STUDIES ON THE INTERACTION OF MYCOBACTERIAL PRODUCTS WITH PHAGOCYTES

by

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ABSTRACT

This study assessed the effects of a 25kDa mycobacterial component on phagocyte functions associated with antimicrobial activity. The 25kDa fraction was obtained by purification of mycobacterial sonicates using Sephacryl S-200 columns. Since the 25kDa fraction could inhibit the intracellular killing ability of polymorphonuclear cells and macrophages, its effects on various mechanisms involved in this process were studied. The mycobacterial fraction inhibited phagosome-lysosome fusion. In addition, it inhibited the release of lysosyme by the granules within the phagocytes. The 25kDa fraction also reduced the production of toxic oxygen products, hydrogen peroxide and superoxide anion (the latter measured by the ability of the phagocytes to reduce nitroblue tetrazolium) and the activity of the hexose monophosphate shunt. On the other hand the 25kDa fraction did not significantly affect the glycolysis pathway used by phagocytes.

Gamma-interferon, but not alpha-interferon, was able to partially reverse the inhibition caused by the fraction, indicating a possible therapeutic role for this cytokine in mycobacterial disease.

Further studies demonstrated an inhibition of lymphocyte transformation to mitogens (PHA, Con A and PWM) and to antigen (PPD) in the presence of macrophages pretreated with the 25kDa mycobacterial fraction. Closely related to this is the inhibition of DR expression by the 25kDa fraction, suggesting potent escape mechanisms employed by mycobacteria to ensure survival within the host.

This is to certify that the dissertation entitled:-
'The interaction of a mycobacterial component with phagocytes'
presented for the degree of Masters of Science at the University of the Witwatersrand, Johannesburg, is my own unaided work and has not been presented at any other university.

.....@lara..... ....6. day of January., 1992

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PUBLICATIONS & PRESENTATIONS

A 25kDa fraction from *Mycobact . ium tuberculosis* that inhibits hexose monophosphate shunt activity, lysosyme release and H_2O_2 production: Reversal by gamma interferon. Infect. Immun. 1989; 57:864-869.

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To my family and my fiance, Mike
for always being there for me.

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Lastly a special word of thanks to Bronwyn for managing to decipher my cryptic notes and converting them into scientific language.

LIST OF ABBREVIATIONS

Aids	Acquired immune deficiency syndrome
APC	Antigen presenting cell
BPI	Bactericidal/permeability increasing properties
CFU-GEMM	Colony forming unit
CSF	Colony stimulating factor
CR1, CR3	Complement receptors (1, 3)
CFPE	Culture filtrate protein extract
cAMP	Cyclic-adenosine monophosphate
DNP-GPA	Dinitrophenyl guinea pig albumin
MFR	D-mannan/fucose receptor
ETZ	Electron-transparent zone
EP	Erythropoietin
F-	Fluoride ion
IFN-gamma	Gamma-interferon
GM-CSF	Granulocyte, macrophage CSF
GM-CFU	Granulocyte, monocyte CFU
G-CSF	Granulocyte CSF
HEL	Hen-egg white lysosyme
HPR	Hexazotized pararosanilin
HMPS	Hexose monophosphate shunt
HIV	Human immuno-deficiency virus
HNP	Human neutrophil peptide
H_2O_2	Hydrogen peroxide
OH-	Hydroxyl radical
Ia antigens	Immune associated antigens
Ir genes	Immune response genes
IL-1,-2,-3,-4,-6	Interleukin 1,2,3,4,6
Ii	Invariant chain
kDa	Kilodalton
LFA-1, LFA-3	Leukocyte function associated antigens (1,3)
MAF	Macrophage activating function
M-CSF or CSF-1	Macrophage-CSF
MHC	Major histocompatibility complex
M-CFU	Monocyte-CFU
MGC	Multinucleated giant cell
MDP	Muramyl dipeptide
MPH	Myeloperoxidase, peroxidase-halide
PPG	Peptidoglycan
PGI	Phenolic glycolipid I
PMA	Phorbol myristate acetate
PMN cells/phagocytes	Polymorphonuclear cells/phagocytes
PKC	Protein kinase C
RES	Reticuloendothelial system
-1	Sulphatide 1
O_2^-	Superoxide anion
TDM	Trehalose dimycolate or cord factor
TNF	Tumour necrosis factor

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CHAPTER 1.
INTRODUCTION &
REVIEW OF THE LITERATURE

1. PHAGOCYTIC CELLS IN INFECTION

Phagocytic cells constitute an important mechanism of host defence against any infection (Werb, 1987). The response of these cells is non-specific and forms the first line of defence against the invading organism. The phagocyte complex falls into two categories: (i) the mononuclear phagocytes, which include tissue macrophages, circulating monocytes and their precursors in bone marrow; and (ii) the polymorphonuclear (PMN) phagocytes (Werb, 1987). The mononuclear phagocytes also play an important role in activating other arms of the immune system, which comprise both T and B lymphocytes (Unanue, 1972).

T lymphocytes which mature in the thymus are responsible for the cell-mediated antigen-specific response to foreign organisms and for activating B lymphocytes (Marchalonis, 1980). B lymphocytes are the antibody producers of the body and are thought to mature in the bone marrow or foetal liver (Stites *et al.*, 1980). On exposure to antigen and T helper cells, B cells develop into antibody producing plasma cells (Marchalonis, 1980).

1.1. HISTORY OF THE MONONUCLEAR PHAGOCYTE SYSTEM

It was as far back as 1892 that Metchnikoff coined the term "macrophage" to identify a group of cells which had the ability to engulf foreign organisms (Langevaart *et al.*, 1970). He recognised the close relationship between the phagocytic cells in the spleen, lymph nodes, bone marrow and connective tissue and grouped these all together into the "macrophage system"

(Langevaart *et al.*, 1970). This concept was then further developed by Aschoff in 1924 who grouped several kinds of cells into the reticuloendothelial system (RES) (Langevaart *et al.*, 1970). This system included endothelial cells; reticular cells of the spleen and lymph nodes; histiocytes; splenocytes and monocytes. This concept was widely criticised since vascular endothelial cells, histiocytes and the reticular cells of the lymph nodes and spleen differ vastly from one another (Langevaart *et al.*, 1970). At a conference on mononuclear phagocytes in Leiden, in September 1969, it was therefore decided to re-define the system as the mononuclear phagocyte system (Langevaart *et al.*, 1970).

1.2. PHAGOCYTE ONTOGENY

1.2.1. Mononuclear Phagocyte Ontogeny

The mononuclear phagocyte originates in the bone marrow as a common progenitor of the granulocyte, erythroid megakaryocytic and macrophage lineages [Colony forming unit CFU - GEMM (Welte *et al.*, 1985) or pluripotent stem cell (Sieff, 1987)]. A group of glycoprotein hormones, termed colony stimulating factors (CSF), will then induce the differentiation of this into the various cell lineages (Sieff, 1987). Pluripotent CSF is the colony stimulating factor which will induce the CFU-GEMM to differentiate into progenitors for each of the separate cell lineages (Welte *et al.*, 1985). It has been postulated that GM-CSF (granulocyte macrophage CSF) governs the rate and percentage of monocytes and granulocytes that emerge from the bone marrow (Sawyer *et al.*, 1989). Differentiation of the

CFU-GM (granulocyte monocyte CFU) into the two different lineages appears to be modulated by the availability of colony stimulating factors. Studies with recombinant GM-CSF indicate that high concentrations favour formation of granulocyte colonies whereas the use of low doses resulted in the predominant formation of macrophages (Metcalf, 1980).

The CFU-monocyte (M-CFU) which results from differentiation of the CFU-GM in the presence of macrophage-CSF (M-CSF or CSF-1 (Sieff, 1987) will differentiate into a monoblast which in turn differentiates into a promonocyte, the first morphologically identifiable cell in the mononuclear phagocyte series (Johnston, 1988). The transit time in the bone marrow from CFU-GM to a mature monocyte is about 6 days (Groopman & Golde, 1981). Newly formed monocytes may remain in the marrow for approximately one day and then enter the blood stream where their half life is thought to be approximately 3 days (Van Furth *et al.*, 1979). Circulating monocytes constitute a mobile pool of incompletely differentiated cells on their way from their site of origin to the tissues. These cells are heterogenous with respect to their cell density, size, morphology and surface antigens (Van Furth *et al.*, 1979). No evidence is available however to suggest that monocytes are destined for any particular tissue and migration to various tissues appears to be a random phenomenon in the absence of inflammation (Johnston, 1988). It has been suggested that once in the tissues, monocytes do not re-enter the circulation but undergo transformation into tissue macrophages. These macrophages

would now demonstrate morphological and functional properties characteristic for the tissue in which they reside (Van Furth *et al.*, 1979) (Figure 1.). The terminal stage of development in the mononuclear-phagocyte line is a multinucleated giant cell (MGC) which characterises granulomatous inflammatory disease, such as tuberculosis (Johnston, 1988). Both monocytes and macrophages appear in the lesions in these diseases before the appearance of giant cells and they are therefore thought to be the precusors to these multinucleated cells (Mariano & Spector, 1974). Monocytes kept in culture for 3-10 days develop general features of tissue macrophages and some of them fuse into large MGC's (Schlesinger *et al.*, 1984). The MGC's are as efficient as macrophages in:

(a) ingesting sheep erythrocytes coated with IgG and *Candida albicans*,

(b) degrading ingested yeast,

(c) reducing nitroblue tetrazolium, and

(d) expressing Ia antigens (Schlesinger *et al.*, 1984).

Although the activity of β-glucosaminidase, acid phosphatase and β-glucoronidase per cell is greater in MGC's, the specific activity of the enzymes is greater in macrophages. These studies indicate that MGC's have the capacity to function like macrophages in host defence against infection (Schlesinger *et al.*, 1984).

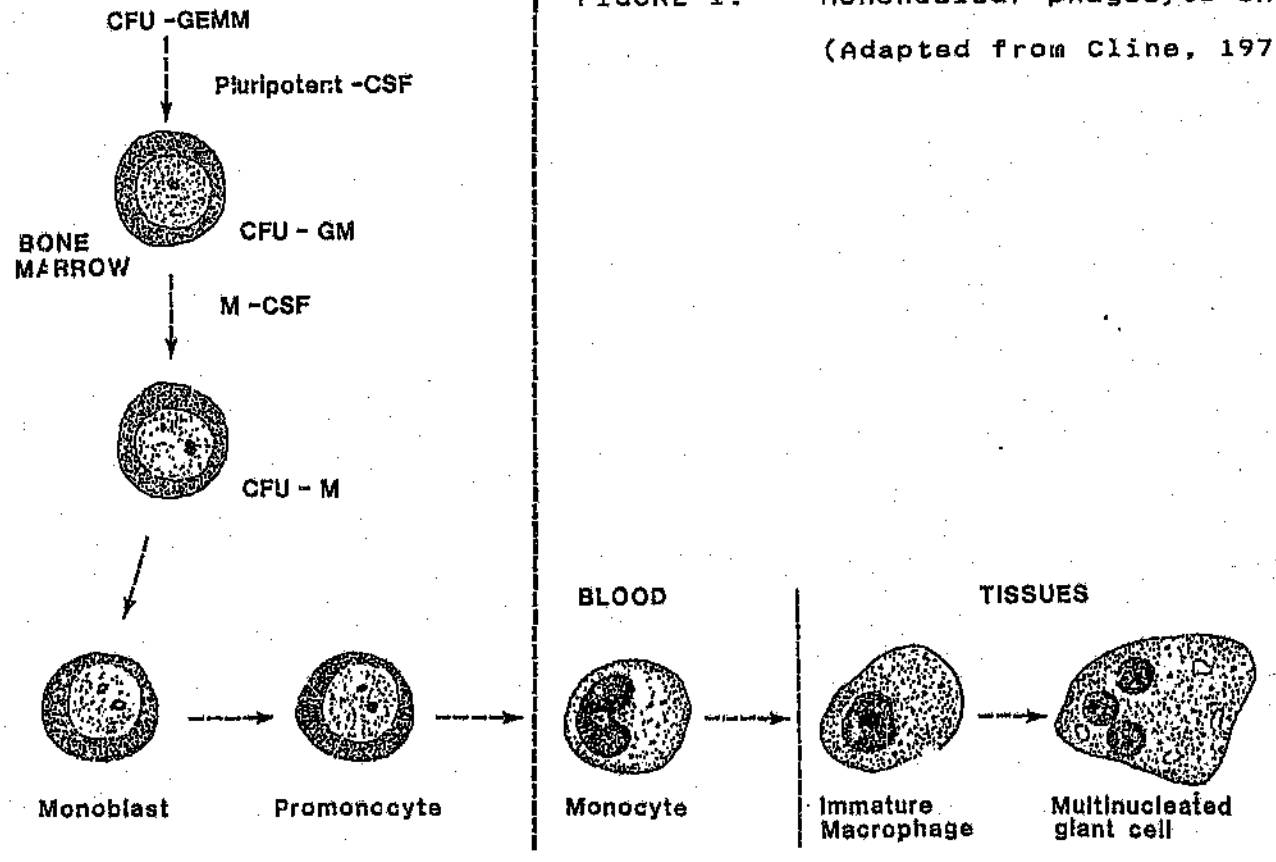


FIGURE 1. Mononuclear phagocyte ontogeny (Adapted from Cline, 1975)

1.2.2. Polymorphonuclear Phagocyte Ontogeny

The PMN or granulocyte originates from the CFU-GM. The CFU-GM initially differentiates in the presence of G-CSF (granulocyte CSF) to form a CFU-G. The CFU-G will further differentiate into a myeloblast. The myeloblast then divides and differentiates into a myelocyte. Subsequently, during a 7 day postmitotic period, cytoplasmic and nuclear changes occur that result in a fully mature and functional PMN cell (Sawyer *et al.*, 1989). The PMN cell acquires 2 major populations of granules:

(i) the primary or azurophilic granules which contain myeloperoxidase, acid hydrolases, lysosyme, cationic proteins and neutral protease; and

(ii) secondary or specific granules which contain lactoferrin, lysosyme, vitamin B_{12}-binding protein, cytochrome b, collagenase and certain receptor molecules (Sawyer *et al.*, 1989). Circulating PMN cells represent only 5% of the total body pool of granulocytes (Sawyer *et al.*, 1989). About half of the intravascular population of PMN cells are not circulating but are adherent to the endothelium of small vessels (margination of PMN cells) (Werb, 1987). PMN cells enter the tissues, where they remain functional for 1-2 days, after which the senescent PMN cells are removed by splenic macrophages or are discharged from mucosal surfaces (Sawyer *et al.*, 1989).

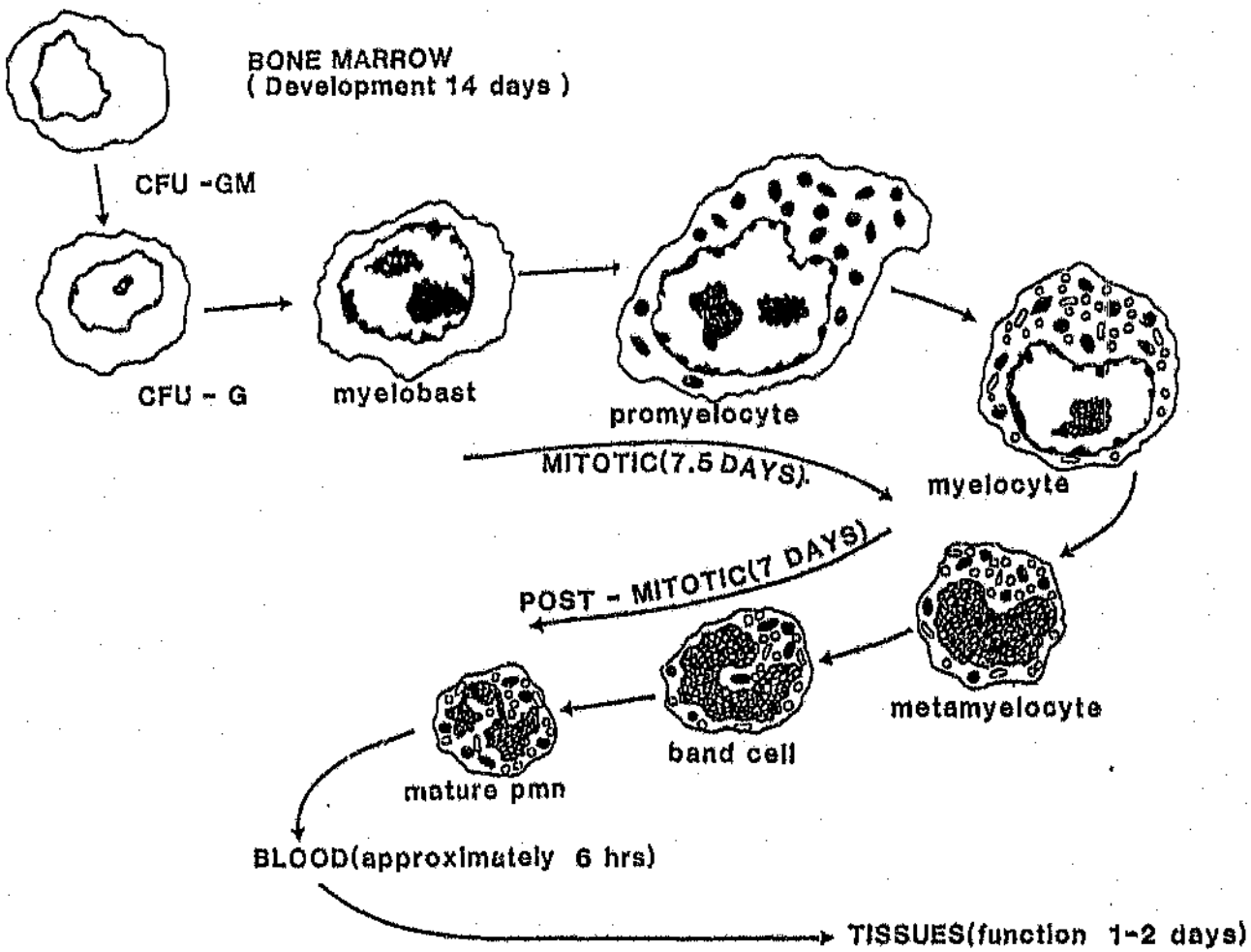


FIGURE 2. Polymorphonuclear phagocyte ontogeny (Adapted from Cline, 1975)

1.2.3. The Colony Stimulating Factors (CSF)

With the development of techniques for the clonal culture of haematopoietic cells in semi-solid culture medium, it became possible to analyse the events occuring during haematopoiesis (Metcalf, 1986). It was found that progenitor cells are intrinsically incapable of unstimulated cell division. A continuous stimulation by appropriate specific regulatory molecules is required for such division (Figure 3.). These molecules or hormones are collectively known as colony stimulating factors (CSF's) (Metcalf, 1986). Five CSF's have been identified:

(i) multi-CSF (or IL-3),

(ii) GM-CSF,

(iii) G-CSF,

(iv) M-CSF (or CSF-1), and

(v) erythropoietin (EP).

These factors are produced by many cells in the body including T lymphocytes, monocytes, endothelial cells and fibroblasts (Sieff, 1987). When monocytes are activated with endotoxin they produce G-CSF (Rennick *et al.*, 1987), and the monokines tumour necrosis factor (TNF) (Beutler, 1990) and interleukin 1 (IL-1) (Dinarello, 1984). These monokines may, in turn, induce T lymphocytes to produce both GM-CSF (Herrmann *et al.*, 1988) and multi-CSF (Kelso *et al.*, 1986) and may also induce fixed bone marrow stromal cells to produce GM-CSF and G-CSF (Sieff, 1986; Zucali *et al.*, 1986). In addition, macrophages are capable of controlling the concentration of their own growth

factor (Bartocci *et al.*, 1987). M-CSF (CSF-1) is produced in most tissues at a constant rate and is removed by receptor-mediated endocytosis by the macrophages of the spleen and liver (Hume *et al.*, 1987). Mature macrophages have been shown to be more effective than proliferating progenitor cells in internalising and degrading M-CSF (Bartocci *et al.*, 1987). It is therefore felt that M-CSF may participate in a feedback l[illegible] whereby macrophages control the production of monocytes by [illegible]ne marrow (Hume *et al.*, 1988).

1 3. ANTIGEN PROCESSING

Once phagocytes recognise a foreign antigen, several processes are initiated. These include binding of antigen to the suface; internalisation of microbes; active metabolic processing and catabolism of macromolecules. In the case of macrophages, antigen is recycled to the surface and presented to T lymphocytes in association with Class II major histocompatibility antigens (Werb, 1987).

1.3.1. Antigen Binding to the Cell Surface

Antigens adhere to the macrophage cell surface either via specific Fc and C3 receptors or via non-covalent interactions, such as the zymosan receptor system (Riches *et al.*, 1988). The latter may represent a more primitive recognition system that recognises yeast and other microbial organisms before a

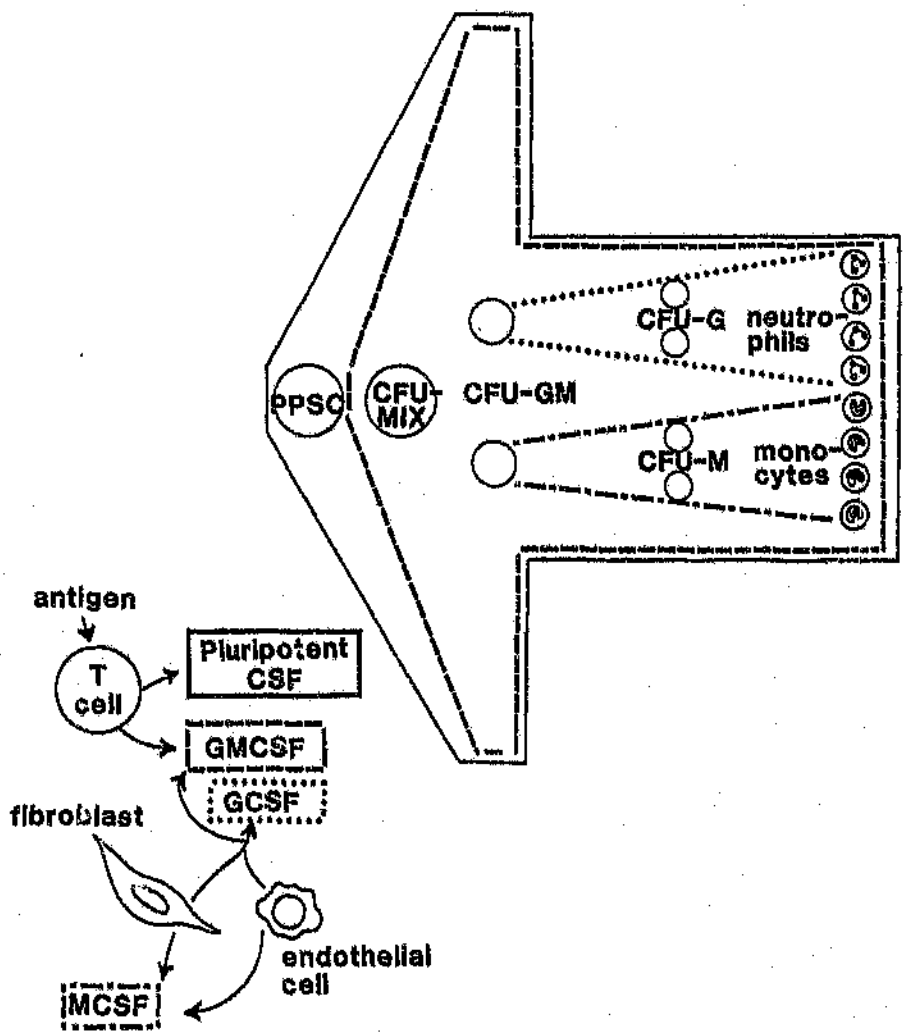


FIGURE 3. The colony stimulating factors and their effect on phagocyte ontogeny.

specific immune response is mounted by the host. The former system indicates an evolutionary advanced stage whereby immunoglobulin and complement enhance the ability of the macrophage to ingest organisms (Riches *et al.*, 1988).

