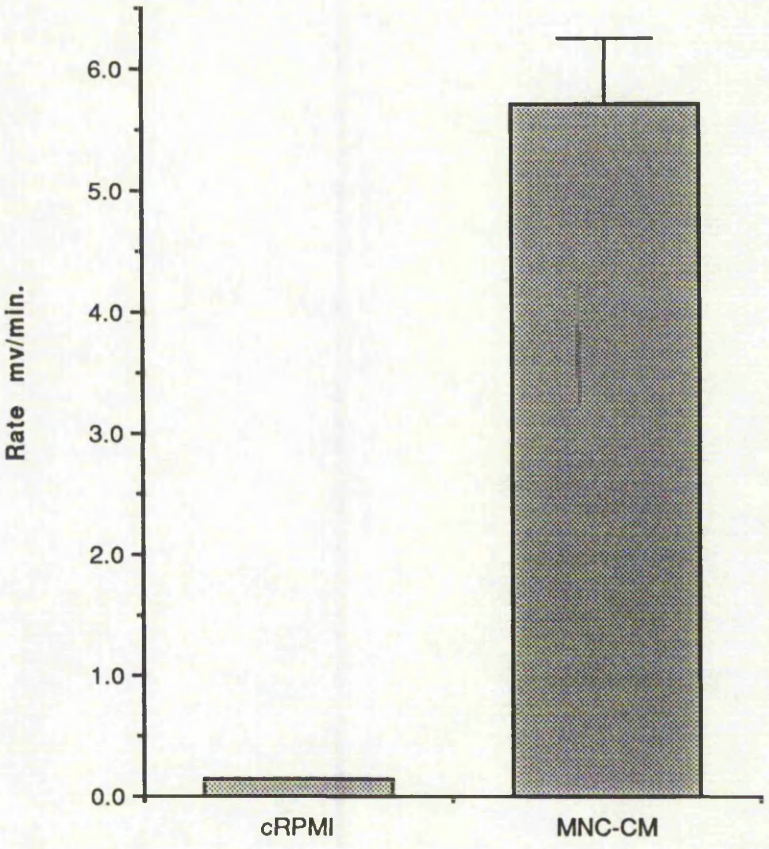

Figure 28. The effect of MNC-CM on the rate of ZAS stimulated chemiluminescence over the first 28 minutes following addition of stimulus.

Results are means +/- SEM from 6 separate experiments using a different MNC and PMN donor for each.

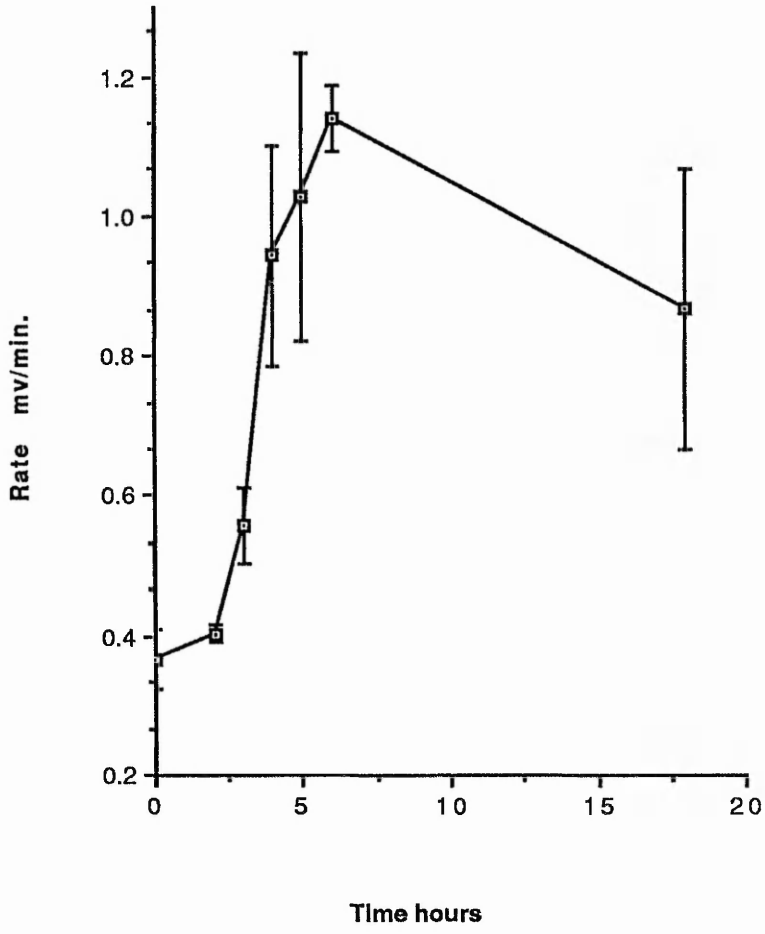

Figure 29. Time course for the production of PMN-PF by MNCs stimulated by 10 ug ml^{-1} LPS.

Results are means +/- SEM from 3 separate experiments with a different MNC and PMN donor for each.

The time required for the priming to occur was next investigated. A pool of 5 hour MNC-CM stimulated by 10 ug ml^{-1} LPS was prepared from five donors, aliquoted and stored at $-70^{o}C$ until required. This was used to incubate neutrophils at $4^{o}C$ for various time intervals. Figure 30 shows that this phenomenon occurred very rapidly with no improvement of priming after 15 minutes. This 15 minute time period was therefore chosen as the standard time. Thus all subsequent experiments were conducted by stimulating mononuclear cells for 5 hours before harvesting the supernatant and using the supernatant to prime neutrophils for 15 minutes at $4^{o}C$, after which they were washed in cold PBS-A before being resuspended in RPMI-A. The cells were then assessed for their chemiluminescence in response to specific stimuli.

Having established the optimal times for the production of PMN-PF and for its effect on neutrophils,the dose dependent stimulation of MNCs by LPS was next investigated. MNCs were prepared as previously described and stimulated by LPS over a concentration range from 1 ng ml^{-1} to 10 ug ml^{-1}. Figure 31 illustrates the dose response curve for LPS to produce PMN-PF. Maximal secretion occurred with the highest endotoxin concentrations. The PMN controls for these experiments consisted of cRPMI containing equal concentrations of LPS as those used to stimulate the MNCs. This revealed that LPS alone was able to prime PMNs. Figure 31 illustrates, however, that the MNC-CM is considerably more effective at priming the neutrophils, thus demonstrating that a factor is indeed released by MNCs in response to LPS. Figure 32 represents the data in Figure 31 expressed as % stimulation over the appropriate control, and

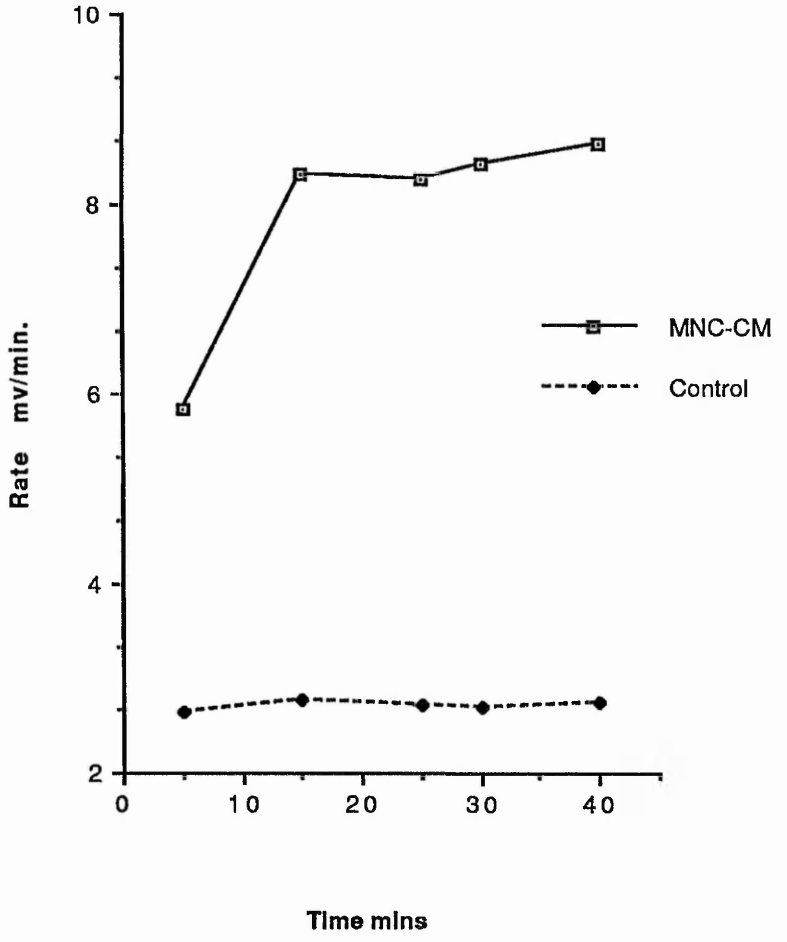

Figure 30. Time course for the priming of neutrophils by MNC-CM at 4°C.

Result of 1 representative experiments from 4, using different PMN donors and a constant pool of MNC-CM derived from 5 MNC donors.

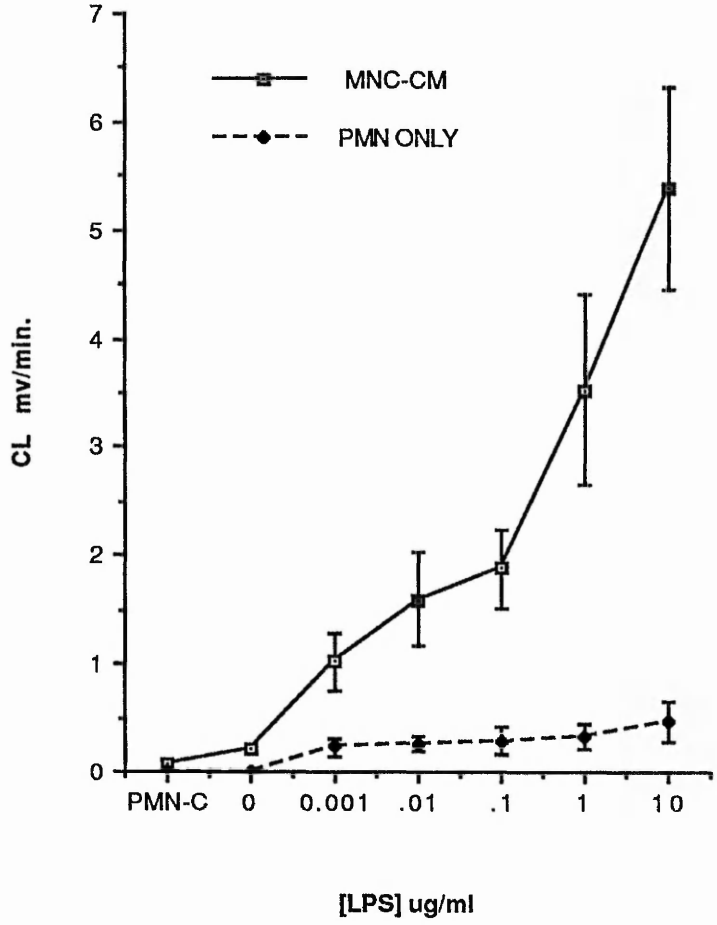

Figure 31. LPS dose response for the production of PMN-PF from 5 hour mononuclear cell cultures compared with the direct priming effect of LPS on ZAS stimulated chemiluminescence.

Results represent means of 4 separate experiments +/- SEM with a different MNC and PMN donor for each.

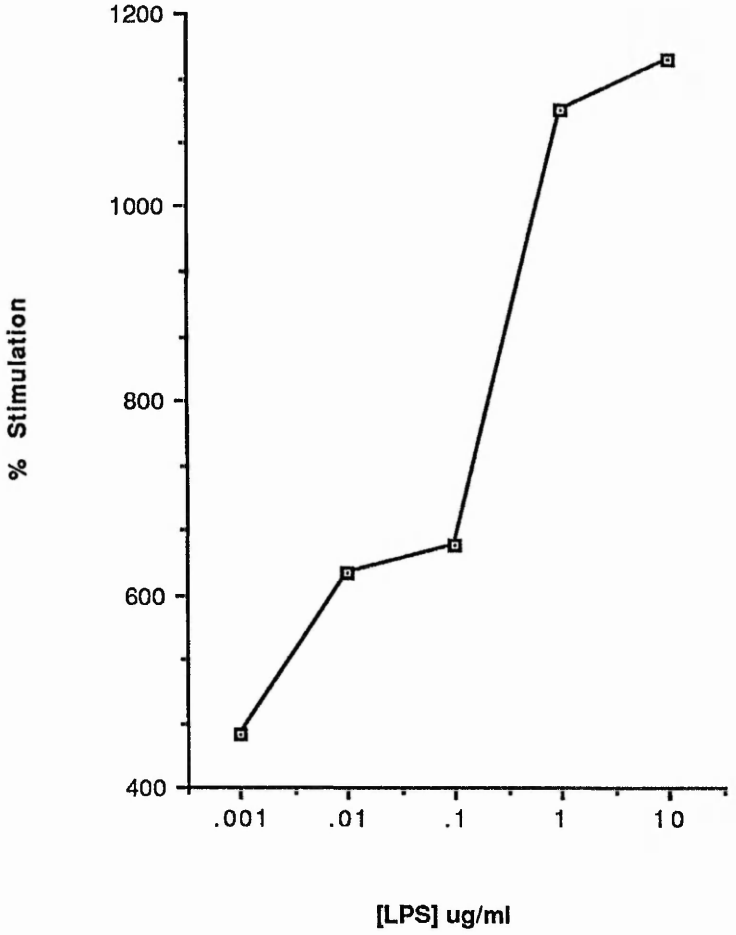

Figure 32. The data from figure 31 expressed as % stimulation of MNC-CM over LPS only controls.

therefore can be considered as a measure of PMN-PF production. It can be seen that maximal secretion occurred at 10 ug ml^{-1} LPS.

5.2. Preliminary characterisation of PMN-PF

The time course for the production of PMN-PF was consistent with that of TNF alpha (Burchett et al. 1988). In addition, a number of reports have demonstrated that pretreatment of neutrophils with TNF is able to enhance their cytotoxic activities (Shalaby et al. 1985; Klebanoff et al. 1986). The priming experiments were therefore repeated using recombinant human TNF alpha (rhTNF-alpha) (a generous gift from Dr Meager, NIBSC, England) in place of MNC-CM. Neutrophils were preincubated at $4^{o}C$ with a range of rhTNF concentrations in cRPMI and treated exactly as described above for the detection of PMN-PF. Figure 33 illustrates the dose response curve for the priming of neutrophils by TNF. At concentrations of TNF greater than 10 U ml^{-1} the ZAS stimulated chemiluminescence was increased.

Klebanoff et al. (1986) demonstrated that TNF dramatically enhanced neutrophils' responses to opsonised Zymosan, and by using monoclonal antibodies to the C3bi receptor, showed that this was due to an increase in receptor expression following exposure of cells to the monokine. The priming experiments were therefore repeated using serum treated Zymosan (STZ) as the neutrophil stimulus in place of ZAS. Figure 34 shows that MNC-CM was able to enhance the rate of chemiluminescence following stimulation by 2 mg ml^{-1} STZ. Figure 35 illustrates that rhTNF increased the rate of STZ induced chemiluminescence in a dose dependent manner. It can be seen from this figure that neutrophil responses to STZ following