1.3.1.1. Zymosan Receptor System

Zymosan particles are a carbohydrate rich cell wall preparation derived from the yeast *Saccharomyces cerevisiae* which are recognised and avidly ingested by monocytes and macrophages (Ezekowitz *et al.*, 1985). This property has resulted in the extensive use of zymosan particles for the study of macrophage responses. Three receptors play a role in its binding: a D-mannan/ fucose receptor (MFR), a β-D glucan receptor and a macrophage-membrane receptor for the third component of complement (CR3) (Riches *et al.*, 1988). The relative importance of these 3 receptors has as yet not been established, but the β-glucan receptor appears to be a common factor among the various monocyte and macrophage populations that respond to zymosan. The MFR however, has been suggested to be expressed only on macrophages (Shepherd *et al.*, 1982). Since human monocytes do not express functional MFR, other recognition mechanisms for zymosan must be operative in these cells. To this end, Czop & Austen (1985) demonstrated that incubation of human monocytes with insoluble β-glucan led to a striking inhibition of zymosan phagocytosis. Such findings indicate that β-glucan receptor is important for phagocytosis.

Opsonisation of zymosan particles may also be mediated by products of complement activation as evidenced by the studies of Ezekowitz *et al.* (1983) who found C3 cleavage products deposited onto the zymosan particles, thus allowing the opsonised zymosan particles to be internalised via the C3 receptor on monocytes and macrophages.

1.3.1.2. The Fc Receptors

Unlike the situation with the binding of zymosan particles, the receptors involved in the binding of IgG-containing antigen/antibody complexes have been more clearly identified. There are 3 IgG Fc receptors:

(i) FcRI which is a high affinity receptor expressed on monocytes and tissue macrophages (Anderson, 1982) but not on PMN cells unless stimulated with IFN-gamma (Shen, 1988), is relatively specific for monomeric and aggregated IgG 2a (Heusser *et al.*, 1977; Unkeless, 1977);

(ii) FcRII is a low affinity receptor for IgG which is specific for aggregated and antigen-complexed IgG 1 and IgG 2b (Diamond & Scharff, 1980). This receptor is the only Fc receptor found on unstimulated neutrophils and is found on monocytes and tissue macrophages (Ross *et al.*, 1989);

(iii) FcRIII is also a low affinity receptor which binds aggregated IgG 3 (Diamond & Scharff, 1980) and is present in high concentrations on neutrophils but is absent in resting monocytes. Tissue macrophages and macrophages in culture express variable amounts of this receptor (Ross *et al.*, 1989).

The Fc receptor most thoroughly investigated is the IgG 1/2b receptor to which two functions have been ascribed, namely that of a Na^+/K^+ ion channel (Young *et al.*, 1985) and that of a phosphalipase A_2 (Suzuki *et al.*, 1982). Both these functions then play an important role in the elicitation of various macrophage and neutrophil functions involved in host defence and inflammation, including the secretion of lysosomal enzymes, the production of reactive oxygen metabolites and arachadonic acid metabolism (Riches *et al.*, 1988).

1.3.2. Ingestion of Antigen

Once foreign particles bind to their respective receptors, the cell membrane invaginates to form a phagocytic vessicle or endosome (Werb, 1987). It has been demonstrated that this is an energy requiring process and is inhibited by sodium azide and 2-deoxyglucose (Sharma, 1986). These agents have been shown to inhibit the oxidative and glycolytic metabolic pathways (Sharma, 1986). Once formed at the periphery of the cell, the endosome flows into the cytoplasm of the macrophage towards the perinuclear area, guided by microtubules in an energy requiring process that utilises the abundant amount of cytoplasmic contractile proteins (Werb, 1987).

1.3.3. Antigen Degradation

In the perinuclear area the phagosome fuses with lysosomes to form a phagolysosome or secondary lysosome (Werb, 1987). At some stage between the initial formation of the phagocytic vessicle membrane and the formation of the secondary lysosomal membrane, the contents become acidified and portions of the membrane, plasma membrane receptors and some of the contents are recycled back to the cell surface (Werb, 1987). The phagocytosed contents are then digested by hydrolytic enzymes within the lysosome (Sharma, 1986).

1.3.3.1. Lysosomal Enzyme Release

1.3.3.1.1. Stimulation of Enzyme Release

The secretory process of lysosomal enzymes by macrophages has been shown to be slower than that of neutrophils, beginning within 6 minutes after the addition of a phagocytic stimulus (Welschar & Cruchaud, 1976) and continuing for 6-12 hours (or longer) before reaching completion (Riches & Stanworth, 1980; Schorlemmer *et al.*, 1977). It has been shown that early lysosomal enzyme release is independent of new protein synthesis as evidenced by studies indicating the ineffectuality of cycloheximide on lysosyme release (McCarthy *et al.*, 1982). It is therefore postulated that early enzyme secretion occurs from preformed intracellular stores (McCarthy *et al.*, 1982). Sustained release of lysosomal enzymes over a prolonged period however, is dependent on protein synthesis (Schnyder & Baggiolini, 1978).

The biochemical requirements of the induction of lysosomal enzyme secretion have not been clearly defined (Riches *et al.*, 1988). It is possible that phospholipid breakdown is a requirement in the initiation of enzyme release since an increased turnover of phosphatidylcholine during exposure of macrophages to zymosan particles has been noted (Channon *et al.*, 1987). Furthermore, dapsone, an inhibitor of phosphatidylcholine turnover has also been demonstrated to inhibit enzyme release (Bonney *et al.*, 1983). However the role played by phospholipid turnover (if any) must still be fully determined since protein kinase C (PKC) and calcium levels appear not to be implicated in inducing enzyme release (Riches *et al.*, 1983; 1988).

Lysosomotropic weak bases, such as ammonium chloride, methylamine or chloroquine, have also been shown to induce the release of lysosomal enzymes (Riches & Stanworth, 1980). Although their mode of action is not known, it has been suggested that alkalinisation of the cytoplasm and/or the lysosome is necessary for lysosomal enzyme release to occur. Segal *et al* (1981) showed that following phagocytosis, there is an elevation of the intravacuolar pH via the electron transport chain which transports electrons from the cytosol to the lumenal surface of the vacuole. The resultant reduction of molecular oxygen to O^{2-} consumes protons during the formation of H_2O_2 (Root *et al.*, 1975), thereby raising the intracellular pH (Segal, 1981). This raised pH facilitates killing and bacteriolysis by granule proteins which are inactive

at low pH values (Segal 1989a). Following this partial degradation by these proteins, the pH of the vacuoles is rapidly reduced to optimise the activities of hydrolases and other proteins that require an acid pH for optimal activity (Hirsch, 1956).

1.3.3.1.2. Lysosomal Enzymes & Proteins Involved in Bacterial Killing and Degradation

It is well documented that a variety of enzymes are released by macrophages and neutrophils (Werb, 1987; Papadimitriou & Ashman, 1989; Johnston, 1988; Unanue, 1976). A list of these enzymes is presented in Table 1. Enzymes derived from phagocytes have been shown to digest bacterial macromolecules such as complex carbohydrates, proteins and lipids to sub-units of molecular weight of 200kDa or less (Werb, 1987). These enzymes include:

(i) Lysosyme: This enzyme has been shown to have a limited spectrum of antibacterial activity, due partly to its ability to cleave a ß 1-4 linkage between the N-acetylglucosamine and N-acetylmuramic acid residues (Unanue, 1976). With regards to its production, lysosyme has been shown to be synthesised within phagocytes (McClelland, 1975). Release of the enzyme into the extracellular milieu appears to be rapid and this rate may be increased many fold in response to phagocyte activation, for example by *in vitro* culture of

macrophages or in alveolar macrophages exposed to inhaled particles (Heise & Myrvik, 1967). The release of lysosyme however appears to be independent of phagocytosis as evidenced by studies demonstrating that a phagocytic stimulus does not alter the amount of lysosyme secreted (Unanue, 1976).

(ii) Neutral Proteinases: Collagenase and elastase, in contrast to lysosyme, are secreted in small amounts by unstimulated phagocytes (Unaue, 1976). Following stimulation of these cells by phagocytosis or endotoxin, the production and release of these enzymes is considerably increased (Werb & Gordon, 1975a;b). Together with gelatinase these enzymes are important in the degradation of connective tissue macromolecules such as elastin, collagen and proteoglycans (Sharma, 1986). They may also promote the migration of macrophages through basement membranes (Werb, 1987). Elastase, in addition, has the capacity to degrade immunoglobulins, alpha-proteinase inhibitor, fibronectin and fibrinogen (Werb, 1987).

(iii) Lysosomal Acid Hydrolases: The hydrolases secreted by phagocytes include ß glucoranidase, n-acetyl-ß glucosaminidase, alpha mannosidase and acid phosphatase. Non-elicited macrophages secrete very low levels of these acid hydrolases. Once activated by cell culture (Schnyder & Baggiolini, 1978) or inflammatory stimuli, such as thioglycollate (Schnyder & Baggiolini, 1978), type-specific polysaccharide and peptidoglycan

TABLE 1. Enzymes Secreted by Phagocytes.

Acid hydrolases

- Phosphatase
- Glycosidase
- Ribonuclease
- Deoxyribonuclease
- Proteases
- Lipases
- Sulphatase
- Other, Cathepsin D

Neutral proteinases

- Collagenase
- Elastase
- Myelinase
- Proteoglycan protease
- Cytolytic proteinase
- Plasminogen activator

Lysosyme

Arginase

Lipoprotein lipase

Angiotensin convertase

Protein kinase

BPI (bactericidal/permeability increasing) protein

(PPG) from group A Steptococci (Davies *et al.*, 1974), products from activated lymphocytes (Pantalone & Page, 1975) or by primary amines (Riches & Stanworth, 1982), the amount of hydrolases released is markedly increased. The secretion of the hydrolases can be divided into two categories. In the first, a variety of stimuli will induce the rapid secretion of the enzymes within 1-4 hours (Musson *et al.*, 1980). This rapid release is similar to the release of these enzymes by neutrophils (Henson, 1980). In the second, a more prolonged secretion of the acid hydrolases in response to lower concentrations of stimuli occurs. This can be maintained for up to 2 weeks in culture and is dependent on protein synthesis since cycloheximide will inhibit their release (Schnyder & Baggiolini, 1978).

(iv) Cationic Proteins: A group of chymotrypsin-like cationic proteins that exhibit microbicidal properties were identified in human granulocytes by Odeberg & Olsson (1976) who also showed that the microbicidal effects of these proteins did not depend on the proteolytic activity. BPI is another cationic protein with anti-microbicidal properties identified in PMN cells (Elsbach *et al.*, 1979) and was named BPI for its bactericidal/ permeability increasing properties. A third group of cationic peptides were identified in rabbit PMN cells (Selsted *et al.*, 1985) and later in human granulocytes (Ganz, 1986). Six of these proteins isolated from rabbit PMN cells have been sequenced and

are cysteine and arginine rich peptides of 32-34 amino acids (Selsted *et al.*, 1985) and are members of a family of proteins known as defensins (Ganz *et al.*, 1985). These molecules kill organisms as diverse as *Staphylococcus aureus, Pseudomonas aeruginosa, Escherischia coli, Cryptococcus neoformans* and even the enveloped virus *Herpes simplex* (Ganz *et al.*, 1985). Three similar low molecular weight peptides have been isolated from human granulocytes and are called human neutrophil peptide (HNP) 1-3 (Ganz *et al.*, 1985) and play an important role in the microbicidal mechanisms employed by the granulocytes. These molecules have, as yet, not been identified in human macrophages (Rook, 1989). The bactericidal activity of cationic proteins is generally at a pH of between 7.0 and 8.0. The pH of the phagosome transiently rises to this level and the proteins can then displace the divalent cations (e.g. Ca^{2+}), which cross-link the endotoxin molecules in the outer membrane of Gram negative organisms (Sawyer *et al.*, 1988). The pH in the phagosome subsequently drops (Segal *et al.*, 1981). In this more acidic environment the major effect seems to be the permeabilisation of the outer membrane which may then allow entry of other microbicidal molecules into the organism (Rook, 1989). In addition to the release of these lysosomal substances, the macrophage enhances its bactericidal capacity by taking up products of

neutrophils such as the defensins and lactoferrin (Pruzanski *et al.*, 1984). The latter is effective in either killing bacteria directly or by interacting with reactive oxygen radicals to facilitate the generation of hydroxyl radicals (Ganz *et al.*, 1985). The partially catabolised protein resulting from these degradative mechanisms can then be released from the cell into the extracellular milieu and be taken up by other macrophages for reprocessing or can remain on the cell surface to be presented to other cells of the immune system (Unanue & Allen, 1986).

1.3.3.2. Oxidative Intracellular Killing Mechanisms

1.3.3.2.1. Activation of the Reactive Oxygen Metabolite Producing System

The term 'respiratory burst' refers to a co-ordinated series of oxygen dependent metabolic events that take place when phagocytes are exposed to appropriate stimuli (Segal, 1989a). It was originally thought that the sole purpose of this rise in oxygen consumption was to provide energy for phagocytosis (Babior, 1978a), but Sbarra & Karnovsky (1959) showed that phagocytosis occured under conditions of either high nitrogen or high oxygen concentrations. These findings left unexplained the reason for the increase in oxygen uptake following activation of phagocytes. However, Iyer *et al.* (1961) demonstrated that at least part of the oxygen consumed in the respiratory burst was converted to hydrogen peroxide (H_2O_2) and they proposed

that this H_2O_2 was used as a bactericidal agent. The initial response to an activating signal by the respiratory burst is activation of a NADPH oxidase complex which catalyses the production of superoxide anion (O_2^-) (Pick *et al.*, 1989). NADPH oxidase is therefore of central importance to the production of reactive oxygen metabolites (Babior, 1984). Activation of the O_2 dependent pathway is induced by opsonised bacteria and zymosan, phorbol myristate acetate (PMA), complement component C5a and fluoride ion (F^-) (Baboir, 1978a). This multicomponent oxidase system forms an electron transport chain in the plasma membrane (Riches *et al.*, 1988).

The components of the electron transport chain that have been identified are a protein of molecular weight between 65 and 67kDa (which may be a flavoprotein) (Segal, 1989b), a 47kDa cytosolic protein (Clarke *et al.*, 1990) and cytochrome b (Riches *et al.*, 1988). The cytochrome b found in macrophages, monocytes and neutrophils occurs in the plasma membrane and becomes incorporated into the wall of the phagocytic vacuole (Segal, 1989b). The cytochrome b consists of 2 subunits - a 22kDa protein and a 91kDa glycoprotein (Clark *et al.*, 1990). The 65-67kDa protein is cytosolic (Riches *et al.*, 1988; Clark *et al.*, 1990) and is therefore translocated from the cell interior to the plasma membrane where it binds to NADPH and probably acts as a proximal component of the entire electron transfer system (Segal, 1989b).

Superoxide generation is generally studied by activation with PMA (Riches *et al.*, 1988). PMA activates protein kinase C (PKC) and causes its translocation to the plasma membrane (Sha'afi, 1988). The activated PKC will, in turn, activate the NADPH oxidase system by phosphorylating one or more proteins (Clark *et al.*, 1990). One of the proteins phosphorylated is a 47kDa cytosolic protein which then associates with cytochrome b in the membrane (Segal 1989b). By this series of reactions an electron is transfered ultimately to molecular O_2 to form superoxide (O_2^-) and NADP+ (Segal 1989a). Figure 4 is a schematic representation of the NADPH oxidase system. The O_2^- produced by this series of reactions then dismutates to form hydrogen peroxide (H_2O_2) (Figure 5) (Segal, 1989a). This dismutation occurs spontaneously at a very slow rate (Becker, 1988). The presence of superoxide dismutase found in the cytoplasm of many cells, however, greatly increases this rate thereby protecting the cell against the ravages of O_2^- (Fridovich, 1986).

Glutathione is a constituent of most, if not all, cells and its function is thought to be the neutralisation of H_2O_2 produced in the cells in order to protect the cell from self-destruction (Meltzer, 1977). In the presence of glutathione peroxide and H_2O_2, glutathione will be oxidised with the release of H_2O (Figure 6). The oxidised glutathione (G-S-S-G) is then reconverted to its reduced state (GSH) by a glutathione reductase reaction (Figure 6) (Reed, 1969) which yields the oxidised NADP+. The NADP+ which accumulates as a

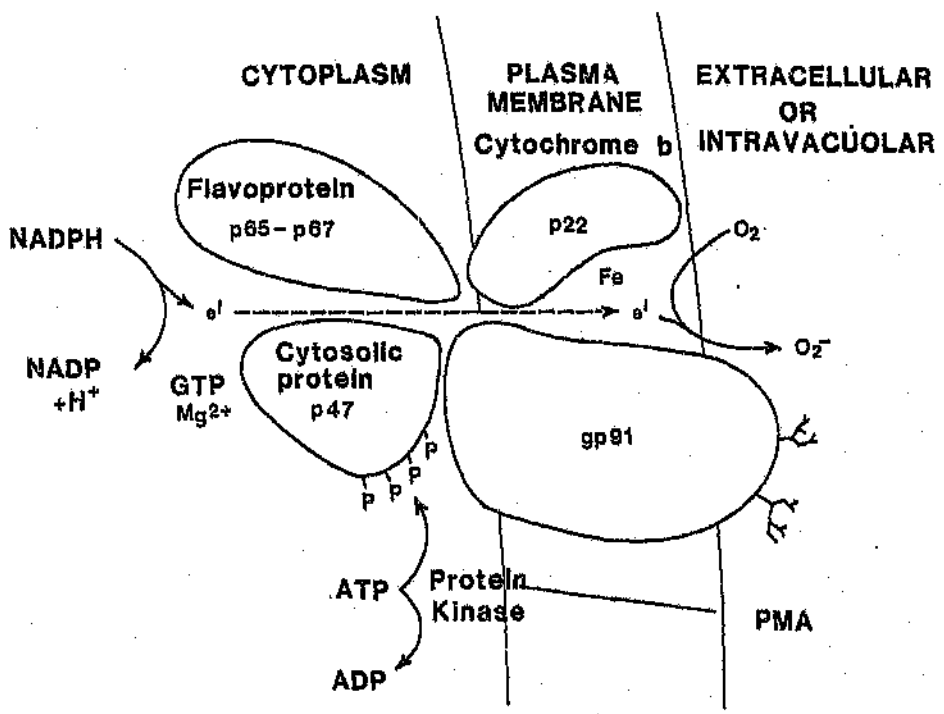


FIGURE 4. Human PMN NADPH oxidase.
(Adapted from Clark, 1990).

FIGURE 5. Production of hydrogen peroxide.

result of these two pathways will then fuel the hexose monophosphate shunt (HMPS) by which glucose-6-phosphate will be oxidised to form 6-phosphogluconate in the presence of the enzyme glucose-6-phosphate dehydrogenase (Figure 6 Reaction 3 - redrawn from Metzler, 1977).

Glucose oxidation through the HMPS is increased during the respiratory burst due to the increased production of NADP+ which is the rate limiting step of the process. Each molecule of glucose which is phosphorylated and subsequently metabolised by the HMPS reduces 2 molecules of NADP to NADPH thus replenishing the supply of reducing agents necessary for the operation of the respiratory burst (Figure 7) (Babior, 1984).

1.3.3.2.2. Bactericidal Mechanisms of the Reactive Oxygen Metabolites

A. Superoxide radical: There is controversy about the microbicidal potential of this anion since Gregory *et al.* (1973), Gregory & Fridovich (1974) and Mandell (1975) could not show microbicidal activity by artificially generated O_2^-. However, it is possible that the concentration of O_2^- production in such systems may be much smaller than that produced in the vicinity of the phagosome (Babior *et al.*, 1975).

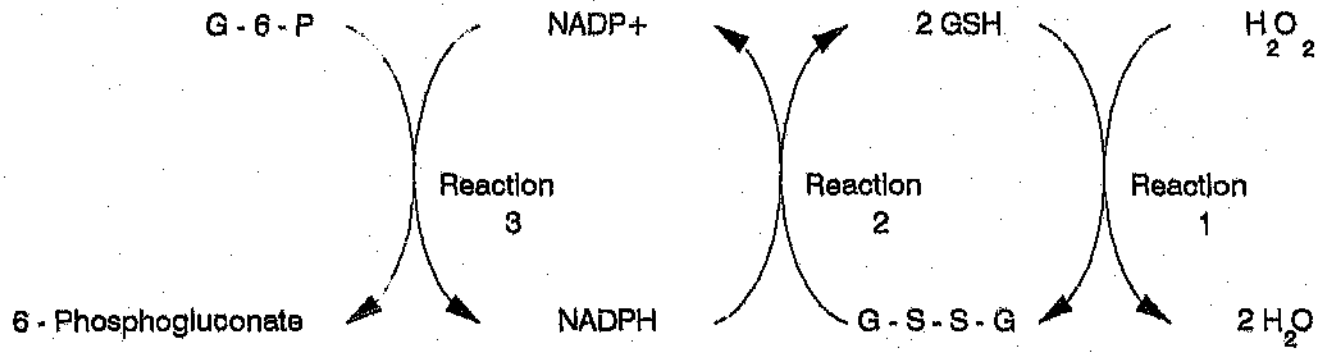


Figure 6: Glutathione - dependent system.