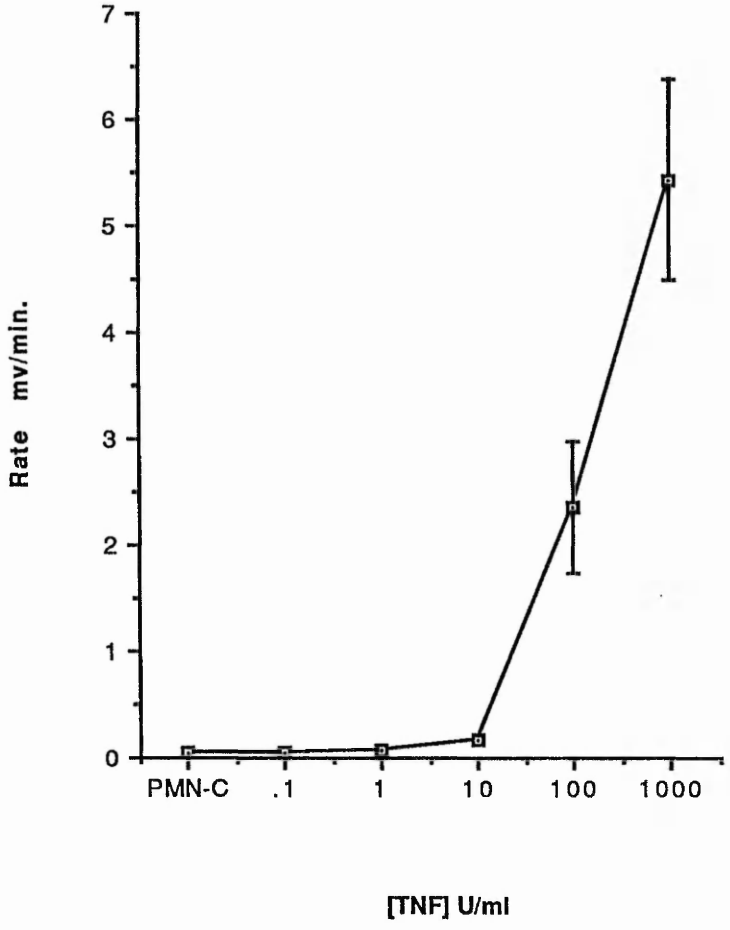

Figure 33. Dose response for the priming of neutrophils with TNF on the rate of ZAS stimulated chemiluminescence.

Results represent means +/- SEM from 3 separate experiments with a different PMN donor for each.

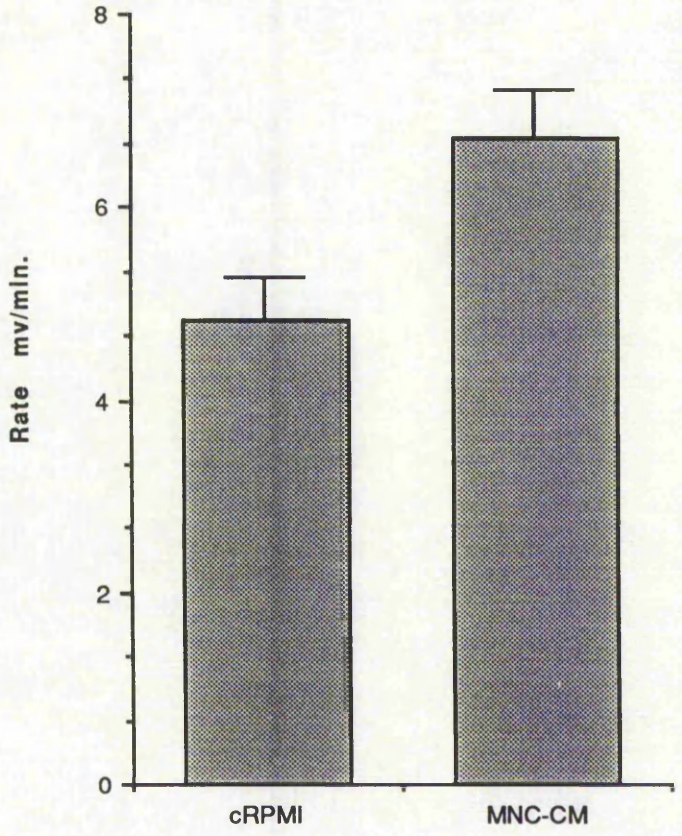

Figure 34. The effect priming neutrophils with MNC-CM on the rate of chemiluminescence following stimulation with 2 mg ml^{-1}STZ.

Results represent means of 5 separate experiment +/- SEM using a different PMN donor for each and a constant pool of MNC-CM derived from 5 MNC donors.

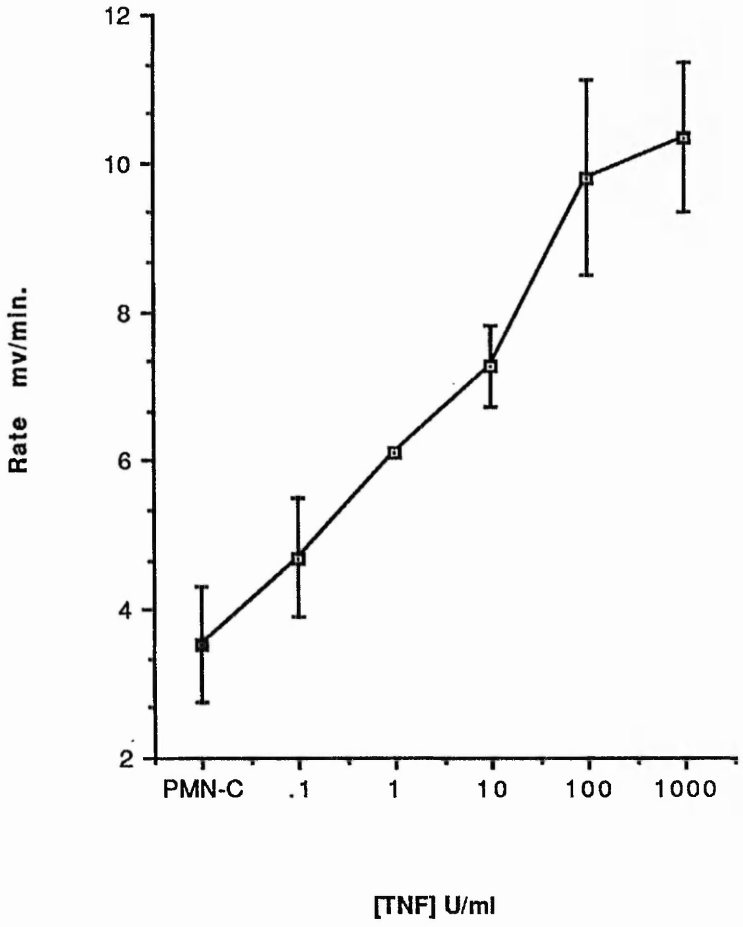

Figure 35. Dose response for the priming of neutrophils with rh TNF on the rate of STZ stimulated chemiluminescence.

Results represent means +/- SEM of 3 separate experiments with a different PMN donor for each.

priming with TNF were sensitive down to 0.1 U ml^{-1} of the monokine. These results are consistent with the data published by Klebanoff. However, the use of STZ as a stimulus to investigate neutrophil priming by MNC-CM was discontinued due to the complex nature of the resultant chemiluminescence curves (Figure 36). A biphasic curve was produced with controls and at low TNF concentrations. This was difficult to quantitate and the control values were high (Figure 34). Therefore ZAS, giving negligible control chemiluminescence, was chosen to continue the investigations.

The direct effect of rhTNF on PMN chemiluminescence in the absence of subsequent stimulation was next investigated. PMN were preincubated with the monokine exactly as described above; following washing, the cells were transferred to the luminometer and chemiluminescence assessed. Figure 37 compares the data from these experiments to that illustrated in Figure 33. The magnitude of the response can be seen to be much lower in the absence of ZAS. Therefore, TNF was shown to have a direct effect on PMN which is enhanced by stimulation with ZAS.

To clarify this observation, the experiments were repeated using MNC-CM to prime the neutrophils, the direct effect of PMN-PF was compared with ZAS stimulation following priming. Figure 38 shows that MNC-CM directly stimulated the PMNs after washing; however, the response was considerably augmented by ZAS.