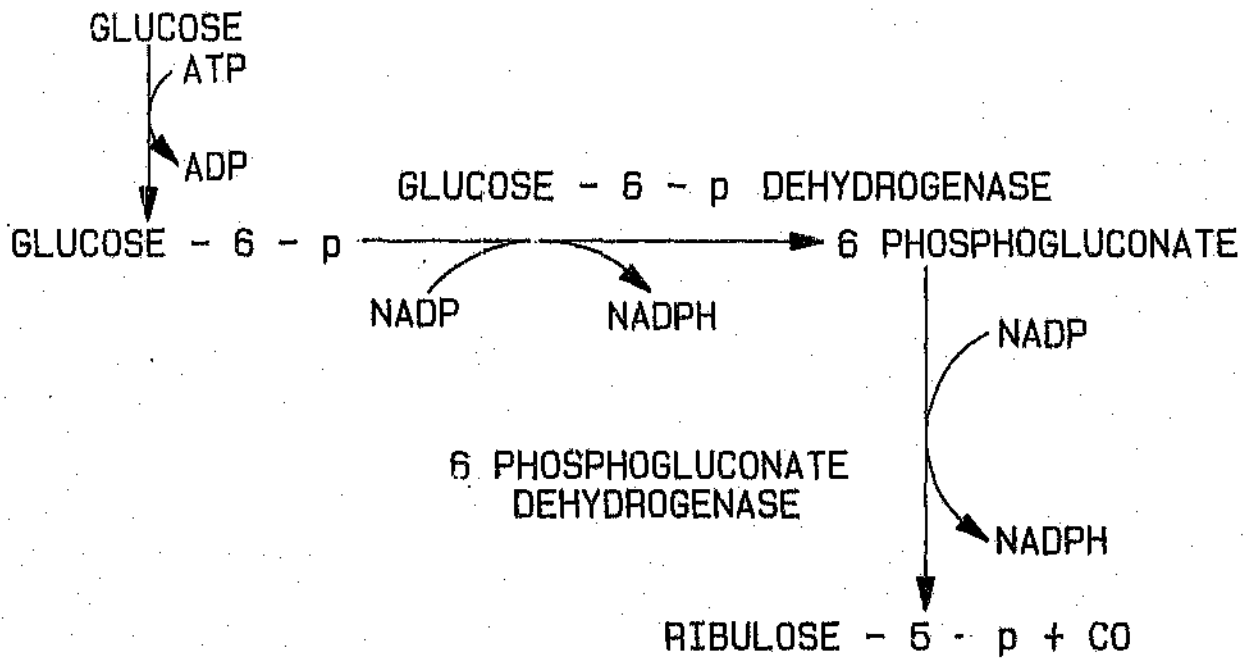


Figure 7: The Hexose Monophosphate Shunt. (Metzler 1977)

B. Hydroxyl radical (OH-): The hydroxyl radical is a highly unstable oxidising species that reacts almost instantaneously with most organic molecules that it encounters (Babior, 1978a). Evidence that both macrophages and neutrophils produce OH- has been obtained by Weiss *et al.* (1977) and Tauber & Babior (1977). The mechanism by which the cells produce OH- is uncertain but one possibility is that OCl-, a product of the myeloperoxidase reaction (discussed below) reacts with H_2O_2 (Figure 8 - reaction 1) to produce singlet oxygen which in turn via the Haber Weiss reaction, forms OH- (Figure 8 - reaction 2) (Klebanoff & Rosen, 1978).

C. Hydrogen peroxide and myeloperoxidase: Although H_2O_2 has a certain amount of bactericidal potency, its potency is greatly augmented through the action of the enzyme myeloperoxidase (Babior, 1978a). This enzyme is present in azurophilic granules of neutrophils and monocytes (Bainton & Farquhar, 1968; Lehrer & Cline, 1969; Salmon *et al.*, 1970) and catalyses the oxidation of halide ions to hypohalite ions by H_2O_2 (Figure 8 - reaction 3).

Cl-, Br- and I- can be oxidised by this enzyme (Klebanoff, 1968; Klebanoff & Shepard, 1984). Several mechanisms have been proposed to explain bacterial killing by the H_2O_2-halide-myeloperoxidase system. The first is the possible halogenation of the bacterial cell wall, which will cause a loss of integrity of the wall leading to cell

death (Zgliczynski & Stelmaszynska, 1975). The second mechanism is the generation of oxidants belonging to a general family of nitrogen-chlorine derivates which are generated via the myeloperoxidase-mediated chlorination of a nitrogenous compound (Figure 8 - reaction 4) (RRNH) (Weiss *et al.*, 1983). Compounds available to react with HOCl are ammonia (produced during neutrophil metabolism) (Grisham *et al.*, 1984), taurine (most abundant free amino acid in leukocytes) (Test *et al.*, 1984); amines; nucleic acids; polyamines and cationic proteins (Test *et al.*, 1984). The N-Cl derivatives produced and released extracellularly are long lived, hydrophilic, low molecular weight compounds with low toxicity but which can react with ammonia to yield a lipophilic oxidising agent monochloroamine (Grisham *et al.*, 1984). The addition of ammonia therefore confers the RNHCl derivatives with bactericidal, cytotoxic and cytolytic properties (Grisham *et al.*, 1984). The third mechanism by which the myeloperoxidase system may be bactericidal is by generating singlet oxygen $^1O_2$ (Figure 8 - reaction 1) (Rosen & Klebanoff, 1977). Singlet oxygen differs from atmospheric oxygen in the distribution of the electrons around the two nuclei. In atmospheric oxygen the electrons form a cylindrical cloud in which the axis is the line joining the nuclei. In singlet oxygen this electron cloud is distorted away from a cylindrical configuration, thereby altering the chemical properties of the molecule.

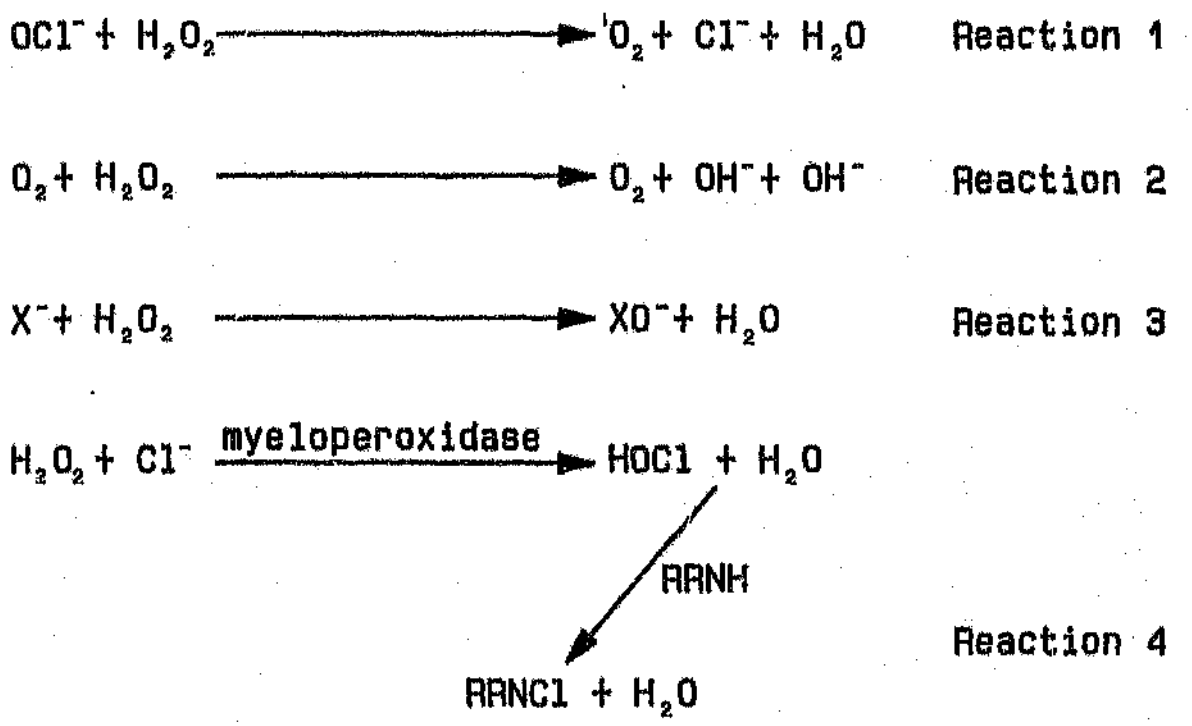


Figure 8: Production of reactive oxygen metabolites

This highly reactive singlet $^1O_2$ has the ability to attack a large variety of double bond containing compounds thereby inflicting lethal damage on the biological systems it comes into contact with (Babior, 1978a).

1.3.3.3. Role of Reactive Oxygen Metabolites

Although the exact mechanisms by which the products of the respiratory burst are involved in bactericidal activity is far from clear, their importance cannot be doubted (Segal, 1989b). Abnormalities of the enzymes involved in the various reactions of the respiratory burst have been demonstrated to result in severe recurrent infections (Holmes *et al.*, 1987). An example of such abnormalities is apparent in chronic granulomatous disease where there is an absence of one of the components of the NADPH oxidase system responsible for the production of O_2^- (Babior, 1978b).

1.4. ANTIGEN PRESENTATION BY MACROPHAGES

An important role of the macrophage is the activation of the antigen-specific arm of the immune response. Fishman & Adler (1963) showed that under certain conditions antibody responses do not occur upon exposure to antigen alone but rather upon interaction with macrophages which had previously phagocytosed the antigen. Work done at the National Institutes of Health, Bethesda, Maryland has shown that the initial antigen binding

cell in a lymphoid cell enriched population is an adherent, phagocytic, radio-resistant cell that appears morphologically to be a macrophage (Rosenstreich & Rosenthal, 1973; 1974; Waldron *et al.*, 1973). Their studies also indicated that antigen recognition involved an initial obligatory macrophage uptake of antigen before interaction of the immunogenic moiety with the T lymphocyte took place. Close contact between the antigen-bearing macrophage and lymphocyte was also shown to be essential since separation of the macrophage bearing antigen and the lymphocyte by a 0.45 micron filter did not allow for initiation of lymphocyte proliferation (Cline & Swett, 1968).

Upon binding to the macrophage, the bulk of the antigen is degraded, but for successful presentation of antigen to T cells to take place only partial catabolism may occur (Unanue & Calderon, 1975). Unanue & Calderon (1975) have shown that antigen may bypass extensive catabolism by 2 mechanisms, either by remaining on the surface membrane for a critical period of time, or by being released from the cell prior to extensive breakdown. Using albumin as an antigen, Schmidke & Unanue (1971) showed that there is heterogeneity in the rate of catabolism of the antigen, with degradation of proteins having a half life varying from 0.6-22 hours. Also 10-25% of the albumin bound resists catabolism and is stored in a stable form with approximately one third of it being on the cell surface (Rosenthal *et al.*, 1975). Ellner & Rosenthal (1975) showed that during the catabolism of 2.4 dinitrophenyl guinea pig albumin (DNP-GPA) 80% of the initially cell bound material was

released from the macrophage, while 20% of the stable residual material remained surface associated. This surface associated residual antigen would then be presented to the T cell.

Ziegler & Unanue (1981) also showed that the macrophage had to be viable, at least for a lag period (the duration depending on the amount of antigen and temperature), presumably during which time antigen processing took place; if the cells were then fixed with paraformaldehyde, antigen presentation to T cells was unaffected. Shimonkewitz *et al.* (1983) further substantiated this by showing that macrophages fixed in low concentrations (0.05% final concentration) of gluteraldehyde were not able to present native or denatured OVA but were able to present proteolytic fragments of the molecule. One other aspect of successful antigen presentation is the genetic control of the entire process. For activation of the T lymphocyte by macrophages ring antigen to occur the 2 cell populations have to be syngeneic (Shevach & Rosenthal, 1973; Rosenwasser & Rosenthal, 1979); if T cells from 1 strain of gu pig were mixed with macrophages of another strain, antigen independent binding would occur, but antigen dependent binding and subsequent activation of the T cells would not (Lipsky & Rosenthal, 1975). In addition, the *in vitro* responses of primed guinea pig T cells to antigens, the response of which is under the control of the histocompatibility-linked Ir genes, would be blocked by antisera directed against the histocompatibility specificities (Shevach *et al.*, 1972).

This indicated that products of the major histocompatibility complex expressed on the surface of the cells were important in macrophage-antigen-T cell interaction.

The histocompatibility-linked Ir genes regulate the response of cells to thymus dependent antigens and this control was thought to be regulated at the level of the T cell (Mitchell *et al.*, 1972), however Shevach & Rosenthal (1973) effectively demonstrated that the macrophage expressed the Ir genes thus suggesting that this was the cell which determined T cell responses. That the Ir genes control the response to certain antigens was shown by Levine *et al.* (1963) where the response of outbred guinea pigs to poly-l-lysine homopolymer [dinitro phenyl (DNP)-PLL] proved to be under the control of a single autosomal gene. Similar control was shown for more conventional antigens, such as insulin (Barcinski & Rosenthal, 1977) where different stains of guinea pigs were shown to recognise and respond to different portions of the insulin molecule.

The availability of inbred strains of mice and guinea pigs permitted the rapid mapping of the Ir genes. The mouse immune response (Ir) genes were mapped by genetic recombination to the MHC between the K and D loci (Figure 9) and the region was thus named the I region for immune reponse (Simpson, 1988). The equivalent region in man is the Class II MHC or HLA-D region (Figure 10) (Trowsdale, 1988). Even after discovering the mapping to the same region of the Ir genes and I region encoded

cell surface antigens, the relationship between the 2 remained unclear. It was hypothesised that the Class II in man, or immune associated (Ia) anitigens in the mouse, were products of the Ir genes (Flavell *et al.*, 1986). Le Meur *et al.* (1985) proved this by showing that transfer of cloned Class II genes into transgenic mice could turn a non-responder strain into a responder one.

The interaction of macrophages with T cells has been suggested to be either in the form of a trimolecular structure with Ia and antigen binding to the T cell independently of one another with Ia serving to stabilise the structure (Herber-Katz *et al.*, 1983; Marfack & Koppler, 1987; Shimonkewitz *et al.*, 1983) or that antigen and Ia interact physically before or after encountering the T cell receptor (Grey & Chesnut, 1985). As hypothesised by Benacceraf (1978; 1981) it was demonstrated that the T cell receptor recognises an antigen-Ia complex and that the antigen binds to the Ia molecule on the antigen presenting cell (APC) (Babbit *et al.*, 1985; Buus *et al.*, 1986). The argument against this was that there was only a limited number of different Ia molecules which had to interact with a myriad of antigens (Grey & Chesnut, 1985). If, however, the binding site for antigen were only a few amino acids long (as suggested by Benacerraf, 1981), many proteins would be able to bind to the same Ia molecule. Grey *et al.* (1989) identified 7 peptides from unrelated proteins which bound to IA^a and showed that these all had a core region of 8 amino acids which were similar to a sequence found in Eαa 327-332 shown to be critical for binding of the latter to IA^a.

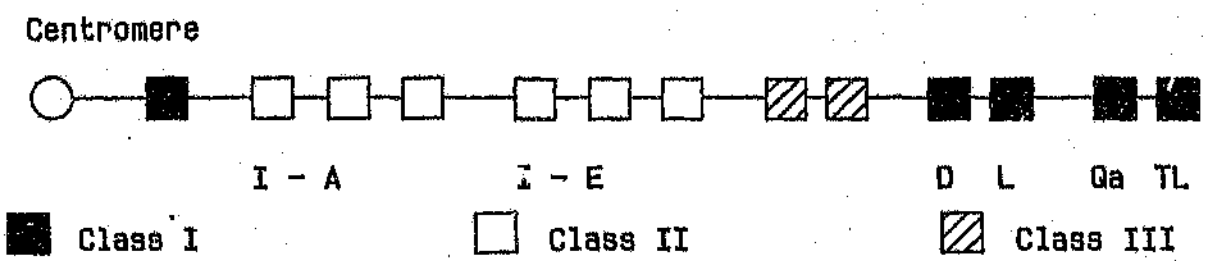


Figure 9: Structural genes for the Mouse Major Histocompatibility Complex.

(Adapted from Barrett, 1988)

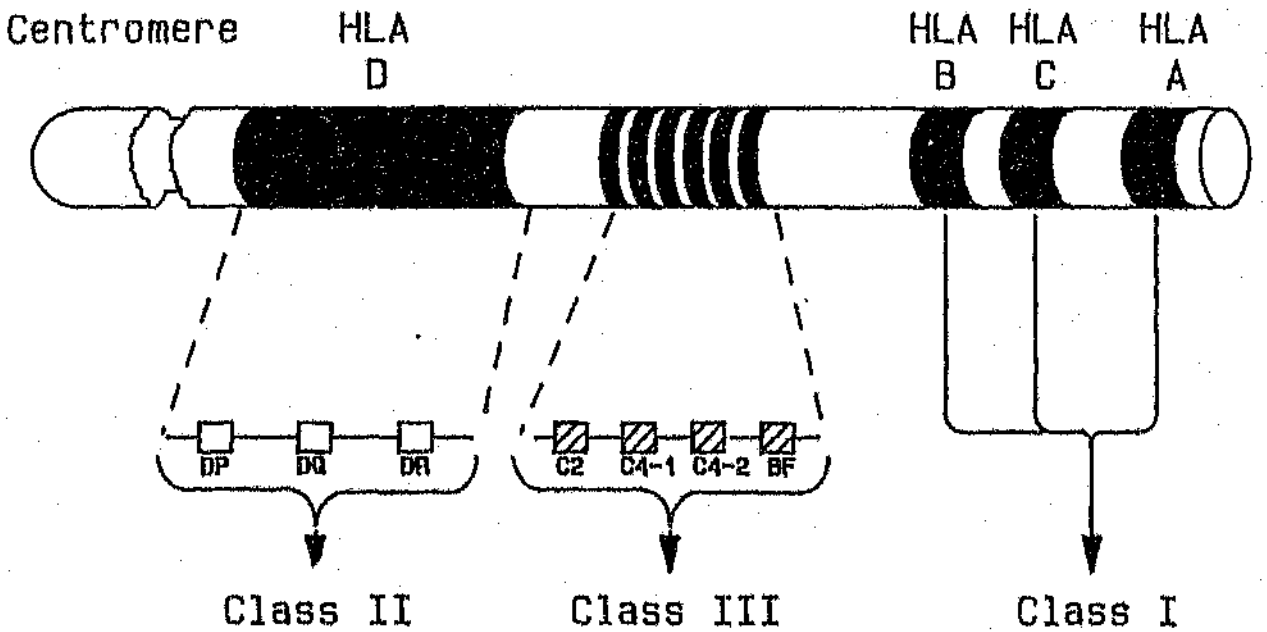


Figure 10: Structural genes for the human major histocompatibility complex on chromosome 6. (Adapted from Arnett, 1988)

Further studies have shown that Ia can present a large fragment of antigen which does not need further proteolytic cleavage, for example hen-egg white lysosyme (HEL) peptide 40-73 could be presented by IA^k APC in spite of the amino acids on either side of the 52-61 binding sequence being present and extending beyond the binding site (Donermeyer & Allen, 1989). Further proof of MHC-antigen interactions was provided by Grey *et al.* (1989) who showed that acid treated Class II MHC (purified from B cells) released peptides which could later recombine to the MHC molecules and Srinivasan & Pierce (1990) isolated a functional peptide-MHC complex from APC's.

Whether interaction of the antigen with Ia occurs on the membrane or in the cytoplasm of the macrophage is not certain, but recent evidence (Leyva-Cobian & Unanue, 1988) suggests that it is a cytoplasmic event and that the Ia-antigen complex is then carried to the cell surface. Donermeyer & Allen (19[illegible]) have lent support to such a theory by demonstrating that once bound to Ia, an antigen becomes resistant to further proteolytic cleavage. This complex, formed in intermediary vessicles in the cytoplasm will be transported to the cell surface where it will then be accessible for recognition by a T cell. Their studies also suggest an explanation of the degree to which antigen degradation within cells occurs. An alternative mechanism suggested by Falo *et al.* (1986) is that processed antigen bind to phospholipid or other carrier molecules, are transported to the cell surface and then interact with Ia.

Stockinger *et al.* (1989) showed a requirement of a variant chain fragment of Ia for antigen processing. [The invariant chain (Ii) fragment is a 31kDa chain which complexes with the polymorphic Ia molecule shortly after its biosynthesis in the endoplasmic reticulum (Simonis *et al.*, 1989).] Fibroblast cell lines which express Ia molecules but do not have the Ii chain, are incapable of presenting native antigen (Shastri *et al.*, 1986). If these lines are transfected with the invariant chain gene, however, successful antigen presentation can then take place (Stockinger *et al.*, 1989). Although the detailed steps involved in this process have not been elucidated, the antigen would be endocytosed, fragmented to peptides and would then interact with Ia (Stockinger *et al.*, 1988). The Ii chain could facilitate binding of the antigen fragment at the appropriate juncture of the Ia molecule. The invariant chain could also participate in intracellular transport and recycling of antigen to the cell surface (Simonis *et al.*, 1989).

CHAPTER 2.

2. TUBERCULOSIS

2.1. INTRODUCTION

Tuberculosis, an infectious disease caused by *Mycobacterium tuberculosis*, is still a major communicable disease especially in developing countries (Wadee, 1989; Schmidt, 1989). In industrialised countries, such as the USA, there has been a rapid decline in the cases of tuberculosis reported amongst the caucasoid population but among other minority groups the decline has been much slower (Snider, 1989). The average annual decline rate in the USA has dropped from 6.7% for the period 1981-1984 to a 0.4% decline rate for 1987-1988 (MMWR, 1990). An increased incidence of tuberculosis amongst inmates of correctional institutes in the USA has recently been noted (Snider & Hutton, 1989; Braun *et al*., 1989). It has been suggested that this may have an impact on the incidence of tuberculosis occuring in the community into which these inmates will be released (Snider & Hutton, 1989).

Although there has also been a decline in the incidence of tuberculosis in England, which is ascribed partly to successful BCG vaccination, tuberculosis is still frequently seen among children, especially those of Asian and West Indian origin (Silverman, 1988). The incidence in these communities living in the UK is estimated at 40/100 000 and 17/100 000 respectively (Silverman, 1988).

Although tuberculosis has always had a bimodal age distribution, occuring in young children and older people, an increased incidence is now being seen in the 24-44 year age group (Bass, 1989). This is the same age group which has an increased

incidence of acquired immune deficiency syndrome (Aids) (Bass, 1989). Current evidence points to a very strong risk factor in immunosuppressed individuals for the progression of latent tuberculosis to clinical disease (Selwyn *et al.*, 1989; Quinn, 1989). This is particularly true in the case of patients with Aids in whom infection with the human immuno-deficiency virus (HIV) is associated with immunosuppression.

In developing contries an even greater problem exists. It is estimated that approximately 3-4 million new smear-positive (pulmonary) and another 3-4 million smear-negative (extra-pulmonary) tuberculosis cases develop anually (Styblo, 1989). About 2-3 million people in these countries die of tuberculosis every year (Styblo, 1989). This occurs in spite of extensive attempts by the World Health Organisation to control tuberculosis (Styblo, 1989). The reasons for failure of the tuberculosis control program in these developing countries are many. These include an inadequate diagnosis of the sources of infection (i.e. people with smear-positive pulmonary tuberculosis) and a chemotherapeutic regimen that is difficult to institute successfully (Styblo, 1989). Since therapy for tuberculosis is a prolonged process, lasting from 6-12 months; compliance is poor and leads to treatment failure and drug resistance (Snider, 1989). Another factor which may affect the irradication of tuberculosis in such countries is the high incidence of patients with Aids. In Africa, unlike Europe, where tuberculosis has a bimodal age distribution, Africa demonstrates a high incidence of individuals between 20 and 50

years of age infected with the tubercle bacillus (Styblo, 1989; Bass, 1989). A relatively high proportion of them are exposed to a substantial risk of exogenous primary infection or reinfection with the tubercle bacillus owing to the high incidence of tuberculosis and to the immunosuppression resulting from AIDS (Quinn, 1989). It is therefore inevitable that the absolute numbers of cases of tuberculosis will increase in developing countries were Aids is a major health problem (Styblo, 1989).

In South Africa current estimates of the incidence of tuberculosis, based on notifications, range from 206-269/100 000 (London, 1989). However, an increased incidence is currently being noted in certain areas of the Cape (London, 1989). In spite of effective diagnosis and treatment, the prevalence of tuberculosis among black miners in South Africa is still very high with an incidence in the order of 500/100 000 (Cowie, 1989). A reason fo' this high rate of tuberculosis may be that infected individuals do not present themselves for treatment and therefore contribute to the perpetuation of the disease in the working population and their home area (Benatar, 1982). Another reason for this high rate of tuberculosis is the migrant labour system. Infected miners who become ill often return home and the disease is easily spread among the extended family and community where medical facilities are often scarce (Cowie, 1989).

It is therefore essential that improved diagnostic techniques, efficient therapy regimens and more effective vaccines be developed if tuberculosis is to be irradicated (Schmidt, 1989).

A more comprehensive knowledge of the host-parasite relationship; the chemistry and functional role of mycobacterial antigens in disease processes and the regulation of the immune response to these antigens may, therefore provide new avenues for the treatment of tuberculosis (Wadee, 1990).

2.2. TUBERCULOSIS: THE DISEASE

Tuberculosis is a chronic infectious disease of long duration in human beings that is caused by *Mycobacterium tuberculosis* (Youmans, 1986). It is usually acquired by inhalation of droplet nuclei containing the tubercle bacilli. The droplets are spread from person to person when infectious material is discharged into the air by coughing or by other means (Farer, 1979). The usual portal of entry is the lower respiratory tract where these particles can reach the most peripheral portion of the bronchial tree and can lodge in the alveoli where a focus of infection can be established (Riley, 1974). Although the airborne route is the main route of transmission, tuberculosis has also been shown to be transmitted via the gastro-intestinal route or by direct inoculation through the skin or mucous membranes (Farer, 1979).

Pulmonary tuberculosis accounts for approximately 85% of all the tuberculous cases (Steigbigel, 1979). The symptoms may range from asymptomatic individuals to those with fever, weight loss, productive cough, haemoptysis and extensive infiltrates with cavitation in the lung (Steigbigel, 1979). The first clinically evident manifestation in man is a granulomatous nodule termed the Ghon focus (Grange, 1980). The centre of the granuloma may often contain necrotic tissue of "cheese-like" appearance (caseation), surrounded by lymphocytes and macrophages (Grange, 1980). Non-healing primary foci in the lung enlarge and the caseous centres frequently liquefy. Discharge of this liquified material by ulceration into the pleural cavity leads to pneumonia (Grange, 1980).

During the progression of the primary disease, the tubercle bacillus can spread to distant sites resulting in tuberculosis of the liver, spleen, kidneys, central nervous system, joints, bone, bone marrow, pericardium, peritoneum, intestine or genito-urinary tract (Steigbigel, 1979).

Miliary tuberculosis is a disseminated form of the disease characterised by a diffuse miliary pattern of tubercles distributed in many organs of the body (Grange, 1980). The symptoms of this form of tuberculosis are usually non-specific. Weakness, anorexia, fatique, weight-loss and fever are very common. Cough is seen in 18-65% of patients, while headaches and abdominal pain often reflect involvement of the central

nervous system and intestinal tract respectively (Proudfoot, 1969). Predisposing factors for this form of disease include alcoholism, pregnancy, neoplastic disease and parental drug abuse (Munt, 1971).

2.3. THE IMMUNOLOGIC RESPONSE TO *MYCOBACTERIUM TUBERCULOSIS*

The earliest reactions in primary tuberculosis are unknown but data accumulated from animal studies indicate that an acute inflammatory reaction occurs in the tissues early in the infection such that organisms are phagocytosed by polymorphonuclear leukocytes (Lucas, 1988). These cells are replaced by alveolar macrophages and by macrophages which mature from monocytes circulating in the blood stream (Dannenberg, 1982). Both cell types are attracted to the site by released bacilli, cellular debris and a variety of chemotactic factors of host origin such as the complement component C5A (Dannenberg, 1989) Macrophages apparently differ in their ability to kill invading mycobacteria (Danneberg *et al.*, 1963). Resident alveolar macrophages do not kill the invading mycobacteria, either because they remain at the periphery of the lesion far away from the bacilli in an established lesion (Danneberg, 1989) or because they have an inability to kill the virulent tubercle bacillus once it has been ingested (Collins, 1989). Blood-derived monocytes which emigrate to the leasion, are activated by lymphokines and can then kill the bacterium (Wiegeshaus *et al.*, 1989). These cells are therefore completely responsible for the fate of the early lesion (Danneberg, 1989).

In the early lesion the tubercle bacillus and the macrophage live in a state of symbiosis (Danneberg, 1989). In 2-4 weeks this balance, however, is disturbed with the gradual development of a specific immune reponse (Danneberg, 1989). Macrophages become activated by antigen-specific T cells or lymphokines released by those cells, and have an increased ability to destroy the tubercle bacillus (Ando *et al.*, 1977). At the same time many of the macrophages are killed either by the large number of multiplying bacilli and their products or by direct cytotoxic action of the bacterium itself (Lucas, 1988). A tubercle thus forms that consists of a caseous necrotic centre surrounded by a lymphocyte mantle consisting mainly of T suppressor cells (Van den Oord *et al.*, 1984), some macrophages and other cell types. These T suppressor cells may have an important role to play in the cellular unresponsiveness which develops in animals and man (Watson & Collins, 1980). Recent evidence in favour of this indicates that macrophages which have phagocytosed mycobacteria release suppressor cell activating factor (SCAF) resulting in activation of suppressor mechanisms locally (Wadee & Rabson, 1981; Wadee *et al.*, 1983).

Even though the pathogenesis of caseation in tuberculosis is not clearly understood, several factors are thought to contribute to its formation (Dannenberg, 1989), viz. high concentrations of lymphokines produced in response to a tuberculous infection by T (and possibly B) lymphocytes may be toxic to the tissues; large amounts of toxic components of the mycobacterium could be

released during the bacterial breakdown process resulting in tissue damage (Dannenberg, 1989); ischaemia from thrombosed blood vessels could cause infarction of the area (Courtade *et al.*, 1975); activation of the complement pathway might damage host cells in the area; hydrolytic enzymes and reactive oxygen radicals released from live and disintergrating macrophages might directly injure the surrounding tissue; and the macrophages might release monokines such as tumour necrosis factor (TNF) which may also damage the surrounding tissues (Dannenberg, 1982; 1989; Lucas, 1988).

Whether the granuloma progresses or regresses depends upon the ability of the macrophages entering the granulating tissue to destroy the invading organism (Collins, 1989). Minute caseous foci can probably be eliminated by surrounding macrophages (Dannenberg, 1989) but larger foci (5-20mm) cannot since macrophages cannot penetrate very far into the caseous centre (Courtade *et al.*, 1975). These foci probably undergo fibrous encapsulation and are isolated within the host (Grange, 1980). It is these large foci which may be reactivated years later as a result of immunosuppression due to a variety of reasons such as stress, steroid therapy or cancer chemotherapy (Wiegeshaus *et al.*, 1989).

Liquefication of the caseous centre of the tubercles is one of the most harmful responses in this disease (Dannenberg & Sugimoto, 1976). It occurs as a result of hydrolysis of the protein, lipid and nucleic acid components of caseous tissue by

the hydrolytic enzymes of macrophages (and perhaps granulocytes) (Weiss & Singer, 1953). The liquefied caseum is an excellent culture medium for the organism, which will now multiply extracellularly resulting in a large antigenic load. This will cause an over stimulation of the delayed type hypersensitivity response of antigen-specific T cells. This response, which in the presence of low concentrations of bacillary antigen is beneficial, will, in the presence of high concentrations of antigen, result in cell death and tissue destruction (Dannenberg, 1982). The walls of nearby bronchi often become necrotic, rupture and form a cavity. The bacilli and small amounts of liquefied tissue can now be discharged into the airways and reach other parts of the lung and environment (Dannenberg, 1989). The organisms may now spread to various organs without any apparent clinical manifestations (Farer, 1979). Intensive dissemination of the organism by the haematogenic route can thus result in serious disease such as tuberculous meningitis and miliary tuberculosis, represented by small lesions spread thoughout the body (Ivanyi, 1986).

During the primary infection there is clonal expansion of antigen-specific T cells which, on re-exposure, rapidly produce lymphokines and thereby accelerate macrophage activation and accumulation at the site of infection (Orme & Collins, 1984). As a result, the lesions remain small and heal rapidly such that secondary tubercles of either haematogenous or lymphogenous origin usually do not progress in immunocompetent adult humans (Dannenberg, 1989). Factors which predispose to post-primary

infection include:- age, concommitant diseases (e.g. Aids), hormonal changes (e.g. pregnancy) and immunosuppressive therapy (e.g. in cancer) (Farer, 1979).

2.4. THE ROLE OF THE PHAGOCYTE IN TUBERCULOSIS

On coming into contact with the tubercle bacillus, the polymorphonuclear cells and macrophages will ingest the organism (Martin *et al.*, 1950; Dannenberg, 1982; Brown *et al.*, 1987; Rook, 1988; Wiegeshaus *et al.*, 1989). The uptake of the mycobacterium requires initial contact between the organism and specialised structures on the membrane of the cells that are capable of triggering the phagocytic process (Piessens, 1989).

The first cells to arrive at the site of infection are the neutrophils which will become 'activated' and ingest and degrade the organisms (Brown *et al.*, 1987). These phagocytes therefore play an important role in the early phases of infection, and are then replaced by alveolar macrophages and macrophages emigrating from the circulating pool of monocytes (Dannenberg, 1989). In addition to degrading the invading organisms, the macrophage also presents the processed mycobacterial antigen on its surface, in context with host MHC Class II molecules to helper T lymphocytes (De Vries, 1989; Bhardwaj & Colston, 1988). The T lymphocytes will respond to this specific antigenic challenge by undergoing clonal proliferation and producing lymphokines (Dannenberg, 1989). The lymphokines will then further activate the macrophages and enhance their tuberculocidal capabilities.

Even though the existence of several adherence receptors have been demonstrated on phagocytic cells; mannose-fucose (Wilson & Pearson, 1986); complement receptors CR1 and CR3 (Payne & Horwitz, 1987); Fc receptors and leukocyte function associated antigens such as LFA-1 and LFA-3 (Springer *et al.*, 1987), the exact receptor involved in promoting the entry of the tubercle bacillus into these cells is not known (Peissens, 1989). There is, however, some evidence from *in vitro* studies on human monocytes which indicates that the CR1 and CR3 receptors on the monocyte mediate the phagocytosis of the bacillus (Schlesinger *et al.*, 1990). Once the organism has bound to the surface of the phagocyte, the plasma membrane will invaginate into the cell and close off around the organism forming a phagosome (Legrange *et al.*, 1983). Consequently the phagocyte undergoes activation and will exhibit profound morphological changes. The cells now demonstrate an increase in surface area, ruffling of the plasma membrane and random migration (Grange, 1980). There is also an increase in the number of mitochondria and lysosomes, an increased metabolic activity, increased phagocytic ability (especially for antibody or complement coated particles; Grange, 1980) and increased tuberculocidal capabilities (Armstrong & D'Arcy Hart, 1971; Wadee, 1990). Within the cytoplasm, enzyme containing lysosomes fuse with the phagosome, forming a phagolysosome (Sharma, 1986). Lysosyme and other hydrolytic enzymes are released by lysosomal granules and will exert their digestive activities on the ingested organisms (Mitchison *et al.*, 1963). In addition, toxic oxygen radicals, such as hydrogen peroxide and

superoxide anion, are produced by the respiratory burst which accompanies phagocytosis (Paton & Ferrante, 1983; Klebanoff & Harmon, 1975; Mitchison *et al*, 1963).

2.4.1. Macrophage Activation by Lymphokines

The lymphokines released by the T lymphocytes have been shown to enhance macrophage function (Nathan *et al.*, 1983). Crude supernatants from antigen or mitogen stimulated lymphocytes have been shown to augment antimicrobial activity (Fowles *et al.*, 1973); enhance HMPS activity (Nathan *et al.*, 1971) and to enhance the secretion of chemically reactive reduced metabolites of molecular oxygen (Nakagawara *et al.*, 1982).

The best characterised lymphokine with macrophage activating function (MAF) is IFN-gamma (Nathan *et al.*, 1983) and the effect of this lymphokine on macrophages will be discussed in Chapter 3. Other lymphokines with MAF activity have, however, been described.

Interleukin 3 (IL-3) or multi-CSF functions as a MAF which selectively regulates the accessory cell characteristics required for antigen presentation, rather than the cytolytic function of the macrophage (Frendl & Beller, 1990). GM-CSF and M-CSF have been shown to increase the ability of macrophages to kill *Leishmania mexicana amazonensis* (Ho *et al.*, 1990). In addition, GM-CSF (together with IL-2 and IL-4) was found to act as a co-factor for IFN-gamma in activating macrophages for antimicrobial function (Belosevic *et al.*, 1988).

IL-4 has also been shown to induce tuberculostatic potential in murine bone marrow-derived macrophages which are infected with *M.bovis* organisms (Flesch & Kaufmann, 1990).

These lymphokines may play an important role in activating macrophages for tuberculocidal activity, although the role played by each has not been determined.

2.5. MECHANISMS OF INTRACELLULAR SURVIVAL OF MYCOBACTERIA

In spite of the many intracellular degradative mechanisms demonstrated by phagocytes, many pathogenic organisms and in particular *M.tuberculosis* have the ability to survive and grow within the cytoplasm of these cells (Nathan *et al.*, 1980). Various escape mechanisms exist which enhance microbial virulence by interfering with the phagocyte's ability to recognise, ingest and kill the organisms (Densen & Mandell, 1990). These include:-

- interference with macrophage activation (Pabst *et al.*, 1988);
- inhibition or neutralisation of oxygen metabolite production (Kusunose *et al.*, 1976; Chan *et al.*, 1989);
- inhibition of phagosome-lysosome fusion (Goren *et al.*, 1974; Armstrong & D'Arcy Hart, 1975);
- disruption of phagosomal membranes and inhibition of degradation (Hooper *et al.*, 1986; Wadee, 1990).

2.5.1. Inhibition of Macrophage Activation

The ability of macrophages to kill mycobacteria depends on their state of 'priming' or 'activation' (Ando *et al.*, 1977). Macrophages primed *in vitro* or activated *in vivo* produce more oxygen metabolites than do unprimed or 'resident' macrophages when they phagocytose mycobacteria (Dannenberg *et al.*, 1972). Agents which cause priming *in vitro* include bacterial LPS, muramyl dipeptide (MDP) and IFN-gamma (Pabst & Johnson, 1980). However, these agents have been shown not to directly stimulate the release of O_2^-, but rather to prime macrophages for enhanced release in response to a subsequent stimulus (Pabst *et al.*, 1988). Sulphatide 1 (SL-1), a tetra-acylated trehalose sulphate is found in the outer cell wall of mycobacteria (Goren *et al.*, 1982; Pabst *et al.*, 1988) and has been shown to inhibit this 'priming' of macrophages (Pabst *et al.*, 1988). In their experiments Pabst *et al.* (1988) showed that SL-1 inhibited several parameters of activation including O_2^- release, phagocytosis and the release of IL-1. Since SL-1 did not directly affect the NADPH oxidase enzyme system or inhibit these three functions in unprimed cells, it is probable that SL-1 affects the processes that convert an unprimed monocyte to an activated macrophage (Pabst *et al.*, 1988).

2.5.2. Derangement of Electron Transport in the Respiratory Chain in Mitochondria

Cord factor, or trehalose dimycolate (TDM), has been shown to be highly toxic to leukocytes (Bloch, 1950) and to cause a derangement of the mitochondrial electron transport system in liver. lung and spleen tissue (Goren, 1972).

Cord factor was originally thought to be responsible for the tendency of virulent *M.tuberculosis* organisms to grow in parallel cords (Bloch, 1950). This activity of cord factor has however never been proven (Goren, 1972). Cord factor is a regular constituent of Wax C in most virulent strains of mycobacteria (Noll, 1957). Wax C consists mainly of long chain fatty acids which are esterified with compounds such as phthiocerol, glycerol and trehalose to form lipids of a molecular weight ranging from 1 000 - 300 daltons (Noll, 1957). Bekierkunst & Artmar (1962) showed that cord factor has been responsible for the attenuation in activity of pyridine nucleotide (NAD) dependent microsomal enzymes. This occurs partly due to the detrimental stimulation of nicotinamide adenine dinuclease activity and the concomitant depression in levels of nicotinamide adenine dinucleotide. In addition to its effect on the electron transport in the respiratory chain, cord factor can directly attack the mitochondrial membranes resulting in swelling and breakage of limiting membranes (Kato, 1969). It is possible that the cord factor could be released from the degraded tubercle bacilli and attack the phagosomal membranes in

the same way it attacks the mitochondrial membrane. Release of high concentrations of the cord factor might then result in membrane rupture and intracellular release of otherwise trapped virulent organisms (Goren *et al*, 1974a).

The final way in which cord factor may contribute to the survival of the organism was suggested by Bloch in 1950 when he showed that cord factor inhibited the migration of leukocytes *in vitro*.

2.5.3. Resistance to Oxidative Killing Systems

Following phagocytosis, phagocytes demonstrate an increase in the oxidative respiratory burst and in the activity of the hexose monophosphate pathway (McRipley & Sbarra, 1967). This results in the production of H_2O_2, superoxide anion, hydroxyl radical and singlet oxygen (McRipley & Sbarra, 1967) which may in themselves be directly toxic to bacteria. Their toxicity may be further enhanced through the generation of more reactive molecules (Lowrie & Andrew, 1988), such as hypohalite ions (Klebanoff, 1968).

Superoxide produced by activation of the NADPH oxidase system (discussed in section 1.3.3.2.1.) is a highly reactive oxygen radical which is capable of breaking down sugar molecules (Chan *et al.*, 1989). The tubercle bacillus, however, seems to be fairly resistant to this molecule (Jackett *et al.*, 1978a). *M.tuberculosis* organisms have been shown to contain

superoxide dismutase (Kusunose *et al.*, 1976) which coverts superoxide to hydrogen peroxide (H_2O_2) and oxygen (Mandell, 1975).

A role for H_2O_2 in killing mycobacteria was suggested as far back as 1963 by Mitchison *et al.*, who showed that resistance of the mycobacterium to H_2O_2 was associated with increased virulence of the organism. More direct evidence for the importance of H_2O_2 in the killing of mycobacteria by phagocytes was supplied by Walker & Lowrie (1981) and Sharp *et al.* (1985). Walker & Lowrie (1981) showed that macrophages activated with phorbol myristate acetate (PMA) could kill *M.microti*. The addition of exogenous catalase, [which decomposes H_2O_2 to oxygen and water (Mandell, 1975).] however protected the organism from intracellular killing, indicating that H_2O_2 was responsible for killing the organism. Sharp *et al.* (1985) provided additional evidence that H_2O_2 played a role in killing mycobacteria. They showed that *M.lepraе* organisms, which are catalase negative and are killed by H_2O_2 *in vitro* are also killed by mouse peritoneal macrophages.

Although susceptibility to H_2O_2 correlates with low virulence in many strains of *M.tuberculosis*, some laboratory-attenuated strains retain their resistance to H_2O_2 (Jackett *et al.*, 1978a). This implies that anti-microbial mechanisms other than direct action of H_2O_2 kill these organisms within the macrophage (Jackett *et al.*, 1980). The myeloperoxidase, peroxidase-halide (MPH) system

(discussed in section 1.3.3.2.2. (C)) has been shown to augment the bactericidal potency of H_2O_2 by forming hypohalite ions (Klebanoff, 1968). The MPH system which was shown to be effective in killing *M.tuberculosis* used either iodide or chloride ions as co-factors (Jackett *et al.*, 1978b).

The peroxidase content of macrophages has been shown to be very low in comparison to PMN cells and monocytes (Lepper & D'Arcy Hart, 1976). However, catalase can replace peroxidase in the killing system, since it can function peroxidatively in the presence of a low steady concentration of H_2O_2, particularly in an acid environment (Klebanoff & Hamon, 1975). Macrophages from various sources, [pulmonary alveolar macrophages of rabbit (Gee *et al.*, 1970) and rat (Davies *et al.*, 1979); and peritoneal exudate macrophages of guinea pig (Simmons & Karnovsky, 1973)], have been shown to contain catalase. It is therefore possible that a catalase, peroxide, halide system may substitute for the MPH system which operates in polymorphonuclear cells and monocytes (Klebanoff & Hamon, 1975). Catalase reacts with H_2O_2 to form an enzyme-substrate complex which reacts with a second molecule of H_2O_2 to form oxygen and water (Mandell, 1975). When the H_2O_2 is maintained at a very low steady state concentration by a H_2O_2-generating system; a number of substances, including thyroid hormones and iodide ions, can successfully compete with the second molecule of H_2O_2 for oxidation by the catalase-H_2O_2 enzyme-substrate complex (Klebanoff & Hamon, 1975). It is under these

conditions that a microbicidal effect is seen (Klebanoff & Hamon, 1975). Jackett *et al.* (1980) showed that the catalase, halide and hydrogen peroxide complex was toxic to *M.tuberculosis in vitro*, but that susceptibility to catalase mediated killing did not correlate with the virulence of the organism.

In contrast to macrophage derived catalase, the presence of the enzyme within mycobacteria has been suggested to have a protective effect for the organism (Lowrie & Andrew, 1988). Catalase from the tubercle bacillus can decompose H_2O_2 to oxygen and water thereby neutralising the toxic effect of H_2O_2 (Mandell, 1975).