To identify the cell type responsible for the PMN-PF, MNCs were separated into plastic adherent and non-adherent populations. A suspension of MNCs was prepared in cRPMI and added to 90 mm petri

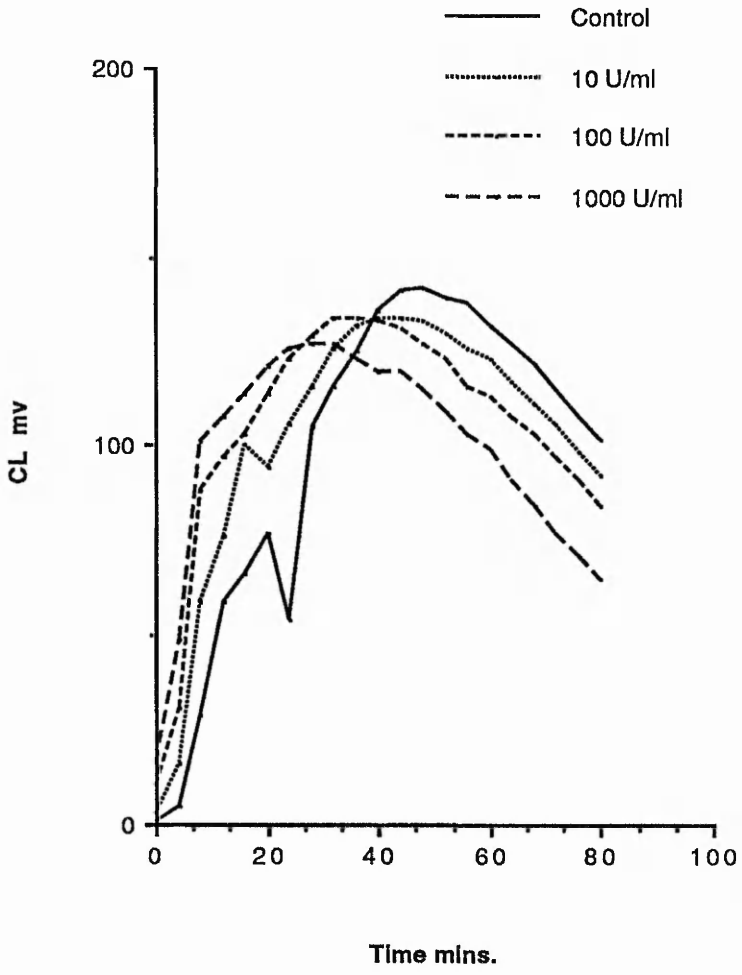

Figure 36. Chemiluminescence curves produced by STZ stimulated PMN following priming with rhTNF.

Results from 1 representative experiment out of 3, using different PMN donors.

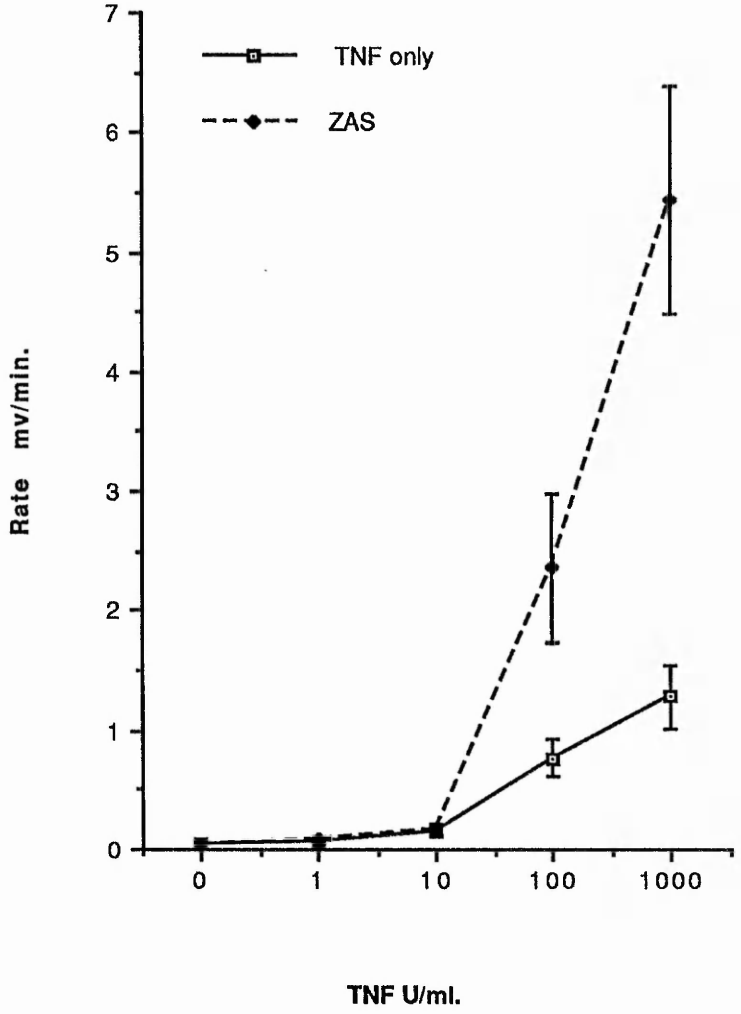

Figure 37. Resultant chemiluminescence following priming of neutrophils with rhTNF in the presence and absence of subsequent stimulation by ZAS.

Results represent means +/- SEM from 3 separate experiments with a different PMN donor for each.

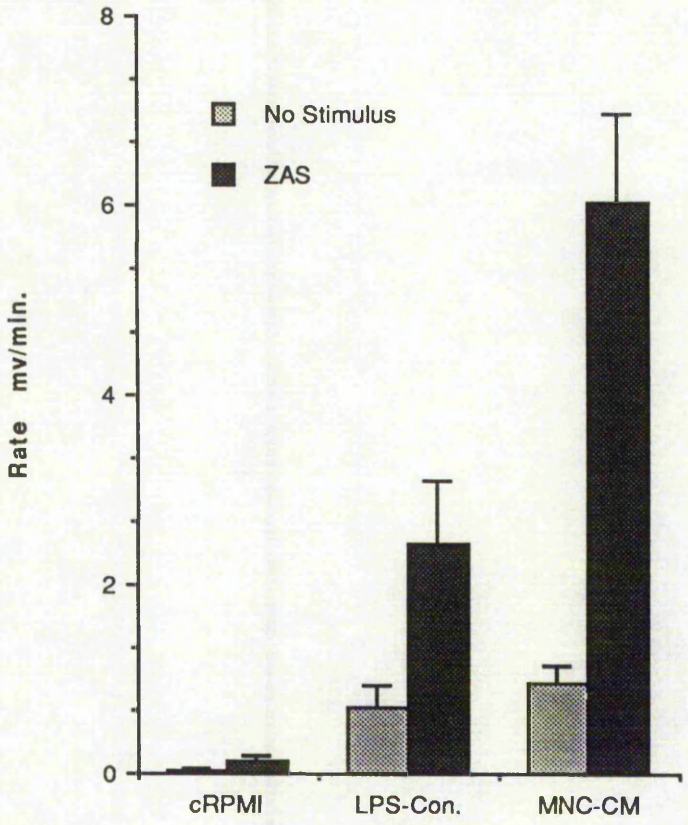

Figure 38. Resultant chemiluminescence following priming of neutrophils with MNC-CM with and without subsequent stimulation.
LPS-Con = Fresh cRPMI containing 10 ug ml^{-1} LPS.
Results represent means +/- SEM from 6 separate experiments with a different MNC and PMN donor for each.

dishes. These were incubated at 37°C for two hours. The non-adherent cells were aspirated, while the adherent monolayer was washed three times in RPMI at 37°C. The adherent cells were then harvested by scraping the dish with a rubber policeman. Both populations were washed once by centrifugation at 400 x g for 10 minutes and resuspended at 10^6 ml^{-1} in cRPMI. A cytospin preparation was made of each population for subsequent assessment of non-specific esterase (NSE) activity. They were transferred to the wells of 12 well tissue culture clusters and incubated for 5 hours with 10 ug ml^{-1} LPS. The supernatants were harvested and stored as previously described. Figure 39 shows the effect of the supernatants on priming of PMN. The adherent cells (92.8% NSE +ve.) produced considerably more chemiluminescence than the non-adherent (2.2% NSE +ve.). Thus it appeared that the monocyte was producing PMN-PF.

5.3 The effect of Lactoferrin on PMN-PF production

Having determined the optimal conditions for the production and detection of PMN-PF and obtained evidence which indicated that the factor was TNF, the effect Lf on the secretion of this priming factor by MNCs was next investigated.