Although most strains of *M.tuberculosis* have been shown to possess catalase, a few strains are devoid of the enzyme (Wheeler & Ratledge, 1988). It is of interest to note that these latter strains are more susceptible to the lethal effects of peroxide and are usually less virulent than strains that contain catalase (Lowrie, 1983). Nevertheless these strains lacking catalase and *M.leprae* (which has no catalase) are still pathogenic organisms. It is therefore possible that the production of toxic oxygen metabolites by the host is not by itself sufficient to kill mycobacteria. It is also possible that pathogenic mycobacteria have other ways of evading toxic oxygen metabolites (Wheeler & Ratledge, 1988).

One such chemically defined mycobacterial component that protects mycobacteria from oxidative degradation is the lipophilic cell envelope of the organisms (Wheeler & Ratledge, 1988).

Chan *et al.* (1989) showed that the phenolic glycolipid I (PGI) of *M.leprae* in infected cells and tissues may protect the mycobacterium from oxygen radical damage. PGI is a major constituent of the ultrastructurally observed electron-transparent zone surrounding the bacillus (Hunter & Brennan, 1981). This zone acts as a scavenger of oxygen radicals in a cell free system (Hunter & Brennan, 1981). Goren *et al.* (1974b), while studying a series of attenuated strains of *M.tuberculosis*, identified one common structural feature that distinguished them from the more virulent strains. The factor was termed 'attenuation indicator lipid' and was shown to be a phenolic phthiocerol diester. This molecule has a structure very similar to a synthetic altered form of PGI produced by Chan *et al.* (1989) which was shown to be unable to scavenge OH-. Attenuated strains of the tubercle bacilli may therefore have on its surface lipids altered in such a way that they are unable to scavenge oxygen radicals and are consequently killed (Chan *et al.*, 1989).

2.5.4. Inhibition of Phagosome-Lysosome Fusion

M.tuberculosis has been shown to escape intracellular degradation by preventing phagosome-lysosome fusion (Armstrong & D'Arcy Hart, 1971). Several mechanisms have been suggested to explain this phenomenon (Rastogi & David, 1988). By coating the surface of the tubercle bacillus with specific antiserum prior to phagocytosis, reversion of non-fusion was observed (Armstrong & D'Arcy Hart, 1975). Later studies by Frêhel & Rastogi (1987) showed similar results using *M.leprae*. This would indicate that the surface properties of the bacterium are responsible for inhibition of phagosome-lysosome fusion. The exact nature of the surface-related inhibitor still remains unknown but the possibilities are that either the mycobacterium produces a 'fusion inhibitor factor' (Armstrong & D'Arcy Hart, 1975) or that the sulpholipids in the outer layer of the bacterium are themselves inhibitory (Goren *et al.*, 1974a & b; 1976). These studies by Goren *et al.* show that the presence of strongly acidic lipids in virulent strains of the tubercle bacilli are responsible for the inhibition of phagosome-lysosome fusion. Later studies by Rastogi & David (1988) confirmed these findings by demonstrating the presence of strongly acidic SO_4^{2-} groups present on the surface of the H37Rv strain of the tubercle bacillus but not on the avirulent strain, H37Ra.

The tubercle bacillus produces other substances which may play a role in inhibiting phagosome-lysosome fusion (Rastogi & David, 1988). One such molecule is cyclic-adenosine monophosphate (cAMP). Lowrie *et al.* (1975) have shown a marked elevation

of cAMP in macrophages ingesting live *M.microti* but not in those ingesting dead organisms. They showed that cAMP was released by the bacterium into the phagosome and that this release inhibited phagosome-lysosome fusion.

Ammonia produced by mycobacteria may also cause inhibition of phagosome-lysosome fusion (Gordon *et al.*, 1980). This group showed that culture filtrates of *M.tuberculosis* inhibit fusion of yeast containing phagosomes with lysosomes. Their studies effectively demonstrated that the inhibition was caused by ammonia present in the culture filtrates. Although ammonia has been shown to raise intralysosomal pH and to inhibit the degradation by lysosomal proteins, it is unlikely that its inhibitory action on phagosome-lysosome fusion operates through interference with pH and protein metabolism (Gordon *et al.*, 1980). This is borne out by studies using chloroquine which demonstrate similar effects on pH and protein metabolism but with an enhancement of phagosome-lysosome fusion (Gordon *et al.*, 1980).

A third factor which is thought to cause inhibition of phagosome- lysosome fusion is the presence of polyanionic compounds (Rastogi & David, 1988). As discussed earlier, Goren *et al.* (1976) showed that strongly acidic sulphated glycolipids found on the surface of the tubercle bacillus were able to induce inhibition of phagosome-lysosome fusion. This was seen both in experiments where the sulfatide was ingested by the phagocyte prior to exposure to yeast, and in experiments

where yeasts used to assess phagosome-lysosome fusion were coated with the sulfatide (Goren *et al.*, 1976). To ascertain if the effect of the sulfatide was due to its polyanionic nature, several *in vitro* studies were performed using polyanionic substances. Draper *et al.* (1979) showed that both suramin and poly-alpha-D glutamic acid (lysosomotropic polyanionic substances) could inhibit phagosome-lysosome fusion in macrophages ingesting *M. lepraemurium*, an organism which does not normally inhibit phagosome-lysosome fusion. Macrophages pre-treated with suramin (D'Arcy Hart & Young, 1975) or poly-alpha-D glutamic acid (D'Arcy Hart & Young, 1978) also showed inhibition of phagosome-lysosome fusion following ingestion of yeast. These studies all seemed to indicate a possible role for these polyanionic agents in preventing phagosome-lysosome fusion in magrophages infected with *M. tuberculosis*. More recently Goren *et al.* (1987) have refuted this hypothesis. These authors suggest that the polyanionic agents concentrate in lysosomes as a gelatinous, sluggishly moving mass that physically entraps particulate electron opaque markers or the acridine orange in a matrix that may not transfer to phagosomes for many hours. Lowrie (1988), however, suggests that this may have resulted in an overestimation of the inhibitory effects of the polyanionic agents, but that "the reality of the phenomenon itself does not seem seriously threatened by thes considerations".

2.5.5. Modulation of Lysosomal pH

The ability of intracellular pathogens to alter pH levels within phagosomes provides an effective mechanism for bacterial survival within the host cell (Black *et al.*, 1986; Horwitz & Maxfield, 1984). This would result in a decrease in the hydrolytic activity of lysosomal enzymes and a probable impairment of fusogenic activity of the phagosome with lysosomes (Kielian & Cohn, 1980). Chicurel *et al.* (1988) showed that a culture filtrate protein extract (CFPE) from *M.tuberculosis* reversibly increased macrophage lysosomal pH. They suggested that an inhibition of phagosome-lysosome fusion mediated by a direct effect of the CFPE on the lysosome rather than on the phagosome would be a probable consequence of this alkalinisation (Chicurel *et al.*, 1988).

2.5.6. Electron Transparent Zone

An additional protective mechanism adopted by mycobacteria is the formation of an 'electron-transparent zone' (ETZ) following phagocytosis (Rastogi & David, 1988). This ETZ consists of c-mycosides (peptidoglycolipids) and has been shown to develop around various mycobacteria following their ingestion by macrophages. These include, *M.avium* (Draper, 1974); *M.lepraе* (Fréhel & Rastogi, 1987) and *M.lepraemurium* (D'Arcy Hart *et al.*, 1972). The lipid-rich zone interferes with the ability of the phagosome to fuse with the lysosome. This is apparently due to the direct interaction of the lipids in the ETZ with the lipids of the phagosome membrane (Lowrie, 1988). It has also been suggested that the ETZ may function by

preventing the hydrolytic enzymes from attacking the cell wall of the engulfed mycobacteria by preventing the normal diffusion of the enzymes, irrespective of successful phagosome-lysosome fusion (Rastogi & David, 1988). The lipids in the ETZ are also a possible site for harmless peroxidation by the reactive oxygen metabolites produced by the phagocyte, thereby protecting the inner bacterial cell wall from the ravages of these lethal toxic molecules (Wheeler & Re ledge, 1988). However studies undertaken by Fréhel *et al.* (1986) showed that following phagocytosis only 7-15% of *M.tuberculosis* formed the ETZ, whereas 85% of *M.avium* did so. This implies that although the ETZ may be important in protecting some species of mycobacteria, it is not an important protective mechanism for the tubercle bacillus (Rastogi & David, 1988).

2.5.7. Interference with Antigen Presentation (T Lymphocyte Activation)

As discussed in section 1.4., an important function of the macrophage is to activate T lymphocytes (antigen-specific cell-mediated response) (Unanue, 1972; Rosenthal *et al.*, 1975). For activation of these cells to occur it is essential that the macrophage process mycobacterial antigens and then present them in conjunction with Ia (or DR antigens) to the T lymphocyte (Bhardwaj & Colston, 1988). Mycobacteria may act at this level by interfering with the antigen presenting process (Ivanyi, 1986). It is possible that poorly degradable polysaccharide or glycolipid constituents persisting in tissues for lon, periods after the killing and disintegration of the organisms could

selectively modulate the Ia-associated events of epitope presentation (Ivanyi, 1986). In this context, arabinomannan, purified from culture filtrates of *M.tuberculosis*, was shown by Ellner & Daniel (1979) to suppress antigen dependent lymphocyte proliferation *in vitro*. This suppression was shown to occur by a disruption of the functional interaction of antigen bearing macrophages with antigen specific lymphocytes. Sibley *et al.* (1990) demonstrated that pre-incubation of the macrophages with lipoarabinomannan, a major constituent of the outer cell wall of the mycobacterium species, blocks activation of the macrophage by IFN-gamma. These authors suggest a blocking of IFN-gamma signal transduction within the cell at some point after receptor occupancy leading to an inhibition of Ia expression and a blocking of macrophage activation. Wadee *et al.* (1983) also showed the mycobacterial phospholipids, released by the macrophages following degradation of the bacterium, activates suppressor cells that non-specifically suppress lymphocyte proliferation to antigens or mitogens. Furthermore, monocytes derived from tuberculous patients have been shown to express significantly lower amounts of Ia on their surfaces (Ellner, 1986). This has been correlated with the suppressed mononuclear cell responses to antigen (PPD) stimulation. *In vitro* culturing of these cells for 24 hours, however, appears to restore the expresion of Ia on these cells. It has been suggested that the expresion of Ia *in vitro* could be due to regeneration of these molecules (Ellner, 1986). A further explanation has been suggested by Tweardy *et al.* (1984), who postulated that immature Ia- monocytes may

have a distinctive immunoregulatory role. This is particulary plausible in light of the studies of Pierres & Germain (1978) who demonstrated immunosuppression mediated by Ia- cells. Evidence also indicates that a raised bone marrow production of monocytes and early release of monocyte precursors may be responsible for the raised number of Ia- monocytes (Schmitt *et al.*, 1977).

For more efficient functioning of the macrophages, activation of these cells occurs via a lymphokine, IFN-gamma, produced by T lymphocytes (Nathan *et al.*, 1984). These activated macrophages have enhanced microbicidal activity and express more Ia on their surface (Nathan *et al.*, 1983; King & Jones, 1983).

By preventing activation of the antigen specific arm of the immune response and by inhibiting the intracellular killing capacity of phagocytes, the mycobacterium has produced an ideal environment for its growth and multiplication.

CHAPTER 3.

3. INTERFERON

3.1. INTRODUCTION

Interferons were first characterised as substances that inhibit virus replication but have subsequently been shown to have an effect on many components of the immune system (Morris, 1988). Interferons are classified into 3 types, alpha, beta and gamma, according to their antigenicity (Morris, 1988). IFN-alpha and -beta are very similar to each other, both biochemically and biologically, and are probably produced by most virally infected cells (Morris, 1988).

The different IFN-alpha molecules have molecular weights ranging from 18 to 23 kilodaltons (kDa) and are not glycosylated (Oppenheim *et al.*, 1987). IFN-beta has a molecular weight of 23kDa and is glycosylated; IFN-gamma has a molecular weight ranging from 20 to 25kDa and is also glycosylated (Oppenheim *et al.*, 1987).

3.2. GAMMA-INTERFERON (IFN-GAMMA)

IFN-gamma (or 'immune interferon') has, over the past few years, become recognised as the major product from lymphocytes that alters phagocyte function (Scheffner & Rellstab, 1988; Nathan *et al.*, 1983; Morrison *et al.*, 1987). It has been shown to act as a macrophage and PMN activating factor resulting in an enhanced ability to destroy facultative and obligate intracellular parasites and tumours (Adams & Hamilton, 1987).

3.2.1. Source of IFN-gamma

Production of IFN-gamma by lymphocytes is induced by antigens, mitogens or the cytokines, tumour necrosis factor (TNF), interleukin-1 (IL-1), interleukin-2 (IL-2) and colony stimulating factors (Balkwill, 1989). T cells (CD3+ cells) have been shown to be the major source of IFN-gamma (Kasahara *et al.*, 1987). Natural killer (CD16+) cells and other IL-2 receptor positive and E rosetting mononuclear cells (which do not fulfill the criteria for either T cell, B cell or monocyte lineages), have also been shown to produce IFN-gamma (Sandvig *et al.*, 1987).

3.3. EFFECTS OF IFN-GAMMA

Much of the work done on the activating effects of IFN-gamma on phagocytes has focused on the monocyte/macrophage system. The dose at which IFN-gamma appears to be effective varies from <1 to 500U/ml (Hoover & Meltzer, 1989). This wide dose range reportedly allows for the fine tuning of monocyte responders, especially in co-operation with other mediators of monocyte activation (Hoover & Meltzer, 1989). Monocyte stimulation has been shown to occur after IFN-gamma binds to specific receptors on the monocyte surface (Celada *et al.*, 1984). Other studies using liposomes have indicated that receptor binding could be bypassed (Koff *et al.*, 1985). Whether the IFN-gamma receptor is the first element of a signal transducing system or whether the receptor functions as a vehicle for transporting its ligand to intracellular receptors therefore

remains to be determined (Aguet *et al.*, 1988). With regard to receptor-mediated binding, recent evidence indicates that freshly isolated monocytes bind the lymphokine via a single type of receptor whereas monocytes cultured for 5 days or more express IFN-gamma receptors with two different affinities (Yoshida *et al.*, 1988). Once bound, IFN-gamma is rapidly internalised and results in the activation of a number of genes, leading to the *de novo* synthesis of numerous surface and secretory proteins by the macrophage (Adams & Hamilton, 1987). These include the production of MHC antigens (Adams & Hamilton, 1987), and the cytokines IL-1 (Newton, 1985), IL-2 (Herrman *et al.*, 1985) and tumour necrosis factor (Beutler *et al.*, 1986).

The exact sequence of events occuring between binding of IFN-gamma to its receptor and gene activation is, at present, not known. However, IFN-gamma has been shown to induce slow rises in intracellular levels of calcium (starting 5-10 minutes after binding and being completed by 15-20 minutes) (Somers *et al.*, 1986) and to enhance the potential activity of protein kinase C (PKC) (Figure 11). Additional stimulation of the cells with a secondary signal (e.g. LPS) results in enhanced phosphorylation of endogenous protein substrates (Adams & Hamilton, 1987). How these factors then activate the interferon-responsive genes remains to be established.

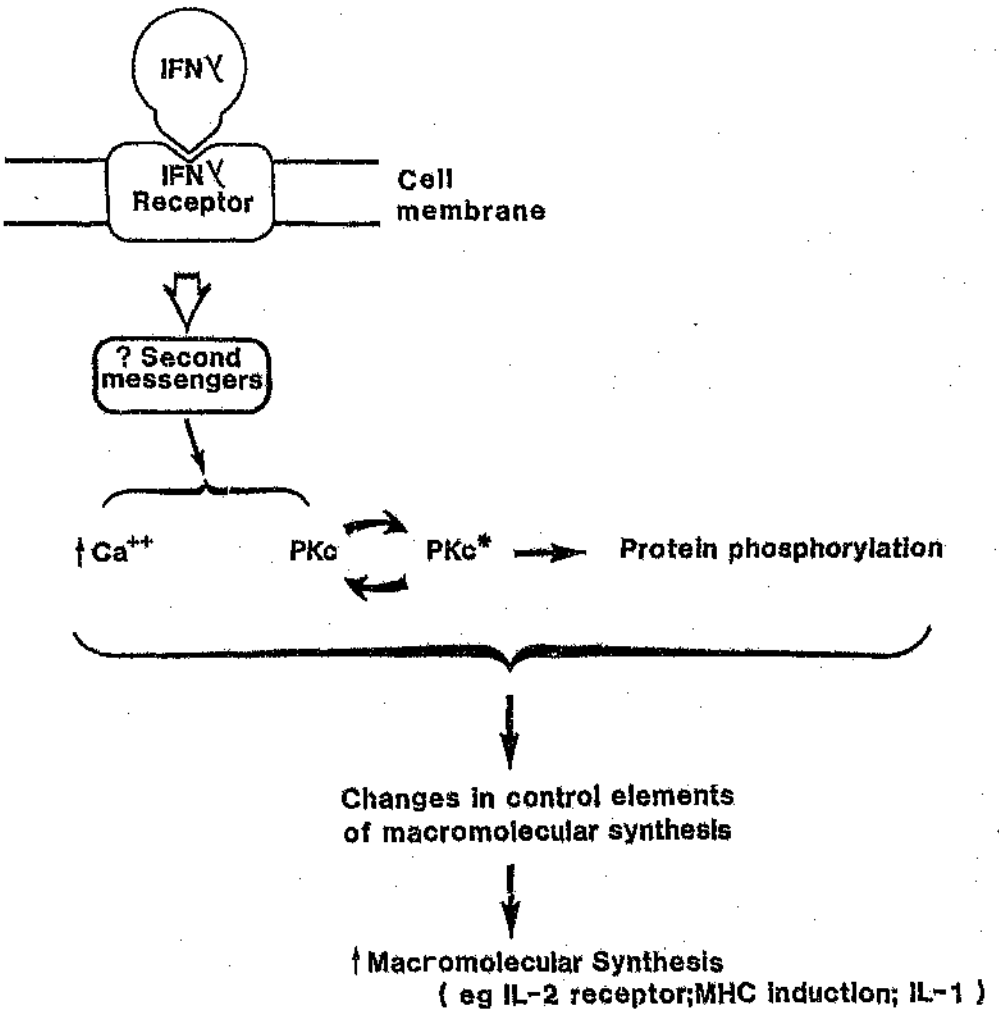


FIGURE 11. A model for signal transduction following binding of IFN-gamma to its receptor.

3.3.1. Effects of IFN-gamma on the Expression of Surface Receptors

Many of the molecules expressed on the monocyte surface, after exposure of the cells to IFN-gamma, have been shown to demonstrate receptor function (Hoover & Meltzer, 1989). Some of the receptors expressed include: IL-2 receptors (Herrman *et al.*, 1985); high affinity Fc-gamma receptors (Guyre *et al.*, 1983); leukocyte function adhesion-1 (LFA-1) molecules (Weinberg *et al.*, 1984); and MHC Class II molecules (King & Jones, 1983). The increased expression of these receptors may have important consequences for cell function:

1) IL-2 may play a role in granuloma formation since macrophages in a granuloma have been shown to exhibit receptors for IL-2 (Hancock *et al.*, 1986).
2) An increased expression of Fc-gamma receptors on macrophages enhances the ability of the macrophage to ingest opsonised particles (Guyre *et al.*, 1983).
3) An enhanced expression of LFA-1 molecules is likely to play a role in the development of multinucleated giant cells since this molecule has been shown to mediate monocyte aggregation (Weinberg *et al.*, 1984).
4) The increased expression of MHC Class II molecules endows the macrophage with the ability to present more processed antigen to T lymphocytes (Rosenthal *et al.*, 1975).

3.3.1. Effect of IFN-gamma on Production of Secretory Products by Macrophages

IFN-gamma has also been shown to induce monocyte production and secretion of soluble factors (Adams & Hamilton, 1987). These include: increased TNF secretion (Phillip & Epstein, 1986); increased IL-1 synthesis in response to LPS (Gerrard *et al.*, 1987); enhanced prostaglandin release (Stevens *et al.*, 1989) and the synthesis of dihydroxy vitamin Ds (Rook *et al.*, 1986) is increased.

TNF, which is released by activated macrophages in developing granulomas may act in an autocrine way to enhance its own synthesis and release. This process would favour further accumulation of macrophages and an increase in the differentiation of these latter cells into epithelioid cells. This leads to the formation of a fully developed granuloma capable of preventing bacterial dissemination and allowing efficient bacterial killing (Kindler *et al.*, 1989).

IL-1 will act, in conjunction with antigen, as an activator of T lymphocytes (Sisson & Dinarello, 1989) and these activated T cells (both helper and cytotoxic) then play an important role in the killing of *M.tuberculosis* (Kaufmann, 1989).

The enhancement of prostoglandin release may, contrary to the other cytokines, act to downregulate the macrophage response to foreign organisms, thereby terminating an active immune response (Stevens *et al.*, 1989).

The increased production of 1,25-dihydroxy vitamin D has been shown to enhance the inhibition of growth of *M.tuberculosis* within macrophages (Rook, 1988).

3.4. THE ROLE OF IFN-GAMMA IN ENHANCING BACTERICIDAL FUNCTIONS OF PHAGOCYTES

Activation of the macrophage and PMN cell intracellular killing ability has been shown to occur in the presence of IFN-gamma (Nathan *et al.*, 1983). This function of IFN-gamma has proven to be important in enhancing the phagocytes capacity of killing several intracellular pathogens, including *Blastomyces dermatitidis* (Morrison *et al.*, 1989), *Listeria monocytogenes* (Peck, 1989) and mycobacteria (Walker & Lowrie, 1981; Crowle & May, 1981; Rook *et al.*, 1985).

The presence of IFN-gamma has been shown to result in an effective enhancement of the oxidative metabolism of both phagocytic cell types (Peck, 1989; Cassatella *et al.*, 1988), resulting in an increased production of toxic reactive oxygen radicals such as hydrogen peroxide, hydroxyl radicals and superoxide (Peck, 1989).

Enhancement of phagosome-lysosome fusion by IFN-gamma has been well documented. Experiments with the intracellular parasite *Salmonella typhimurium*, that inhibits phagosome-lysosome fusion, demonstrated that IFN-gamma could effectively reverse this inhibition (Kagaya *et al.*, 1989). A similar enhancement of phagosome-lysosome fusion was shown in

bone-marrow macrophages infected with *Mycobacterium bovis* (Flesch & Kaufmann, 1988). Recombinant IFN-gamma activated bone-marrow macrophages have been shown to inhibit mycobacterial growth by 98%. Addition of increasing concentrations of suramin, an inhibitor of phagosome- lysosome fusion (D'arcy Hart & Young, 1975), practically reversed this growth inhibition. In addition, Flesch & Kaufmann (1988) showed that inhibition of mycobacterial growth was not reversed significantly by oxygen scavengers. Together these two findings suggest that enhancement of phagosome-lysosome fusion might be another mechanism by which IFN-gamma activates macrophage antimycobacterial activity.

An inhibition of the intracellular multiplication of *Legionella pneumophila* within monocytes treated with IFN-gamma has also been documented. In this system IFN-gamma limited the availability of iron and down-regulated transferrin receptors (Byrd & Horwitz, 1989). *L. pneumophila* has a definite metabolic requirement for iron and phagocytes acquire iron via transferrin receptors. By down-regulating the number of transferrin receptors on the cell surface, IFN-gamma limits the amount of iron within the cell and thereby limits *L.pneumophila* multiplication (Byrd & Horwitz, 1989).

Inhibition of intracellular growth of both *Chlamydia psittaci* and *Toxoplasma gondii* within fibroblasts has been shown to depend on treatment with IFN-gamma (Byrne *et al.*, 1986; Pfefferkorn, 1984). It has been postulated that the

mechanism by which IFN-gamma enhances killing in this system is by the induction of indoleamine 2,3-dioxygenase which catalises the degradation of tryptophan. IFN-gamma has been shown to induce indoleamine 2,3-dioxygenase activity in monocytes and macrophages, thereby possibly enhancing the host defences against these organisms (Carlin *et al.*, 1989).

Another role of IFN-gamma in augmenting intracellular killing is its ability to stimulate the release of lysosyme, a potent anti-bacterial agent, from lysosomes (Lewis *et al.*, 1990). The studies carried out by Lewis *et al.* (1990) showed that IFN-gamma, in the presence of suboptimal concentrations of LPS, stimulated monocytes to release greater amounts of lysosyme.

3.5. ROLE OF IFN-GAMMA IN MYCOBACTERIAL INFECTION

In view of the ability of IFN-gamma to activate the various intracellular killing mechanisms, a role for IFN-gamma in activating phagocytes to kill mycobacteria can be envisaged. Several recent conflicting reports have appeared examining these effects (Douvas *et al.*, 1985; Rook *et al.*, 1986a; Flesch & Kaufmann, 1987).

Douvas *et al.* (1985) showed the IFN-gamma pretreated macrophages became tumouricidal and leishmanicidal but that there was an enhanced replication of mycobacteria in these macrophages. Other groups (Walker & Lowrie, 1981; Rook *et al.*, 1985) have shown that murine peritoneal macrophages can

be activated by unpurified T cell products, which may contain IFN-gamma. This resulted in the intracellular destruction of *M.microti* (Walker & Lowrie, 1981) or the ihibitition of *M.tuberculosis* multiplication within macrophages (Rook *et al.*, 1985). Rook *et al.* (1986) have also shown that IFN-gamma has variable effects on mycobacterial growth in human monocytes. These vary from enhancement of growth of mycobacteria (Douvas *et al.*, 1986) to significant inhibition of growth of the organisms (Flesch & Kaufmann, 1987). This differed from the effect of IFN-gamma on mouse peritoneal macrophages where consistant inhibition of growth was seen (Rook *et al.*, 1986a). Such discrepencies may be due to the fact that there are distinct subpopulations of macrophages at different stages of maturation in the human system (Rook *et al.*, 1986a). Flesch & Kaufmann have done an extensive amount of work with the anti-mycobacterial function of murine bone marrow-derived macrophages. Their data shows that:-

(i) when treated with IFN-gamma, the macrophages have increased anti-mycobacterial activity (1987);

(ii) different strains of *M.tuberculosis* have different susceptibility to IFN-gamma activated macrophages (1987);

(iii) inhibition of *M.bovis* by IFN-gamma activated macrophages is an oxygen-independent process since the inhibition was not reversed by scavengers of reactive oxygen metabolites (1988);

(iv) enhancement of phagosome-lysosome fusion to due to IFN-gamma treatment might result in this increased inhibition of mycobacterial growth (1988).

3.6. CONCLUSION

The exact role played by IFN-gamma in tuberculosis is therefore still uncertain. This uncertainty is underscored by two recent review articles by Kaufmann (1989) and Rook (1990) which emphasise the opposing roles of IFN-gamma. Kaufmann (1989) has suggested that IFN-gamma plays an important role in protection against the tubercle bacillus, whilst Rook (1990) has suggested that IFN-gamma has only a partial restorative effect in humans and may in fact play a more important role in the immunopathology of tuberculosis rather than in immunity to the disease.

CHAPTER 4
MATERIALS AND METHODS

4.1. PREPARATION OF *MYCOBACTERIUM TUBERCULOSIS* ORGANISMS

4.1.1. Bacterial Isolation and Preparation

M.tuberculosis organisms from human sputum specimens were grown on Lowenstein-Jensen slopes for 2-3 weeks at 37°C. Colonies were scraped off into physiological saline and the suspension was centrifuged at 200g for 5 minutes to remove any of the Lowenstein-Jensen medium that may have contaminated the bacterial suspension during the isolation procedure. The supernatant was collected and centrifuged at 800g for 30 minutes at 4°C, followed by 2 successive washings of the pellet in saline. The mycobacterial suspension was homogenised with a homogeniser using an 18K probe (Janke & Kunkel KG, Staufen, Breisgau). The mycobacteria were then autoclaved and sonicated in an MSE Soniprep 150 sonicator (18um peak to peak). All homogenisation and sonication was carried out at 4°C. The sonicated material was centrifuged at 800g for 15 minutes, the supernatant carefully collected, and the protein concentration of the sonicate determined using the Biorad protein estimation kit (Biorad Laboratories, Richmond, California, USA) according to the manufacturers instructions. The mycobacterial sonicate was aliquoted and stored at concentrations of 1mg/ml at -20°C until required.

4.1.2. Fractionation of *M.tuberculosis* Extracts by Gel Filtration Chromatography

Sonicated *M.tuberculosis* extract was passed through a sephacryl S-200 column to separate various fractions according to size.

4.1.2.1. Preparation of Sephacryl S-200 Column

Sephacryl S-200 (Pharmacia Fine Chemicals, Uppsala, Sweden) was packed in a 940 x 150mm Pharmacia column by gravity. This column was equilibrated with PBS (pH 7.4) (appendix I) at a flow rate of 0.5ml/minute for a further 2 days at 4°C. Even packing of the column was confirmed by observing the passage of Dextran blue T2000 (Pharmacia, Uppsala, Sweden) through the column. The void volume of the column, as determined by Dextran blue exclusion, was found to be 60ml.

4.1.2.2. Standardisation of Columns

Columns were standardised by using proteins of known molecular weight. These included: ribonuclease (14kDa), chymotrypsin (25kDa), carbonic anhydrase (30kDa), bovine serum albumin (67kDa), alcohol dehydrogenase (150kDa); aldolase (158kDa) and catalase (232kDa) (all from Pharmacia Fine Chemicals, Uppsala, Sweden). The proteins were dissolved in PBS to a final concentration of 5mg/ml. Two millilitres of each protein solution were layered onto the column and the fractions collected in 1ml volumes in a Spectra/chrom fraction collector (Spectrum Medical Industries, Los Angeles, USA). Fraction collection was initiated when half the protein solution was judged to have entered the column. The elution volume of various standards were determined and a standard curve drawn therefrom.

4.1.2.3. Fractionation of *M.tuberculosis* sonicates on Sephacryl S-200 Columns

To fractionate *M.tuberculosis* antigens, 2ml of mycobacterial extract at a concentration of 1mg/ml was loaded onto the column. One millilitre fractions eluting from the column were collected and protein elution profiles of the fractions were assessed. By comparison with elution profiles of the known standards, eight major protein peaks (arbitrarily defined and alphabetically termed A - J) of molecular size ranging from 669 to 14 kilodaltons (kDa) were obtained.

4.1.3. Concentration of *M.tuberculosis* Fractions

The major protein peaks obtained from the column have previously been assessed for their ability to inhibit the intracellular killing capacity of phagocytes (Wadee *et al.*, 1987). To confirm these results, and to determine more precisely its mode of action, the effect of the 25kDa fraction on other phagocyte functions was investigated. The aliquots in peak H (elution volume 106-125ml) corresponding to proteins of a molecular weight of 25kDa, were pooled, poured into spectrapor dialysis tubing (Spectrum Medical Industries Inc, USA - molecular weight cut off point 6-8 000 daltons) and concentrated by placing the dialysis tubing in Acquacide II (Calbiochem Corp., La Jolla, USA) at 4°C. Protein estimation was done on the concentrated fraction using the Biorad protein assay. A standard curve was plotted by measuring the absorbance values of known concentrations of BSA (ranging from 2-200mg/ml). The concentrated fraction at 1mg/ml was then aliquoted and stored at -70°C.

4.2. ESTABLISHMENT OF MONOCYTE MONOLAYERS

Venous blood was collected into preservative free heparin (10units/ml - Sigma, St Louis, MD, USA), separated by centrifugation on hypaque-ficoll gradients and the mononuclear cell fraction at the plasma-ficoll interphase collected. The cells were washed 3 times in physiological saline and resuspended in RPMI-1640 (Highveld Biologicals, Johannesburg) containing 10% fresh autologous human serum. Mononuclear cells at a concentration of $3 \times 10^6/ml$ were then plated in 16 wells of lab-Tech tissue culture chamber/slides (Miles Scientific, Naperville, Illinois, USA) each well containing 0.5ml.

Cell cultures were incubated for 60 minutes at 37°C in a humidified incubator containing 5% CO_2 and 95% air. At the end of this culture period non-adherent cells were removed by washing 3 times with warm (37°C) saline. The remaining adherent cells were then cultured in RPMI-1640 containing 10% fresh autologous serum and 100 units of penicillin and streptomycin. Cultures were maintained by removing half of the supernatant fluid and replenishing with an equivalent amount of RPMI-1640 medium containing serum every 4 days. Growth of cells was examined using an inverted microscope and wells were expanded when the monolayer had reached 75% confluency. Expansion of cells was performed by vigorous pipetting of cell cultures followed by plating into equivalent wells with an equal volume of medium containing 10% fresh autologous serum. Cell viability was assessed by trypan blue exclusion and cell

cultures were maintained for 2 months or longer. To ascertain the purity of the macrophage cultures, the cells were incubated with the monoclonal antibody OKM1 (Ortho Diagnostic Systems Inc., Raritan, NJ, USA), stained with the esterase stain and assessed for their phagocytic ability.

4.2.1. Assessment of Macrophage Purity

4.2.1.1. Assessment of OKM1 Binding by Macrophages in Culture

Macrophages grown in tissue chamber slides were fixed onto slides by adding methanol into the chamber and incubating at room temperature for 1 minute. The slides were washed and 200ul of the monoclonal antibody OKM1 (Ortho Diagnostic Systems Inc., Raritan, NJ, USA - 1:50 dilution) were added to each chamber and the slides incubated at room temperature for 2 hours, after which they were washed 3 times in PBS. Fluorescein isothiocyanate labelled rabbit anti-mouse antibody (Boehringer Mannheim, Frankfurt, Germany - 1:50 dilution) was added to each chamber and the slides incubated for 60 minutes in the dark. The unbound conjugated antibody was washed off with 3 washes in PBS and the slides viewed under a fluorescent microscope.

4.2.1.2. Assessment of Esterase Activity of Adherent Cells

Esterase activity was assessed by the naphthyl acetate method (Davis & Ornstein, 1959). A solution of pararosanilin was made by adding 2g of pararosanilin hydrochloride (Chroma, Stuttgart, Germany) to 5ml of 2M hydrochloric acid (Merck, Darmstadt,

Germany). This solution was filtered and stored at 4°C until used. Sodium nitrite solution was made by adding 400mg of sodium nitrite (Analar-BDH, Poole, UK) to 10ml distilled water. This solution was stored at 4°C for no longer than 2 days. The incubation mixture for the esterase staining was prepared freshly each time by adding 0.4ml of pararosanilin solution to 0.4ml of sodium nitrite solution. The reagents were allowed to react for 1 minute to form hexazotized pararosanilin (HPR) which was then added to 7.25ml of phosphate buffer solution (2.75% $NaH_2PO_4H_2O$). Two hundred and fifty microlitres of freshly made alpha-naphthylacetate (1% in acetone) was added to the incubation medium. The pH was adjusted to 7.4, after which 2.5ml of distilled water was added to the mixture.

Cells were fixed on slides in 2% gluteraldehyde (BDH Chemicals Ltd., Poole, UK - diluted in PBS) for 10 minutes. The slides were then washed in water for 20 minutes and the water changed 10 times in the course of the washing procedure. Slides were stained for 60 minutes at 37°C using the incubation mixture for esterase staining, followed by washing for 10 minutes under running water and then dehydrated using increasing concentrations of alcohol (50%, 70%, 90%, 96% and 100%), immersed in xylene and mounted with DePex (Gurr, BDH Chemicals Ltd., Poole, UK). The number of esterase positive cells were enumerated under oil immersion (x100 lens) and expressed as a percentage of 200 adherent cells counted.

4.2.1.3. Phagocytic Properties of Adherent Cells

Bakers' yeast prepared as described in Section 4.4 were used. To adherent cells, grown on chamber slides, 0.1ml fresh human AB serum and 0.1ml Bakers' yeast (6×10^6/ml) were added. The chamber slides were then placed on a shaker and incubated at 37°C for 30 minutes. At the end of the incubation period the slides were fixed in methanol (BDH Chemicals Ltd., Poole, UK) for 5 minutes, washed in water and stained with a 1:10 dilution of Giemsa (Gurr Ltd., London, UK) in physiological saline for 5 minutes. Slides were dried and 200 cells counted under oil immersion lens. The number of cells that had phagocytosed Bakers' yeast were expressed as a percentage of the total number of cells.

4.2.1.4. Preparation of Adherent Monocytes for Studies of Bactericidal Ability

For bactericidal studies, assessment of lysosyme production, H_2O_2 production and glycolysis, where large numbers of adherent cells are needed, adherent cells were cultured on plastic tissue culture plates (145/20mm - Greiner Labor-technik, Solingen). Twenty millilitres of mononuclear cells at a concentration of 3×10^6/ml were added to each plate and the plates incubated in a humidified incubator at 37°C overnight.

Further preparation of the adherent cell cultures was described for the cells isolated on culture slides (Section 4.2.). Cultured monocytes were removed by treatment with trypsin-EDTA. The monolayer was washed with warm (37°C) physiological saline and 15ml of a mixture of Trypsin-EDTA containing 0.125% Trypsin, 0.05% EDTA in phosphate buffered saline (Highveld Biologicals, Johannesburg) was added to the monolayers and the cultures incubated for a further 15 minutes at 37°C. Cells were collected, pooled and washed 3 times with cold RPMI containing 10% heat inactivated foetal calf serum (Highveld Biolcgicals, Johann °C. They were then counted and reconstituted in , ratio of 1 x 10^7/ml.

4.3. ISOLATION OF POLY .ONUCLEAR LEUKOCYTES (PMN CELLS)

PMN cells were obtained from normal volunteers following centrifugation of heparinised peripheral blood on hypaque-ficoll density gradients. The cell pellets obtained were resuspended in 38ml of physiological saline and mixed with 12ml of 3% gelatin (Difco, Detroit, MI) in saline. The cell-gelatin suspension was incubated at 37°C for 30-60 minutes and the leukocyte rich supernatant removed, centrifuged and washed twice in saline. Residual erythrocytes were lysed with 0.83% ammonium chloride (Appendix 2) at 4°C for 10 minutes. The remaining PMN cells were then centrifuged at 100g, washed, resuspended in saline and brought to a concentration of 10 x 10^6/ml in PBS.

4.3.1. Assessment of Purity of the PMN Cell Suspensions

To ascertain the purity of the PMN cell preparations, the cell suspensions were counted in Turks' white cell counting fluid and in a Sysmex K1000 (Milsh, Japan) cell counter. Evidence of PMN cell purity was assessed by adding 50ul of cell suspension to 450ul of Turks' white cell counting fluid and the percentage PMN cells ascertained microscopically. The cell suspensions were also assessed by differential count in the Sysmex K1000 cell counter.

4.4. PREPARATION OF *SACCHAROMYCES CEREVISIAE* (BAKER'S YEAST)

Commercially available Bakers' yeast was prepared by suspending a small pellet in physiological saline, washing 3 times by centrifuging 50ml suspensions at 400g and resuspending in saline each time. The yeast were killed by heating at 80°C for 60 minutes. The organisms were then washed with saline, resuspended to a concentration of 6 x 10^7/ml in saline and stored at -20°C until used. For experiments to assess phagosome-lysosome fusion, yeasts were washed but not heat killed.

4.5. PREPARATION OF *STAPHYLOCOCCUS AUREUS (S.AUREUS)* ORGANISMS

Colonies of *Staphylococcus aureus* Cowan Strain A (*S.aureus*) organisms were innoculated into 50ml of Muller-Hilton medium and allowed to grow for 3-4 hours at 37°C. The organisms were then washed by 3 centrifugations at 400g in physiological saline. The *S.aureus* organisms were then resuspended in PBS to a final concentration of 1 x 10^8/ml.

4.6. PHAGOCYTIC ABILITY OF PHAGOCYTES

When the phagocyte comes into contact with an invading micro-organism it will engulf the organism and form a phagosome around it (Werb, 1987). Initial experiments were therefore designed to evaluate the effect of the the 25kDa mycobacterial fraction on the phagocytic ability of PMN cells and cultured monocytes. Concentrations of cultured monocytes or PMN cells were adjusted to 1 x 10^7/ml and 0.3ml of this suspension was incubated with 0.1ml of Bakers' yeast suspension, 0.1ml of human AB serum and 0.1ml of the 25kDa fraction (50ug/ml - final concentration) in 5ml tubes. The non-phagocytic control consisted of 0.1ml of Bakers' yeast suspension, 0.1ml of serum and 0.3ml of PBS. Cultures were incubated at 37°C on a rotating wheel for 50 minutes and 0.2ml aliquots were removed every 10 minutes and added to 0.5ml of Turks leukocyte counting fluid. Phagocytosis was expressed as the percentage of yeast which had been ingested. This was calculated by counting the number of extracellular yeast particles on a Neubauer heamocytometer and using the formula:

$$\% \text{ phagocytosis} = 100 - \frac{\text{No. of extracellular organisms}}{\text{No. of organisms in non-phagocytic control}} \times 100$$

4.7. BACTERICIDAL ABILITY OF PHAGOCYTES

To assess the bactericidal ability of phagocytes, staphylococcus organisms were incubated with monocytes or PMN cells for a period of time after which the number of organisms not killed was assessed by culturing supernatants from lysed cells on blood agar, using a modified technique of Quie *et al.* (1967).

Each test system contained 0.5ml of 10 x 10^6/ml PMN cells or macrophages; 0.1ml of *S.aureus* and 0.1ml fresh autologous serum. To assess the effect of the 25kDa fraction on the bactericidal ability of phagocytes, 0.1ml of different concentrations of the 25kDa fraction (10,50,100mg/ml) was added to each culture system.

One hundred microlitres of IFN-gamma (rIFN-gamma - Amersham International, Buckinghamshire, UK) at different concentrations (50,100,200mg/ml) were added together with 100ul of the 25kDa mycobacterial fraction to some test systems. In addition, a cell free control system contained 0.1ml serum; 0.1ml of *S.aureus* and 0.8ml PBS. The final volume in all culture systems was brought to 1ml with sterile PBS. Cultures were incubated at 37°C for 90 minutes on a rotating wheel. At the end of this period, 0.1ml aliquots from each system were added to 9.9ml sterile distilled water and then serially diluted in distilled water to a dilution of 1 x 10^{-4} and 1 x 10^{-5}. Of these dilutions, 0.1ml was plated out on blood agar plates and the plates incubated overnight at 37°C, after which the colonies were counted. The difference in counts between the test and control systems were assessed after approximately 18 hours and the percentage of bacteria killed intracellularly was determined using the formula:

$$\% \text{ killing} = 100 - \frac{\text{No. of colonies in test system}}{\text{No. of colonies in cell-free control system}} \times 100$$

4.8. PHAGOSOME-LYSOSOME FUSION

When microorganisms are ingested by phagocytes, they are enclosed within phagosomes (Werb, 1987). Lysosomes present in the cytoplasm of the phagocyte are capable of fusing with and discharging their contents into the phagosome such that the foreign particles within the phagosome are degraded (Armstrong & D'Arcy Hart, 1971). To assess the process of phagosome-lysosome fusion D'Arcy Hart & Young (1971) described a technique whereby phagocytes are incubated with acridine orange, a lysomotropic fluoresent dye, which will be transferred to the phagosome if fusion of the lysosome and phagosome occurs. Phagocytosed yeast particles will then fluoresce green if they are killed, indicating successful phagosome-lysosome fusion.

Monocytes or freshly prepared PMN cells at 1 x 10^6/ml were used to assess the effect of the 25kDa on phagosome-lysosome fusion. Monocytes grown in culture on 8 chamber Lab-Tek slides were prelabelled with acridine orange (Merck, Darmstadt, Germany). Two hundred millilitres of the acridine orange solution (5ug/ml) were added to each chamber. Cultures were then incubated for 15 minutes at 37°C, after which the acridine orange was poured off, fresh RPMI (maintained at 37°C) added to each chamber and the cells incubated for a further 15 minutes at 37°C to allow for the dye to concentrate in the lysosomes. The cells were then washed 3 times with warm (37°C) medium and used in assays to visualise phagosome-lysosome fusion.

To label PMN cells, acridine orange (5ug/ml) was added to 2ml of a PMN cell suspension, and the tube incubated at 37°C for 15 minutes. Cells were then centrifuged at 200g, resuspended in RPMI (maintained at 37°C) and incubated for a further 15 minutes at 37°C.

Acridine orange labelled phagocytes were incubated with opsonised yeast (100ul) (Appendix 3) for 30 minutes at 37°C, after which the cells were extensively washed with warm PBS to remove non-phagocytosed yeast cells. Fusion was allowed to progress for a further 30 minutes at 37°C.

In experiments in which the effects of the mycobacterial fraction on the fusion process were examined, 100ul of the fraction at a final concentration of 50 or 100ug/ml were added to the cells. In some test systems, IFN-gamma (50, 100, 200, 500U/ml) was added with the 25kDa fraction (50ug/ml) or alone (control). The final volume of each system was maintained at 400ul. After the last 30 minute incubation period the plastic chambers of the macrophage cultures were removed from the slides and wet mounts were made and examined under a UV microscope. For the PMN cells, drops of the cell suspension were placed onto slides and examined under a UV microscope (Leitz, Westlar, Germany). Using the criteria of Goren *et al.* (1984), yeasts were regarded as having been killed when they appeared uniformly bright green; cells in which fluorescence was restricted to the periphery of the yeast particle were placed in the intermediate category, implying an early stage of phagosome-lysosome fusion

and yeasts that appeared non-fluorescent were regarded as not having acquired acridine orange by phagosome-lysosome fusion. This indicated that fusion had been delayed or was inhibited. Viability, non-viability and intermediate killing of yeast cells were scored independently by two investigators.