Since Broxmeyer et al. (1978) demonstrated that the inhibition of GM-CSF production by Lf was dependent upon the relative concentration of LPS and Lf, the initial experiments involved different stimulus and inhibitor concentrations. The example illustrated in Figure 40 shows that PMN-PF production was inhibited at every LF concentration; however, unlike Broxmeyer's observation, maximal inhibition by Lf occurred at the highest LPS concentration.

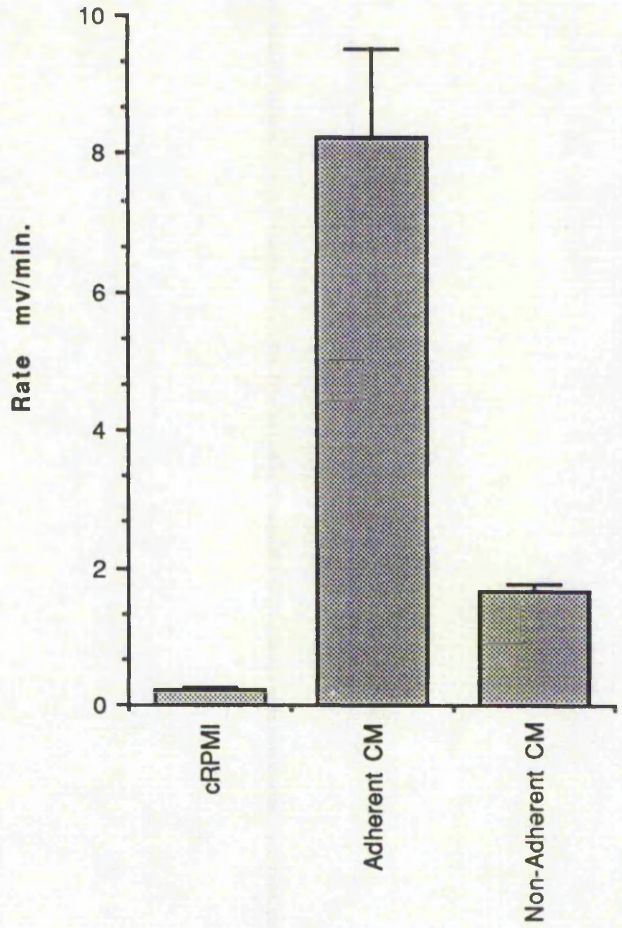

Figure 39. Production of PMN-PF by adherent and non-adherent mononuclear cells stimulated with 10 ug ul^{-1} LPS for 5 hours.

Results represent means +/- SEM from 3 separate experiments with a different MNC and PMN donor for each.

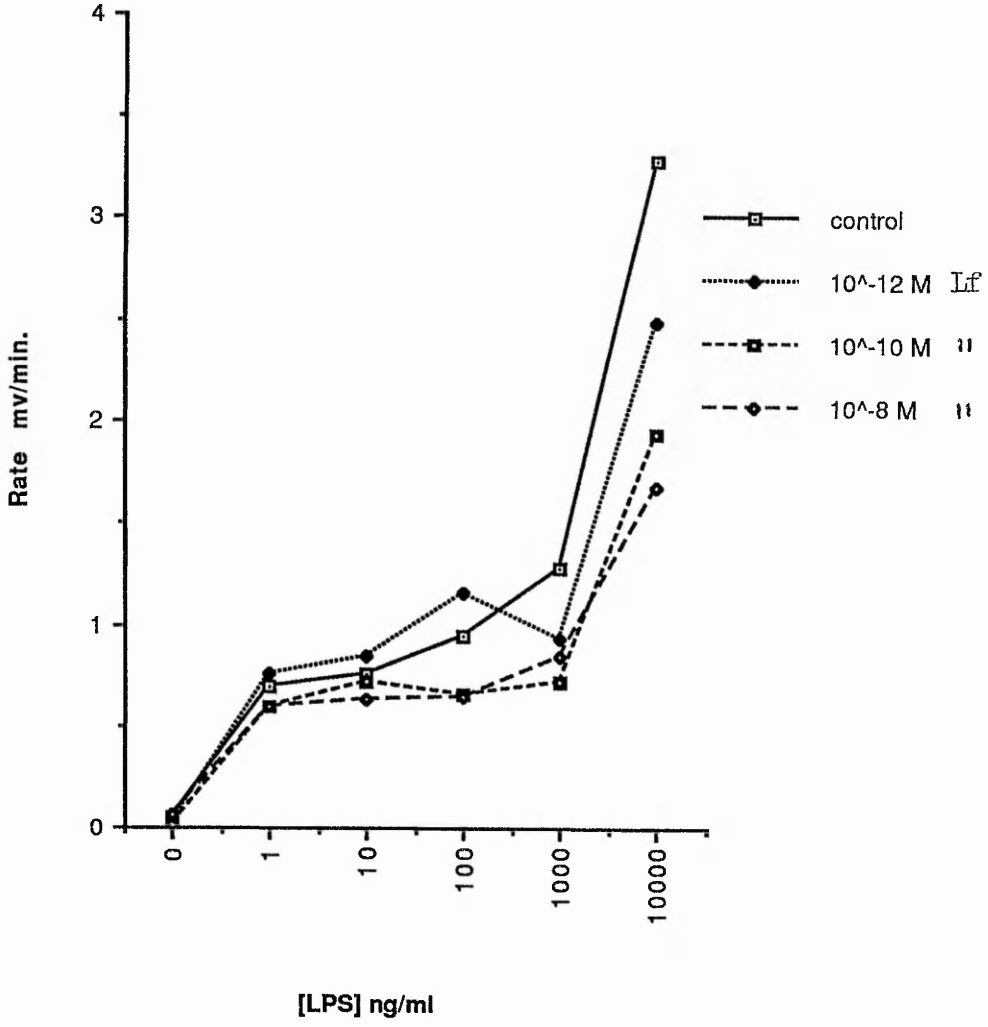

Figure 40. The effect of different Fe-Lf and LPS concentrations on PMN-PF production by cultured mononuclear cells.

Results are from 1 experiment.

Control experiments, whereby Fe-Lf was added to MNC-CM before priming neutrophils, revealed no inhibition; thus demonstrating thatthe production, not the effect,of PMN-PF was influenced by Lf.

Whilst conducting these experiments, it was observed that individual responses to Lf were extremely variable, with a large number of donors not responding to Lf at all. It can be seen from table 4 that individuals appear to fall into two main categories: those who respond to Lf and those who do not.

Donor	Rate mv Min^{-1} Control	10^{-10} M Fe-Lf	% Difference
Responders.			
1	3.083	1.049	-66.0
2	4.288	2.003	-53.3
3	15.340	11.415	-25.6
4	3.245	1.749	-46.1
5	3.287	2.449	-25.5
6	2.802	1.103	-60.6
7	4.592	3.797	-17.3
8	7.890	6.830	-13.4
Mean	5.566	3.799	-31.7
SEM	1.511	1.277	
Non-Responders.			
1	9.663	10.465	+1.1
2	9.898	10.034	+1.4
3	2.340	2.841	+21.4
4	2.882	2.713	-5.9
5	4.442	4.775	+7.5
Mean	5.845	6.166	+5.55
SEM	1.664	1.708	

Table 4. The effect of 10^{-10} M Fe-Lf on PMN-PF production from different individauls' MNCs stimulated by 10 ug ml^{-1} LPS.

Discussion

The data presented in this chapter has clearly demonstrated a positive interaction between mononuclear cells and neutrophils. A factor produced by MNCs in response to LPS has been shown to enhance PMN chemiluminescence considerably following stimulation by both ZAS and STZ. Enhancement of neutrophil chemiluminescence with lucigenin is considered to be a measure of O_2^- production (Allen 1986); therefore, the MNC derived PMN priming factor (PMN-PF) functions by enhancing the cells' oxidative metabolism. The significance of this will be discussed later.