4.9. LYSOSYME PRODUCTION AND ASSAY

4.9.1. Lysosyme Production

Lysosyme is a cationic enzyme, present in both macrophages and PMN cells, which has the ability to hydrolyse N-acetyl muramic β-1, 4 N-acetyl glucosamine linkages in bacterial cell walls (Gordon *et al.*, 1974). It may therefore have an important role to play in the mycobactericidal ability of phagocytes. Since the 25kDa fraction has been shown to inhibit intracellular killing by phagocytes, it was of interest to examine the effects on the production of lysosyme.

Macrophages grown on 145mm tissue culture plates for 6 weeks or longer were removed by Trypsin-EDTA treatment (Section 4.2.4.). The cells were resuspended in RPMI to a concentration of 10×10^6/ml. PMN cells, isolated as described in Section 4.3., were also reconstituted in PBS to a concentration of 10×10^6/ml.

The production and release of lysosyme was initiated by incubating 1ml of cell suspension (either macrophages or PMN's) with 200ul of yeast particles at a concentration of 60×10^6/ml

in the presence of 200ul of fresh human serum and 100ul of varying concentrations of the 25kDa fraction, IFN-gamma or RPMI The tubes were incubated for varying periods of time (10,15,30 and 60 minutes), after which they were centrifuged at 300g for 5 minutes and the supernatant collected was stored at -20°C until used.

4.9.2. Assay of Lysosyme Production

Lysosyme production was assayed by the method of Gordon *et al.* (1974) by measuring the initial rate of lysis of a suspension of *Micrococcus lysodeikticus* (M3770, Sigma, St Louis, MO, USA) with the aid of a PYE Unicam PU 8800 spectrophotometer fitted with an automatic reader. Standards of lysosyme (Seikagaku Kogyo Co. Ltd, Tokyo) rich materials were diluted in PBS. Reaction conditions were as follows: 1ml of sample was mixed at room temperature with 2ml of a suspension of *M.lysodeikticus* at a concentration of 0.25mg/ml in 0.067M phosphate buffer, pH 6.3. The rate of decrease in turbidity was measured at 540nm, with a ratio switch position of 0.5 and with a full recording scale equal to 0.3 optical density units. The initial reaction was linear for at least 1 minute and was directly proportional to eg. lysosyme concentrations in the range 0.2 to 2ug/ml.

4.10. ASSESSMENT OF NITROBLUE TETRAZOLIUM REDUCTION

4.10.1. Introduction

Stimulation of phagocytes (either by an agonist or by phagocytosis) results in a marked increase of the oxygen consumed by these cells (respiratory burst) (Sawyer *et al.*, 1989; Babior, 1978a). This increase of oxygen consumption results from the activation of an NADPH oxidase which reduces oxygen to a superoxide radical (O_2^-) (Reaction 1, page 100).

Associated with the respiratory burst is the activation of the hexose monophosphate shunt, a high energy yielding metabolic pathway which generates the NADPH used by the cells to produce superoxide (Pick, 1985) (Reaction 2, page 100).

Nitroblue tetrazolium (NBT) acts as a non-specific electron acceptor in this series of reactions, such that the yellow dye becomes reduced to an insoluble blue formazan (Hudson & Hay, 1980). These insoluble granules can then be seen in the cytoplasm of the cell (Hudson & Hay, 1980). Production of reactive oxygen radicals by phagocytes is reflected by enumerating the percentage of cells demonstrating reduced tetrazolium within their cytoplasm (Baehner *et al.*, 1976).

4.10.2. NBT Assay Procedure

Cells present in buffy coats obtained by sedimentation of heparinised whole blood were used in the NBT assay. Cell systems comprised 200ul of buffy coat derived cells incubated with 200ul of endotoxin activated serum (EAS) (Appendix 4) in

the presence of the 25kDa fraction (100ul); 100U/ml of IFN-gamma (100ul) or both. Unstimulated cells (control) were incubated with 200ul human serum (not EAS) and 200ul of PBS.

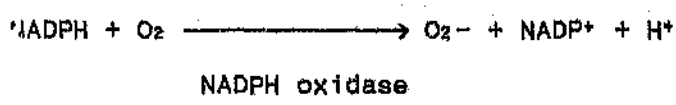
After an incubation period of 30 minutes at 37°C, 400ul of NBT (Sigma Chemical Co, St Louis, MO, USA) solution (Appendix 5) was added to each tube and the cell suspension allowed to incubate for 15 minutes at 37°C, followed by a further 15 minutes at room temperature. Smears of each sample were then made, allowed to air dry, fixed in methanol for 1 minute and stained with well filtered haematoxylin for 3 minutes (Appendix 6). The slides were washed well under running water, allowed to dry and the smears examined under oil immersion. Cells with a large bluish precipitate were regarded as having reduced NBT.

4.11. HYDROGEN PEROXIDE PRODUCTION

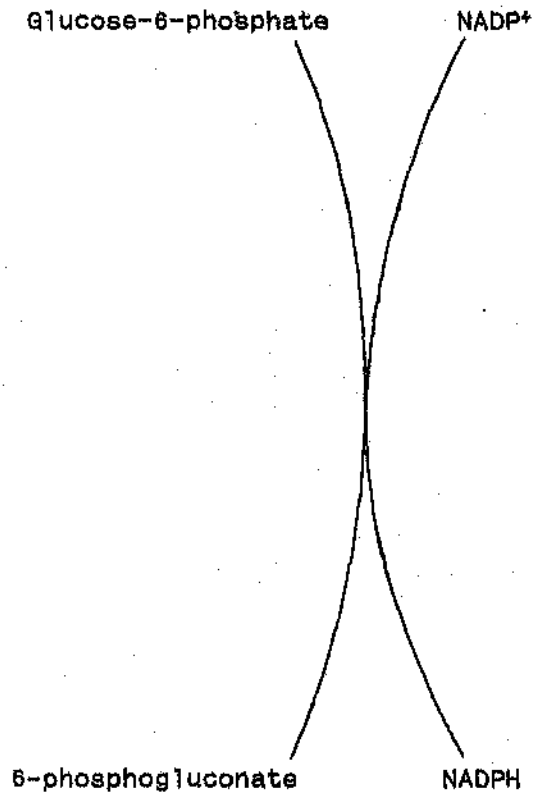
4.11.1 Introduction

By spontaneous dismutation, the O_2^- produced by the oxidation of NADPH, reacts with the hydrogen protons to produce H_2O_2 (Pick, 1985). The rate of this reaction can, however be increased 10 fold by the action of superoxide dismutase, present within phagocytes (Pick, 1985). Hydrogen peroxide (H_2O_2) is another bactericidal agent used by phagocytes to destroy invading microorganisms (McRipley & Sharra, 1967).

(Reaction 1)



(Reaction 2)



4.11.2. Measurement of H_2O_2 Production

To measure the H_2O_2 produced by monocytes and PMN cells a microtechnique using 96-well flat-bottomed microtitre plates (Sterilin Ltd., Middlesex, UK), essentially as described by Paton & Ferrante (1983) was used. To each well 70ul PMN cells or monocytes (which were prepared as described in Section 4.2.1. and 4.3, respectively); 20ul horseradish peroxidase (250ug/ml type IV - Sigma Chemical Co, St Louis, MO, USA); 70ul phenol red (500ug/ml - Merck, Germany) (Appendix 7) and 40ul opsonised yeast (Appendix 5) was added. All solutions were made up in PBS. Test or contol systems contained 20ul of the 25kDa mycobacterial fraction, or PBS respectively. The 25kDa fraction was used at varying concentrations ranging from 10ug/ml to 100ug/ml. In some experiments, IFN-gamma was included in the culture system whereby 20ul of IFN-gamma were added at a concentration of 200U/ml. The plates were incubated at 37°C for 60 minutes after which the reaction was stopped by adding 20ul of 2M NaOH. The absorbance at 620nM was measured in a Titertek Multiscan MC spectrophotometer (Flow Laboratories, Bioggio, Switzerland). The assay was standardised with freshly diluted H_2O_2 (BDH Chemicals Ltd, Poole, UK) and was linear over the range 0 - 60uM.

4.12. HEXOSE MONOPHOSPHATE SHUNT (HMPS) ACTIVITY

4.12.1. Introduction

On activation, phagocytes change their metabolic processes from a low energy yielding, oxygen independent glycolytic pathway to the aerobic, high energy yielding HMPS (Babior, 1978a).

The pathway provides a mechanism for splitting the carbon chain of a glucose molecule one carbon at a time, releasing CO_2 and energy and regenerating NADPH which acts as an electron donor for NADPH oxidase (Babior, 1978a).

4.12.2. Assessment of HMPS Activity

HMPS activity was assessed by the production of $^{14}CO_2$ from glucose-1-^{14}C (New England Nuclear, Boston, MA, USA) by the method of Wood *et al.* (1963). Cells were suspended to a final concentration of 2 x 10^7/ml in 0.15M glucose-free PBS. The test apparatus was a 10ml glass scintillation vial (Packard Instruments, Illinois USA) which served as the outer chamber, stoppered with a tightly fitting perforated metal lid. Inside this chamber was placed a 1.0ml autoanalyser cup which served as the inner chamber. The reaction mixture consisted of: 700ul glucose-1-^{14}C (0.07uCi); 100ul Bakers' yeast (20 x 10^8/ml); 100ul fresh serum; 100ul 25kDa mycobacterial fraction (50ug/ml); 100ul IFN-gamma (200U/ml). Appropriate volumes of PBS was added to control cultures which did not contain the 25kDa fraction or IFN-gamma. This reaction mixture was placed in the outer chamber and 600ul of a solution of 1M KOH (Merck, Darmstadt, Germany) was placed in the inner chamber (autoanalyser cup). The inner chamber was carefully lowered into the scintillation vial and allowed to stand at 37°C for 10 minutes. The reaction was initiated by introducing 200ul of cell suspension (prepared as described in Section 4.2.4. and 4.3) into the outer chamber by injection through the rubber lined cap with a syringe

to which a long-needle was attached. The reaction was terminated by the additon of 2ml of 2M HCl after 60 minutes. The chambers were allowed to stand at room temperature for a further 60 minutes to permit the release of ^{14}C labelled CO_2 and its subsequent absorption by the KOH. Aliquots of 500ul of the inner chamber mixture were then transferred to scintillation vials containing 10ml of acidified instagel (Appendix 8) (Packard) and counted in a LKB liquid scintillation spectrometer (Stockholm, Sweden) for 10 minutes each.

Controls consisted of the reaction mixture devoid of the cell suspension, and the counts so obtained (background counts) were subtracted from the corresponding experimental values. All tests were performed in duplicate and expressed as corrected (minus background) counts per minute (c.p.m.).

4.13. MEASUREMENT OF THE GLYCOLYTIC PATHWAY

4.13.1. Introduction

Glycolysis is a pathway used by cells to supply energy required for normal cell functioning. This pathway involves the conversion of glucose via a series of reactions to pyruvate which is then reduced to lactate by lactate dehydrogenase (Metzler, 1977). The extent of glycolysis by both resting and stimulated cells was measured by lactate production (Hohorst, 1962), using recognised procedures (Boehringer Mannheim biochemical test combination).

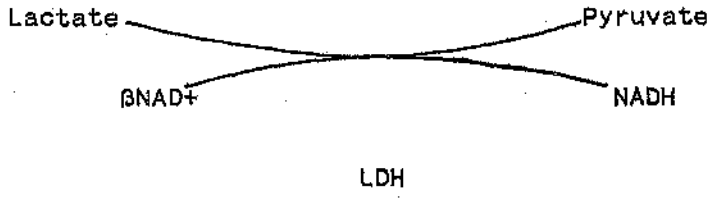
4.13.2. Method

4.13.2.1. Production of Lactate

This assay was performed in two parts. In the first, lactate rich supernatants were produced and in the second aliquots of these supernatants were assessed for the amount of lactic acid produced. Monocytes maintained in culture were removed from the plastic tissue culture plates as described in Section 4.2.1. and PMN cells were isolated as described in Section 4.3. Both cell types were resuspended in 0.15M PBS containing 10mM glucose (BDH, Poole, UK). Each assay was performed in duplicate. The reaction mixture consisted of: 300ul cells (1 x 10^7/ml); 200ul serum (control) or EAS (25%); 100ul 25kDa mycobacterial fraction; 100ul IFN-gamma (200U/ml). PBS was added as required to each tube to make up the final volume to 800ul. Tubes were incubated at 37°C for varying periods of time (10, 20 and 30 minutes) and the reaction was stopped by deproteinisation with the addition of 1ml of cold 0.6N perchloric acid (PCA). The tubes were mixed well, vortexed and centrifuged at 400g for 10 minutes. Supernatants were collected and used to measure the lactic acid produced.

4.13.2.2. Assessment of Lactate Produced

Aliquots of supernatants from each tube were assayed for lactic acid by monitoring the spectrophotometric change accompanying the reduction of the enzyme cofactor β nicotinamide adenine dinucleotide (NAD+) to NADH during the conversion of lactate to pyruvate by lactate dehydrogenase (LDH).



Each tube contained the following: 2ml assay buffer (Appendix 9); 200ul 27mM NAD; 20ul LDH (2mg/ml) diluted in 2.1M NH_4SO_4; 200ul supernatant fluid. Control tubes contained 0.6N PCA instead of supernatant fluid. The tubes were incubated at 25°C for 60 minutes and the change in optical density at 340nm (ΔE 340nm) was assessed spectrophotometrically using a PYE UNICAM PU8800 spectrophotometer (Phillips Analytical). Results were expressed as ug lactate/6 x 10^6/ml/unit time by the conversion factor 1.25 x ΔE 340 x 1 000 as described by Hohorst (1962).

4.14. ANTIGEN PRESENTATION BY MACROPHAGES TREATED WITH THE 25kDa MYCOBACTERIAL FRACTION

4.14.1 Introduction

Previous studies (Wades *et al.*, 1987) and the present work demonstrate that the 25kDa mycobacterial fraction inhibited the intracellular killing ability of the macrophage. In addition, these studies have also shown its effects on the intracellular degradative functions of the macrophage. It was of interest, therefore, to determine whether the 25kDa mycobacterial fraction had any effect on the antigen presenting capacity of the macrophage. This was assessed by lymphocyte proliferation assays to both mitogens and antigens.

4.14.2. Method

Mononuclear cells from PPD positive donors, separated on Hypaque-Ficoll density gradients, were plated onto 96-well flat-bottomed microtitre plates (Starilin Ltd., Middlesex, UK) at a concentration of 1 x 10^6/ml (200ul/well). The non-adherent cells were removed by washing 3 times with warm (37°C) medium. The adherent cells were then treated with the 25kDa fraction at a final concentration of 50ug/ml (this concentration was used as it optimally inhibited the various pathways of phagocyte function) for 60 minutes, followed by washing 3 times with warm medium to remove the 25kDa mycobacterial fraction.

The non-adherent cells removed from the 96-well plates were re-adhered for one hour on plastic tissue culture plates (145/20mm - Greiner Labortechnik, Solingen) in order to remove any remaining adherent cells. The non-adherent cells were then collected, washed and resuspended in RPMI 1640 containing 10% autologous serum to a concentration of 1 x 10^6/ml, so as to reconstitute a ratio of 20:80 (macrophages to lymphocytes). Two hundred microlitres of this cell suspension was then added to each well containing macrophages treated with the mycobacterial fraction. The lymphocytes were then stimulated with purified protein derivative (PPD) (Connaught Medical Research Laboratories, Canada) at a final concentration of 1, 5 and 10ug/ml; phytohaemagglutinin (PHA) (Wellcome Laboratories,); Concanavilin A (con A) (Pharmacia, Uppsala, Sweden) or pokeweed mitogen (PWM) (Boehringer Mannheim, Frankfurt, Germany). The

cultures stimulated with the mitogens were incubated for 3 days while the cultures stimulated with PPD were incubated for 5 days at 37°C in a humidified incubator containing 5% CO_2 and 95% air. Eighteen hours prior to harvesting 20ul (1uCi) of tritiated thymidine (methyl-^{3}H thymidine, specific activity at 24Ci/mMole - Radiochemical Centre, Amersham, England) were added to each well. Cells were then harvested onto glass fibre filters (Whatman Ltd., Maidstone, UK) and placed in individual plastic scintillation vials (Packard Instruments, Illinois, USA). Filters were dried overnight and 5ml of Instafluor (Chemlab, South Africa) was added to each vial. Radioactivity was measured in an LKB Rackbeta liquid scintillation spectrometer (Stockholm, Sweden). Counts were expressed as mean counts per minute (c.p.m.) of triplicate cultures.

4.15. EFFECT OF THE 25kDa MYCOBACTERIAL FRACTION ON THE INDUCTION OF MHC CLASS II ANTIGEN (HLA-DR) EXPRESSION BY CULTURED MACROPHAGES

4.15.1. Introduction

It is well documented that for macrophages to activate antigen specific T lymphocytes, they must present processed antigen particles in conjunction with MHC Class II (HLA-DR) molecules (Schroer & Rosenthal, 1980; Shimonkevitz, 1983; Shevach & Rosenthal, 1973). The effect of the 25kDa mycobacterial fraction on the expression of HLA-DR by stimulated macrophages was assessed in conjunction with the previous experiments.

4.15.2. Assessment of DR Expression
4.15.2.1. Induction of DR Expression

Mononuclear cells separated by centrifugation on a hypaque-ficoll density gradients were washed in saline, resuspended to a concentration of 3 x 10^6/ml in RPMI 1640 containing 10% autologous serum and added to each well of a 96-well flat-bottomed microtitre plate (200ul/well) (Sterilin Ltd, Middlesex, England). The cells were allowed to adhere for 60 minutes after which the non-adherent cells were removed by washing three times with warm saline. The adherent cells (macrophages) were then grown in RPMI 1640 containing 10% autologous serum for 3-6 weeks. Purity of cells was assessed with the OKM1 monoclonal antibody (Section 4.2.1.). DR expression was induced by incubation of the cells with *E.coli* lipopolysaccharide (LPS) (*E.coli* 0127:B8 - Difco Labs, Detroit, USA) at a concentration of 20ug/ml, yeast particles at a concentration of 1 x 10^6/ml or IFN-gamma at a concentration of 200U/ml. The cells were then incubated for a further 5 days at 37°C. Cells were fixed by the addition of 100ul of 0.5% paraformaldehyde to each well and plates were incubated for 30 minutes at room temperature. Plates were washed three times with phosphate buffered saline (PBS) (Appendix 1). DR expression was then assessed using a modified cellular ELISA.

4.15.2.2. Cellular Enzyme-Linked Immunosorbent Assay (ELISA) for DR Expression

After the cells had been fixed with paraformaldehyde, the wells were treated with 0.5% BSA in a carbonate buffer, pH 9.6 (Appendix 10) for 60 minutes. This blocking step was aimed at

preventing non-specific binding of the monoclonal antibody. After washing the plates three times with PBS-Tween, 100ul of 1:100 dilution of the monoclonal antibody to DR (Ortho Diagnostic Systems Inc., Raritan, NJ, USA) were added to each well and the plates were incubated in a humidified chamber for 120 minutes at room temperature. The plates were washed three times with PBS-Tween followed by the addition of 100ul of a peroxidase labelled anti-mouse IgG (Amersham International, UK). The plates were incubated for 60 minutes at room temperature after which unbound conjugated antibody was removed by washing the trays three times with PBS-Tween. One hundred microlitres of peroxidase substrate (Appendix 11) were added and the reaction stopped after 30 minutes with 50ul of 2.5M sulphuric acid (BDH Chemicals Ltd., Poole, UK). Optical densities were measured at 492nm in a Titretek Multiscan MC spectrophotometer (Flow Laboratories, Bioggio, Switzerland).

CHAPTER 5
RESULTS

5.1. SEPARATION OF STANDARDS OF KNOWN MOLECULAR WEIGHT ON A SEPHACRYL S-200 COLUMN

Figure 12 demonstrates a plot of the elution volume of known molecular weight standards off a Sephacryl S-200 column against the log molecular weight for ribonuclease (14kDa), chymotrypsin (25kDa), carbonic anhydrase (30kDa), bovine serum albumin (67kDa), alcohol dehydrogenase (150kDa); aldolase (158kDa) and catalase (232kDa). The experimental points lie close to a straight line.

5.2. SEPARATION OF MYCOBACTERIAL EXTRACT ON A SEPHACRYL S-200 COLUMN

Figure 13 demonstrates that unabsorbed mycobacterial extracts can be separated into several fractions by column chromatography. The protein elution profile of extracts off such sephacryl columns is shown (mean of 10 experiments). The major protein peaks obtained from the column were arbitrarily identified alphabetically from A-I. The position of molecular weight markers used is indicated on the figure.

5.3. ASSESSMENT OF PURITY OF CELL SUSPENSIONS

5.3.1. Assessment of Purity of Macrophage Cell Cultures

In order to ascertain whether adherent cells grown in culture were macrophages, the cultures were treated with the monoclonal antibody OKM1, assessed for esterase staining and their phagocytic ability determined.