Figure 31 clearly shows that a factor was produced by mononuclear cells and that the phenomenon was not simply due to carry over of LPS in the conditioned medium. It can be seen that LPS did itself have a dose dependent priming effect, but in comparison to MNC-CM this was very slight. However, the importance of keeping LPS contamination of buffers and media as low as possible was illustrated in a report by Guthrie et al. (1984). They showed that LPS at 10 ug ml^{-1} was able to prime PMNs to release up to 50% more O_2^- after stimulation with fMLP, PMA or fixed immune complexes. Their work demonstrated that this effect required at least 30 minutes incubation at 37°C with LPS to be observed. Whilst conducting the priming experiments described here the PMNs were incubated at 4°C for only 15 minutes, which would explain why comparatively little LPS priming occurred in this work. However, all the reagents used were maintained sterile to avoid the build-up of endotoxin, and hence prevent high backgrounds due to its priming effect.

A number of observations suggest the identity of the factor to be TNF. The time course for its production as illustrated in Figure 29, is consistent with that of TNF (Burchett et al. 1988). In addition, the effect of PMN-PF on neutrophils is very similar to the reported effects of TNF. Shalaby et al. (1985) demonstrated that rhTNF alpha and TNF beta were able to enhance the ability of human neutrophils to phagocytose unopsonised latex beads after exposure of the cells to the monokine for 20 minutes. They also found that TNF stimulated antibody dependent cell mediated cytotoxicity (ADCC) to SRBCs, and suggested that this was due to enhanced PMN oxidative metabolism.

Further to this, Berkow et al. (1987) showed that preincubation of PMN with rhTNF at concentrations greater than 10 U ml^{-1} enhanced O_2^- by up to 278% in response to fMLP. This increase was due to a more rapid initial rate. Similar to the results presented here, no further priming effect was achieved if the PMN was incubated with TNF for longer than 10 minutes. They also found that the enhancement was not lost when the cells were washed following priming. Thus, the data of Berkow is in very close agreement with that obtained using MNC-CM. The results obtained with rhTNF for the enhancement of chemiluminescence in response to ZAS and STZ (Figures 33 and 35) show that above 10 U ml^{-1} TNF increased responses to both stimuli, which is also in accordance with Berkow's data. However, Figure 37 shows that at high TNF concentrations, PMN chemiluminescence was stimulated directly, while Berkow found no direct effect of TNF on neutrophil O_2^- production. This discrepancy may be a result of the different TNF

preparations used, since many workers have demonstrated that the monokine directly effects the range of PMN functions, notably Tsujimoto et al. (1986b) who showed that TNF alone stimulated O_2^-.

A number of workers have investigated the mechanism of PMN priming by TNF. Atkinson et al. (1988), who also found O_2^- production following priming and subsequent stimulation with fMLP, showed that following priming there was a change in fMLP receptors from a low affinity to a moderate affinity state. Receptor numbers, however, were unaffected by TNF. As with the work presented here, and that of Berkow, they also found that the optimal time for priming was around 15 minutes.

Using opsonised Zymosan as a stimulus, Klebanoff et al. (1986) were able to block the priming effect of TNF with monoclonal antibodies against the PMN C3bi receptor (CR3). Furthermore, Gamble et al. (1985) demonstrated increased neutrophil adherence and expression of CR3 in response to TNF. These findings help to explain the priming phenomena to a range of stimuli: by increasing either receptor affinity (fMLP) or expression (CR3), the activating signals to a fixed ligand concentration will be greater providing the latter is not limiting.

All the reports on priming PMN with TNF were conducted by incubating the cells with the monokine at 37°C. Whilst conducting the preliminary investigations into the priming effects of MNC-CM it was found that preincubation could be conducted at 4°C without loss of the subsequent response. This facilitated the washing of the PMN without causing them to clump; this in turn enabled the

chemiluminescence to be assessed in FCS free medium, which was found to increase chemiluminescence by eliminating the quenching effect of the serum. Therefore, priming at 4°C followed by washing was found to be far more satisfactory. The implications of this are, however, important. The priming factor must bind to its receptor at 4°C, remain bound during the washing procedure and then have a rapid effect when the cells are warmed to 37°C such that a response can be observed 10 minutes after addition of stimulus (Figure 27).

Although considerable evidence exists to suggest that PMN-PF is actually TNF, other candidate cytokines cannot be discounted. A large amount of data has been published clearly demonstrating that GM-CSF has potent PMN priming activities.

Weisbart et al. (1986) showed that GM-CSF increased high affinity fMLP receptor expression by a factor of three following a 5-15 minute exposure to the growth factor, and that this was accompanied by an 85% increase in chemotaxis. Similarly, Lopez et al. (1986) reported that recombinant human GM-CSF enhanced phagocytosis and ADCC by human neutrophils. Although the role of GM-CSF in PMN priming is clear, it is unlikely that it is involved in the phenomenon described in this chapter, since its production in mononuclear cell cultures does not begin until after three days of incubation. Also, English et al. (1988) demonstrated that recombinant GM-CSF primed neutrophils for enhanced oxidative responses to fMLP, but only when the cells were incubated at 37°C for 90 to 120 minutes, no effect was observed with incubation at

4°C.

Another important cytokine in this priming phenomenom is IFN gamma. Perussia et al. (1987) reported that immune interferon had a potent synergistic effect in the enhancement of ADCC induced by IFN alpha and beta and of GM-CSF. Although of potential importance in further amplifiying PMN functions, it is unlikely that IFN gamma was involved in the priming data presented here, since an effect with IFN was only detected after incubating the PMN at 37°C for more than four hours.

Thus, it is highly likely that PMN-PF is TNF. It may therefore be deduced from the data in Figure 40 and that in Table 4 that Lf is able to inhibit the production of TNF. Obviously this suggestion will require investigation using specific assays for TNF. However, the implications for the inhibition of PMN-PF regardless of its exact nature are potentially important. That Lf is able to inhibit the production of a factor which dramatically enhances neutrophil responses to subsequent stimulation may be relevant in the control of inflammation. The inhibition of priming factor production could function as a protective mechanism to prevent excessive neutrophil activation which could be deleterious to the host. If Lf does indeed inhibit TNF production, then the mechanism could be extended to the control of chemotaxis to an inflammatory site, since it has been proposed that TNF is involved in directing PMN migration to infected tissues (Ming et al. 1987).

Whilst conducting the investigations into the effect of Lf on PMN-PF production, it became apparent that some individuals' MNCs were

not inhibited by Lf (Table 4). Unfortunately, at the time of writing, follow-up data was not available to determine whether this represents a true difference between individuals' responses to Lf or a variation in response within a donor, with some giving blood whilst their MNCs were in an unresponsive state. Broxmeyer and colleagues have reported data which could support either suggestion. As discussed in Chapter 3, they have shown that Ia molecules may function as Lf receptors (Broxmeyer and Platzer 1984), in which case, certain HLA-DR types might respond to Lf and others not. If this proves to be true, then the proposed in vivo functions of Lf as a regulator of inflammation would be questionable, since such a mechanism can hardly apply to one individal and not to another. Broxmeyer and Platzer (1984) have also shown that inhibition of GM-CSF production in mice by Lf was lost if the animals were infected with Friend Leukaemia virus. Therefore, the observed variation of the inhibition of PMN-PF production by Lf could reflect in some way the health and immune status of the individual at the time of donating blood.

A third explanation is also provided by the work of Broxmeyer and Platzer, who showed that the inhibition of CSA production by Lf was dependent upon the concentration of monocytes used. At greater than 10^5 monocytes ml^{-1} no inhibition occured, and variable responses were observed around this concentration. They showed that this phenomenon could be overcome by indomethacin, and was due to a concentration dependent production of PGE_2 which masks the effect of Lf. Preliminary investigations demonstrated no enhancement of PMN-PF production by inclusion of indomethacin in to

the MNC cultures, and therefore, it was not used in subsequent experiments. The absence of indomethacin could explain the variable results obtained. Obviously further experimentation is required to resolve this issue. By finding the explanation to this variation in the response to Lf, some insight could be given into the controversy surrounding the various inhibitory effects of Lf.

Summary

A factor produced within five hours of culture by LPS stimulated mononuclear cells was found to enhance neutrophil chemiluminescence dramatically in response to zymosan activated serum. This PMN priming factor (PMN-PF) was found to have optimal effect after preincubation of the neutrophils for 15 minutes at 4°C, and was not lost by washing the cells. This could be duplicated by recombinant human tumour necrosis factor. The kinetics of PMN-PF secretion, combined with its enhancing effects on neutrophil chemiluminescence, would suggest its identity to be TNF. Lf was found to inhibit PMN-PF production from some individuals' MNCs, but not from others. This would imply that Lf inhibits TNF production; however, the reasons for the variable effects remains unanswered.

CHAPTER SIX.

CONCLUSIONS: LACTOFERRIN AS A NEGATIVE FEEDBACK REGULATOR IN THE CONTROL OF INFLAMMATION

The work discussed in the preceding chapters demonstrated that Lf, released from the secondary granules of phagocytosing neutrophils, is able to inhibit a variety of mononuclear cell functions in response to a range of stimuli. The current understanding of mononuclear cell proliferation has revealed a complex network of interacting cytokines, passing positive and negative signals between participating cells. When considered in the wider context of inflammation, the scenario becomes much more complex. This is in part due to the pleotropic nature of a number of factors, namely those of central importance to inflammation, IL-1 and TNF.

The inhibition of mononuclear cell proliferation in response to mitogens and allo-antigens by a single factor, Fe-Lf, is probably due to inhibition of a common mechanism. The data in Chapter 4 has demonstrated an effect of Lf on IL-1 production, and in Chapter 5, of a monocyte derived neutrophil priming factor which is probably TNF. Both of the monokines are produced in response to the same stimulus, although their production is modulated by different mechanisms (Burchett et al. 1988). Burchett demonstrated that LPS-stimulated monocytes rapidly produce and secrete TNF from a pool of pre-existent mRNA. IL-1 (as discussed in Chapter 4) is secreted by monocytes in response to LPS, but usually remains cell associated. They suggest that the rapid secretion of TNF may amplify IL-1 production and its local effects. This is supported by Dinarello

et al. (1986), who demonstrated that rTNF alpha stimulated IL-1 production from human MNCs in vitro. Thus, it may be suggested that TNF (or a similar cytokine) is the central factor being inhibited by Lf. The demonstrated effects of Lf on IL-1 production in LPS stimulated MNC cultures, and on IL-2 in crowded MLCs, could all be due to inhibition of TNF. Evidence to support this suggestion was recently published by Shalaby et al. (1988). They were able to detect TNF alpha in one-way MLC supernatants within one hour after initiation of the cultures, which reached a peak by four hours. In addition, they found that rTNF alpha and beta increased expression of IL-2 receptors on the responding cells in the MLC. Addition of TNF alpha or beta to MLCs increased ^{3}H-TdR incorporation by up to six times. Their investigations led them to suggest that TNF alpha secretion may function as the initiating cytokine in MLC. The enhancement of MLC by TNF could also be due to an increase in HLA-DR expression on T cells in response to the monokine (Scheurich et al. 1986).

Further evidence to support that TNF is the central factor being affected in the MLC by Lf is provided in a paper by Yokota et al. (1988). They demonstrated that rTNF alpha directly enhances T cell proliferation in response to antigens and mitogens in the absence of AC. However, when AC were present (paraformaldehyde fixed antigen bearing macrophages), the enhancement only occurred when the cells were cultured in flat bottomed wells. When the experiments were repeated in RBWs, T cell proliferation was much higher and could not be enhanced by addition of exogenous rTNF-alpha. This phenomenon is very similar to that reported here for

the MLC, and may be explained by the enforced contact between macrophages and T cells resulting in the induction of TNF secretion by the former.

The actions of TNF are very similar to those of IL-1, which has been clearly shown to be inhibited by Lf. Therefore, the potential consequences for the inhibition of either monokine, by Lf released at an inflammatory locus, will be similar. For example, both TNF alpha and IL-1 have been shown to enhance lymphocyte adhesion to human umbilical vein endothelial cells (Cavender et al. 1987), augment proliferation and differentiation of highly purified human B cells (Jelinek and Lipsky 1987a), increase neutrophil adhesion to endothelial cells (Pohlman 1986) and stimulate GM-CSF production from endothelial cells (Broudy et al. 1987; Munker et al. 1986) and from fibroblasts (Zucali et al. 1988; Zucali et al. 1986).

When the events are considered sequentially in the context of the host response to infection, the importance of an inhibitor of IL-1 and/or TNF becomes apparent. The invasion of peripheral tissues by a bacterial pathogen results in the activation of local macrophages by released endotoxin. This stimulates the cells to secrete TNF and IL-1, which stimulate local endothelial cells and fibroblasts to secrete colony stimulating factors and become adhesive for neutrophils and lymphocytes. The CSFs may themselves act locally by enhancing neutrophil functions such as adhesion and oxidative metabolism (as discussed in Chapter 5) or exhibit distal effects on the bone marrow to increase haematopoiesis (reviewed by Cannistra and Griffin 1988). In addition, TNF alone could induce chemotaxis

of neutrophils and monocytes to the site of infection (Ming et al. 1987) and enhance the neutrophils' killing capacity (Shalaby et al. 1985). Thus, the inhibition of either TNF or IL-1 by Lf released from neutrophils at an inflammatory site, could potentially lead to a reduction in these related phenomena and hence introduce a control mechanism in the process; thereby preventing excessive neutrophil accumulation and activation, which if allowed to continue unchecked could be potentially very harmful.

A report by Koivuranta-Vaara et al. (1987) has provided evidence in support of this proposed negative feedback mechanism. They demonstrated that LPS stimulated neutrophils to release Lf. The amount of Lf released was markedly enhanced when monocytes were added to the PMN suspension. The observed effect of LPS on neutrophils was secondary to the endotoxin stimulating monocyte secretion of TNF alpha, which then induced degranulation by the PMN. Thus it could be argued that TNF produced at a site of infection could modulate its own production by inducing the release of Lf from arriving neutrophils. There is no data available indicating whether the Lf released in response to TNF is carrying iron or not.

At the time of writing there are no reports concerning inhibitors of TNF. However, inhibitors of IL-1 and 2 have been demonstrated. The majority of reports have focused on inhibitors of Interleukins in pregnancy, where immune suppression would obviously be desirable to prevent rejection of the foetus. Domingo et al. (1985) demonstrated that pregnancy serum was able to inhibit T cell proliferation in response to PHA and to allogeneic cells. They

showed that this was due to the serum inhibiting IL-2 production in MLC. There is evidence to show that neutrophil activation occurs in pregnancy (Maallem and Fletcher 1980), which is likely to increase the serum Lf level. However, Domingo et al. incubated their MLCs in FBWs, conditions which (according to the data in Chapter 3) are inappropriate to demonstrate inhibition by Lf; this suggests that their inhibitor was some other factor.

An inhibitor of IL-1 activity has been isolated from the urine of pregnant women and was termed Uromodulin by Muchmore (1986). Uromodulin was found to be a heavily sialylated glycoprotein of molecular weight 85 Kda. The molecule was shown to bind to IL-1 with high affinity and down regulate the activity of the monokine in a classic LAF assay.

Another IL-1 inhibitor has been reported by Seckinger et al. (1987). This is of molecular weight 18-25 Kda, and was found in the urine of patients with monocytic leukaemia or febrile conditions. Unlike the role postulated for Lf, this inhibitor was specific for IL-1 and did not affect the action of TNF on cultured human fibroblasts.

An inhibitor of the action of IL-1 has been found in human peripheral blood neutrophils (Tiku et al. 1986). This was released from PMN incubated for 18 hours with zymosan or from sonicated cells. Gel filtration of inhibitor containing supernatants revealed activities with molecular weights of approximately 70 and 160 Kda. Inhibitory supernatants were specific for IL-1, having no effect on IL-2 stimulated proliferation of CTLL cell lines. The

inhibitor functioned by blocking the effect of IL-1 in the classic LAF assay. It is conceivable that their 70 Kda inhibitory factor was Lf, since Broxmeyer et al. (1978) obtained active Lf from disrupted (freeze/thawed) neutrophils, whilst incubation with zymosan could be considered analogous to the preparation of PNCM detailed in this report. It is also possible that the 160 Kda fraction was the dimer, as postulated by Bagby et al. (1982). Although Bagby showed that Lf polymers were inactive as inhibitors of CSA production, the function measured by Tiku may be affected by dimeric Lf.

Tiku found that the neutrophil derived inhibitor blocked the effect of IL-1 in the classic LAF assay, and suggested that this was due to the inhibitor binding and deactivating either IL-1 or its receptor. However, when Lf was added with IL-1 to EL4-NOB.1 (Chapter 4) no inhibition was observed. This apparent discrepancy between the findings of Tiku and those reported here can be explained in one of two ways. The most obvious of these is that Tiku was not dealing with Lf; however, as it was present in neutrophils, was released by phagocytosis or disruption of the cells and had a molecular weight of around 70 KDa, this would suggest that it could indeed be Lf. Nevertheless, Tiku's supernatants would undoubtedly contain Lf. These concentrations would have been considerably higher than those used here. They prepared the inhibitor from 5 x 10^6 PMNs which were either incubated for 18 hours or sonicated, as compared to 10^6 PMNs incubated with C guilliermondii for 30 minutes in this report. It is quite conceivable that higher concentrations of Lf would inhibit

the action of IL-1.

It is highly likely that Lf binds to IL-1, since Muchmore and Decker (1987) showed that the blocking of IL-1 activity by uromodulin was dependent on intact glycosylation of the inhibitor, and that binding of uromodulin to IL-1 could be blocked by N-linked oligosaccharides or monosaccharides. This led them to suggest that IL-1 was behaving similarly to an endogenous lectin. The carbohydrate structure on uromodulin responsible for binding IL-1 appeared to be a complex, mannose rich, heavily sialylatedmolecule. Spik et al. (1985) investigated the primary structure of glycans on Lf and demonstrated that they are involved in the recognition of macrophage membrane lectins. Thus, the glycan residues on Lf could potentially recognise and bind to the lectin like structures on IL-1. It may therefore be suggested that Lf does bind to IL-1 and that at high Lf concentrations it inhibits IL-1 activity and may therefore block systemic effects. At low concentrations, Lf could inhibit the production of IL-1 from monocytes and provide a negative feedback for local effects as discussed.

The demonstration that Lf inhibits production of IL-1 and possibly TNF could link many of its various reported functions. The consequent inhibition of IL-2 production could explain the inhibitory effects of Lf on natural killer cell activation (Nishiya and Horwitz 1982). The inhibition of antibody formation by Lf (Duncan and McArthur 1982) could also be explained by effects on IL-1, since B cell activation can be stimulated either directly or indirectly (via T cells) by IL-1. Considered together with the

effects of IL-1 and TNF on GM-CSF production by fibroblasts and endothelial cells as previously discussed, Lf has the potential to function as a potent negative feedback regulator in inflammation and immunity, particularly since it is delivered to the site of infection by neutrophils responding to inflammatory mediators. Whether these postulated functions can be demonstrated in in vivo experiments remains to be seen. Further work is obviously required since little consensus of opinion has been reached over the role of lactoferrin after 10 years of research.

Appendix 1: Buffers and Media

a) Buffers for Lactoferrin ELISA

1. Bicarbonate Coating, buffer pH 9.6

   Stock soloution : (stored at 4°C)

Na_2CO_3	31.8 gl^{-1}
$NaHCO_3$	58.6 gl^{-1}

   Dilute 1:20 with distilled water for use (adjust to pH9.6).

2. Conjugate Buffer, (Borate saline) pH 8.6 (Store 4°C)

Boric acid	5.5 gl^{-1}
Sodium tetraborate	9.54 gl^{-1}
NaCl	29.22 gl^{-1}

3. Phosphate buffered saline (PBS) pH7.4.

   Stock solution : (stored at 4°C)

NaCl	80 gl^{-1}
KCl	2 gl^{-1}
Na_2HPO4	11.5 gl^{-1} - Dissolve first
KH_2PO_4	2 gl^{-1}

   Dilute 1:10 with distilled water for use.

4. Washing buffers:

   i) PBS wash buffer - PBS containing 0.01% w/v gelatin, 0.1% v/v Tween 20, prepare fresh each time.

   ii) Borate wash buffer - Borate saline containing 0.1% v/v Tween 20 (store 4°C).

b) Buffers and media for leucocyte preparation

PBS-A:

PBS containing 1mg ml^{-1} BSA, 1mM Ca Cl_2 and 0.7mM Mg Cl_2.

cRPMI:

RPMI 1640 containing 2mM L-Glutamine, 100 IU ml^{-1} penicillin, 100 ug ml^{-1} streptomycin and 10% v/v Foetal calf serum.

Appendix 2. Reagents and Plastic Disposables

All less specialised reagents were obtained from BDH Limited. Details of the suppliers are listed at the end of this appendix.

Reagents:

Reagent	Catalogue No.	Supplier
Activated Charcoal	33204	BDH
Antilactoferrin	A186	Dako-Patts
Avidin-peroxidase conjugate	622521	ICN Biomedical
Bovine serum albumin	A9543	Sigma
Concanavalin A	C7275	Sigma
Cyanogen bromide activated Sepharose 4B	C9142	Sigma
Diff Quick	130832	Merz and Dade
Dimethy sulphoxide	103234L	BDH
Ethylenediaminetetra-acetic acid (EDTA) Dipotassium salt	ED2P	Sigma
Feotal calf serum	F4010	Sigma
Ferrous ammonium sulphate	F3754	Sigma
Gelatin	G2625	Sigma
L-Glutamine	16-801-49	Flow Labs
Indomethacin	I7378	Sigma
Interleukin 2	663581	Boehringer
Lactoferrin	L8010	Sigma
Lactoferrin Standard	(discontinued)	Calbiochem
Lipopolysaccharide from E.coli serotype 005.B5	L2880	Sigma
Lucegenin	M8010	Sigma

Lymphocyte Separation Medium	16-922-54	Flow Labs
2-Mercaptoethanol	M6250	Sigma
Mitomycin C	M0503	Sigma
Mono-Poly Resolving Medium	16-980-49	Flow Labs
Nutrient Broth No.2	CM67	Oxoid
Penicillin/Streptomycin	16-700-49	Flow Labs
Phosphate buffer saline tablets	28-103-05	Flow Labs
Phytohemagglutinin	L 8754	Sigma
Ready Protein scintillation cocktail	158727	Beckmann
RPMI 1640	12-602-54	Flow Labs
Salicylic acid	S3007	Sigma
Sigmacote	SL-2	Sigma
Trizma Base	T1503	Sigma
Trypan Blue	T6146	Sigma
Tween 20	P1379	Sigma
Zymosan A	ZA4250	Sigma

Radio Chemicals:

^{3}H-Thymidine	TRK61	Amersham
^{3}H-Uridine	TRK410	Amersham
^{59}Fe ammonium sulphate	IF.19	Amersham

Plastic Disposables:

Cryotubes	3-6656A	Gibco Nunc
50ml tissue culture flasks	3010	Falcon

250ml tissue culture flasks	3023	Falcon
0.2 um minifilters	64-001-04	Flow Labs
100 um Petri dishes	1029	Falcon
96 well microeliza plates	M129B	Dynatech
12 well tissue culture clusters	C2512	Costar
96 well tissue culture clusters FBW	C3598	Costar
96 well tissue culture RBW	C3799	Costar
2 ml polypropelene vials	72.694	Sarstedt

Suppliers:

Amersham International plc, Buckinghamshire, UK. (020404) 4444

BDH Ltd, Hampshire, U.K. (0703) 619171

Beckman - RIlC Ltd, Buckinghamshire, UK. (0494) 41181

BCL, Beohringer Mannheim, East Sussex, UK. (0273) 471611

Calbiochem, Cambridge, UK. (0223) 316855

Costar. Distributors: Northumbria Biologicals Ltd, Northumberland UK. (0670) 732992

Dako Patts Ltd, Buckinghamshire, UK. (0494) 452016

Dynatech Laboratories Ltd, Sussex, UK. (0403) 814565

Falcon Becton Dickinson UK Ltd, Oxford, Uk. (0865) 777722

Flow Laboratories Ltd, Hertfordshire, UK. (0923) 774666

Gibco Nunc Ltd, Paisley, UK. (041-889) 6100

ICM Biomedical, Buckinghamshire, UK (0494) 443826

Oxoid Ltd, Hampshire, UK. (0256) 841144

Sigma Chemical Company Ltd, Dorset, UK. (0202) 733114

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