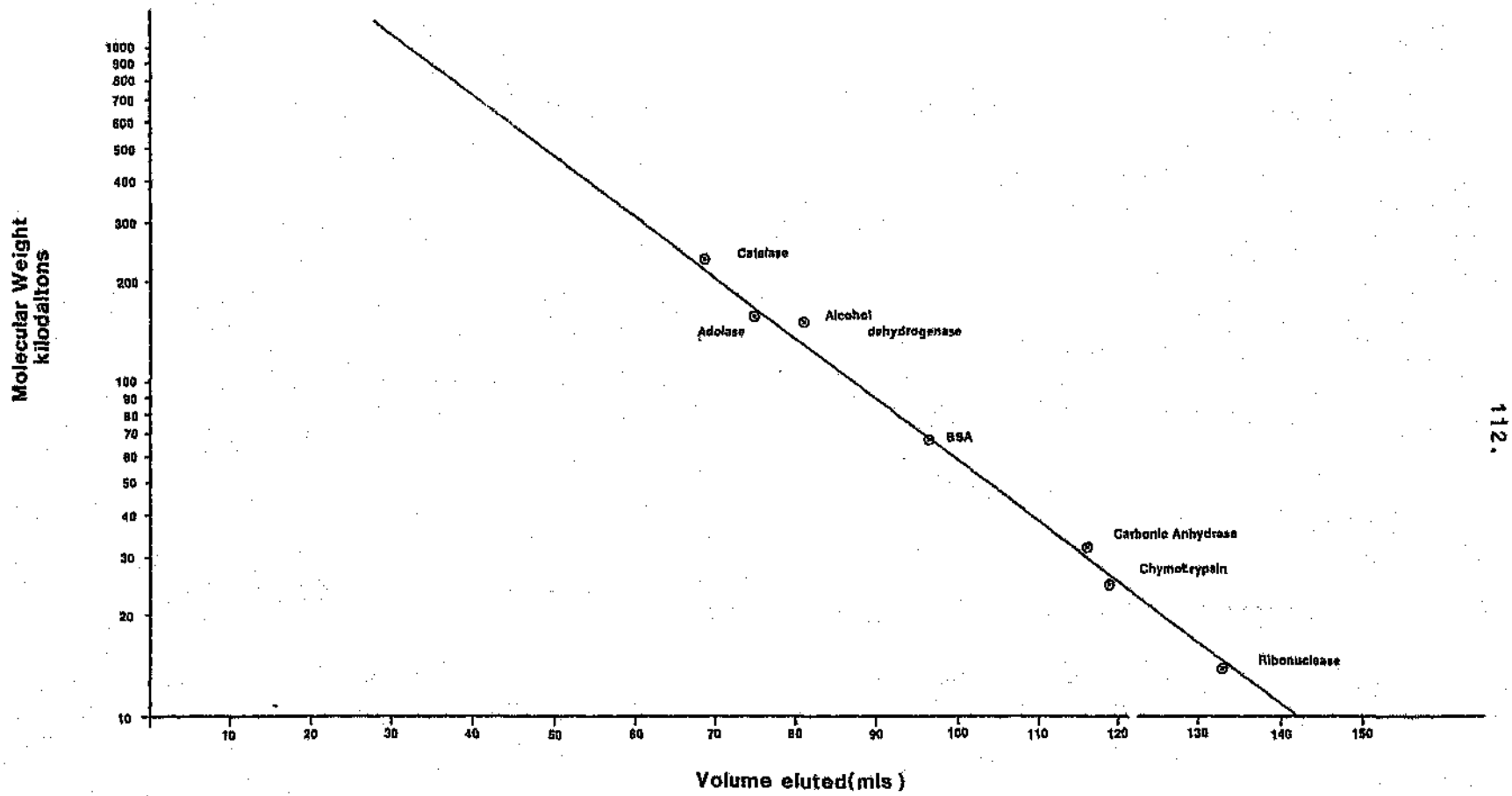


Figure 12: The relationship of proteins with known molecular weights and elution volumes off a Sephacryl S - 200 column.

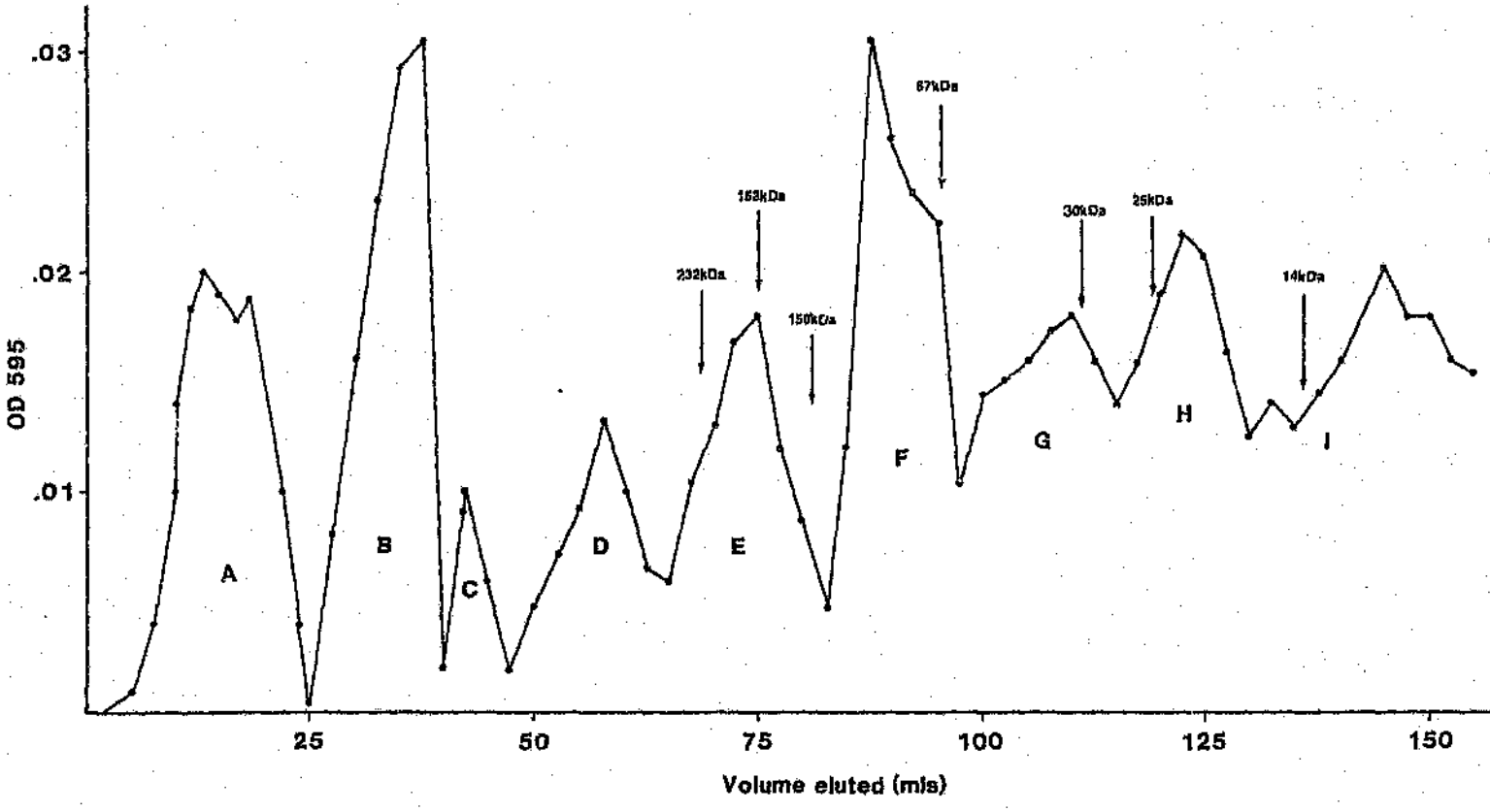


Figure 13: Protein elution profile of M.Tuberculosis extracts off a Sephacryl S - 200 column.

5.3.1.1. Binding of OKM1 Monoclonal Antibody By Cells in Culture

Binding of OKM1 by cells grown on tissue culture chamber slides was assessed by counting the number of cells demonstrating fluorescence using an indirect sandwich immunofluorescent assay. Repeated tests consistently demonstrated a greater than 95% fluoresence of the cell cultures, indicating binding of the OKM1 to these cells. These results were confirmed by 3 different observers.

5.3.1.2. Esterase Staining of Cell Cultures

Cells grown in culture on tissue culture chamber slides were assessed for esterase staining as described in Chapter 4, Section 4.2.1.2. The results indicate that of 200 cells visualised microscopically 100% were esterase positive. Similar results were obtained for each experiment performed. Figure 14 is a photograph representative of one such experiment.

5.3.1.3. Phagocytic Ability of Cultured Monocytes

Ninety five percent of the cells on tissue culture slides showed the ability to phagocytose yeast particles. Figure 15 shows monocytes which have phagocytosed yeast particles. Similar results were obtained for each experiment performed. Figure 15 is a representative figure of one such experiment.

Because of the finding that >95% of cells were recognised by antibodies to monocytes, were esterase positive and were actively phagocytic, the techniques employed for isolating and culturing monocytes were used throughout these studies.

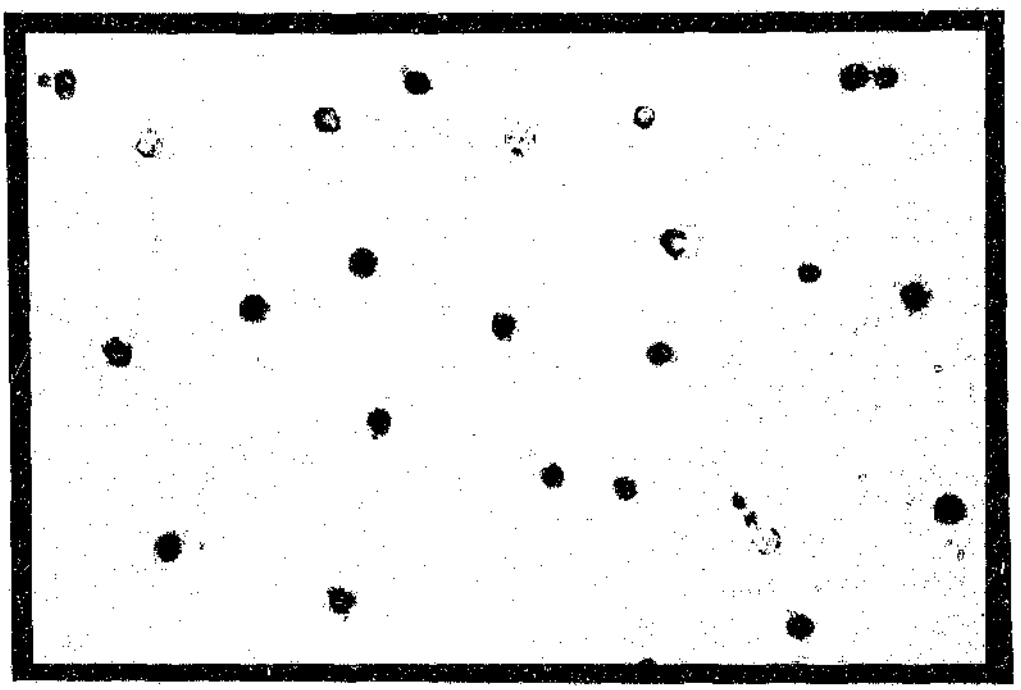


FIGURE 14. Esterase staining of cell cultures.

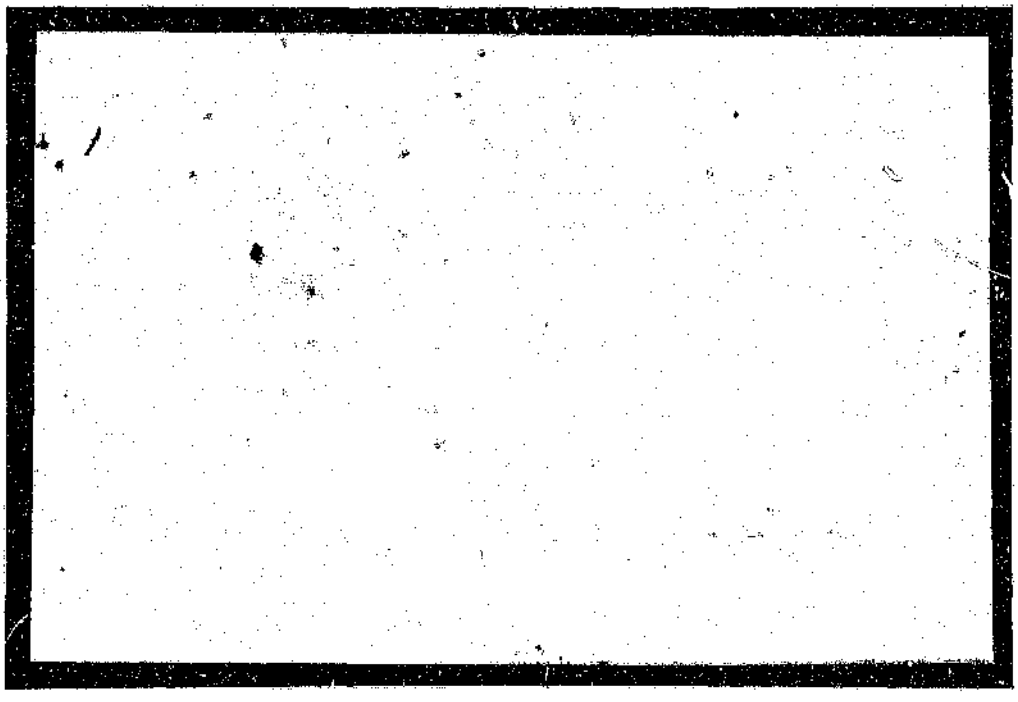


FIGURE 15. Phagocytic ability of cultured monocytes

5.3.2. Assessment of Purity of PMN Cell Suspensions

Visual assessment and differential counting done by a Sysmex K1000 (Milsh, Japan) cell counter indicated a >95% purity of the PMN cell suspension. Such results were repeatedly obtained from each experiment. Figure 16 demonstrates results obtained for one such experiment.

5.4. THE EFFECT OF THE 25kDa MYCOBACTERIAL FRACTION ON THE PHAGOCYTIC ABILITY OF CULTURED MONOCYTES AND PMN CELLS

To determine whether the 25kDa fraction of *M.tuberculosis* had any effect on the phagocytic capacity of phagocytes, cultured monocytes and PMN cells were incubated with Bakers' yeast in the presence or absence of 50ug/ml of the mycobacteral extract for varying periods of time. Table 2 (mean of 5 experiments) demonstrates that the 25kDa had no effect on the phagocytic ability of either macrophages in culture or PMN cells. Increasing the concentration of the mycobacterial fraction to 100-200ug/ml also was without effect (results not shown).

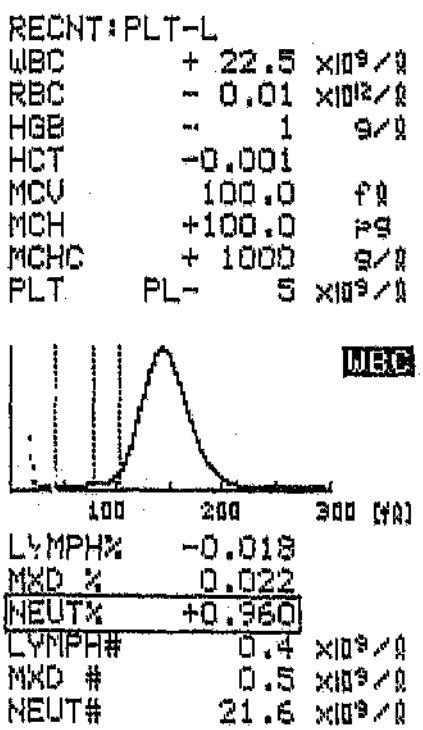


FIGURE 16. Purity of PMN cells in cell suspensions as determined by Sysmex K1000 cell counter.

TABLE 2 Effect of the 25kDa Mycobacterial Fraction on Phagocytic Function.

	% Phagocytosis (mean and range of 3 experiments)			
	PMN Cells		Cultured Monocytes	
Time (minutes)	Without extract	With extract	Without extract	With extract
10	28(24-32)	25(21-29)	24(22-26)	25(22-27)
20	33(30-36)	26(23-29)	28(26-30)	28(25-30)
30	78(73-84)	78(76-80)	74(71-75)	76(72-78)
40	92(90-96)	93(91-95)	90(88-94)	89(88-90)
50	96(94-97)	94(93-98)	96(93-97)	96(94-98)

5.5. EFFECTS OF THE 25kDa MYCOBACTERIAL FRACTION OF THE BACTERICIDAL ABILITY OF PHAGOCYTES

Both cultured monocytes and PMN cells were capable of killing the majority of *S.aureus* organisms within 90 minutes (Table 3). These results are also shown graphically in Figure 17A). In the presence of various concentrations of the 25kDa fraction (ug/ml), a significant decline in the intracellular killing ability was noted for both cell types (Table 3 & Figure 17). The effect of the 25kDa fraction was optimum at a concentration of 50ug/ml had no significant additional effect on the killing ability of the cells. The 25kDa fraction was therefore used at a concentration of 50ug/ml.

When PMN cells and monocytes were treated with the 25kDa mycobacterial fraction in the presence of various concentrations of IFN-gamma, a significant recovery in bactericidal activity was seen (Figure 17C). Adding IFN-gamma to the system reversed the inhibitory action of the 25kDa fraction, with an optimum reversal being seen at a concentration of 100U/ml. Increasing the concentration further did not have any added effect. IFN-alpha at a concentration of 100U/ml had no such effect (Figure 17D). Increasing the concentration of INF-alpha to 200U/ml still failed to restore the inhibitory effects of the 25kDa mycobacterial component (results not shown).

TABLE 3 Intracellular Killing Ability of PMN Cells and Cultured Monocytes.

		S.aureus mean colony count ± SD (% killed)		
Cell Type	Experiment Number	Medium	Phagocytic Cells	Phagocytic Cells + 25kDa Fraction (50ug/ml)
PMN cells	1	350	70 (80)	250 (29)
	2	296	4 (98)	136 (54)
	3	312	47 (85)	168 (46)
	Mean ± SD	319 ± 28	40 ± 33	186 ± 59[a]
Monocytes	1	360	47 (87)	181 (50)
	2	424	85 (80)	200 (53)
	3	390	66 (83)	187 (52)
	Mean ± SD	391 ± 32	66 ± 19	189 ± 10[a]

[a] – P <.001

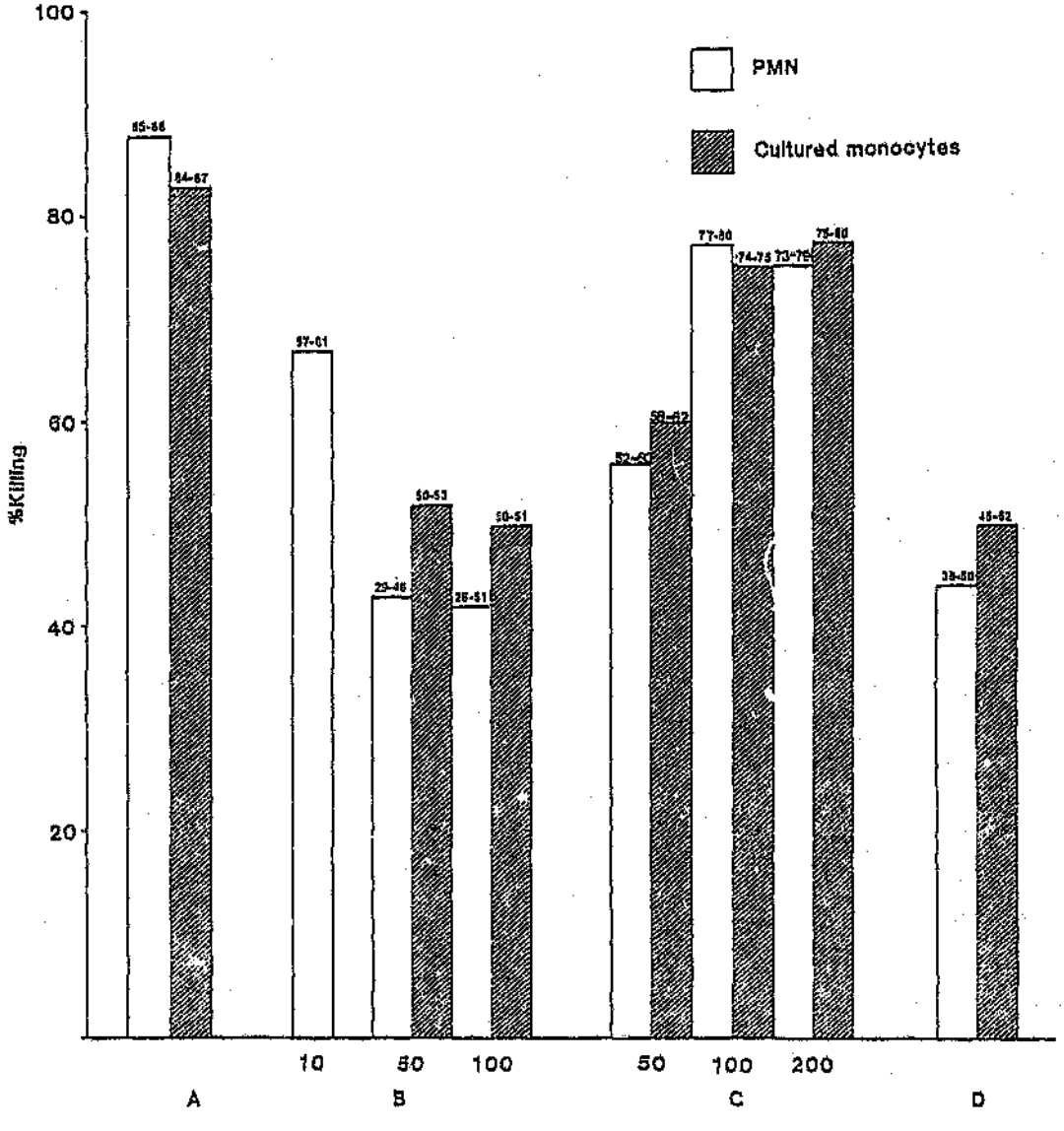


FIGURE 17. Intracellular killing ability of PMN cells and cultured monocytes in the presence of:
(A) Medium; (B) Varying concentrations of the 25kDa fraction (ug/ml); (C) 25kDa fraction + varying concentrations of IFN-gamma (units/ml); (D) 25kDa fraction + 100U/ml of IFN-alpha.

5.6. PHAGOSOME-LYSOSOME FUSION IN THE PRESENCE OF THE 25kDa MYCOBACTERIAL FRACTION

Lysosomes of both PMN cells and macrophages in culture were prelabelled with acridine orange and the phagocytes were permitted to ingest live Bakers' yeast in the presence or absence of the 25kDa mycobacterial fraction. A mean of four experiments showed that the 25kDa fraction significantly inhibited this process (Table 4, Figure 18B). The effect of the 25kDa fraction was optimum at 50ug/ml.

In Figure 18C the effects of varying concentrations of IFN-gamma on the inhibition of phagosome-lysosome fusion mediated by the 25kDa mycobacterial fraction are shown.

Treatment with IFN-gamma partially reversed the inhibitory action of the 25kDa mycobacterial fraction, with optimum effects at a concentration of 200U/ml. IFN-gamma on its own did not affect phagosome-lysosome fusion. The addition of IFN-alpha at a concentration of 200U/ml also had no effect on reversing the inhibition caused by the 25kDa fraction (Figure 18D).

TABLE 4. Percentage of yeast containing PMN cells and cultured monocytes demonstrating phagosome-lysosome fusion (as evidenced by dye uptake) in the presence or absence of the 25kDa mycobacterial fraction.

Cell System	% Fused	% Non-Fused	% Intermediate
PMN cells + yeast	78	14	8
Monocytes + yeast	73	12	15
PMN cells + yeast + 25kDa fraction (50ug/ml)	30	54	16
Monocytes + yeast + 25kDa fraction (50ug/ml)	34	49	17
PMN cells + yeast + 25kDa fraction (100ug/ml)	29	52	19
Monocytes + yeast + 25kDa fraction (100ug/ml)	36	46	18

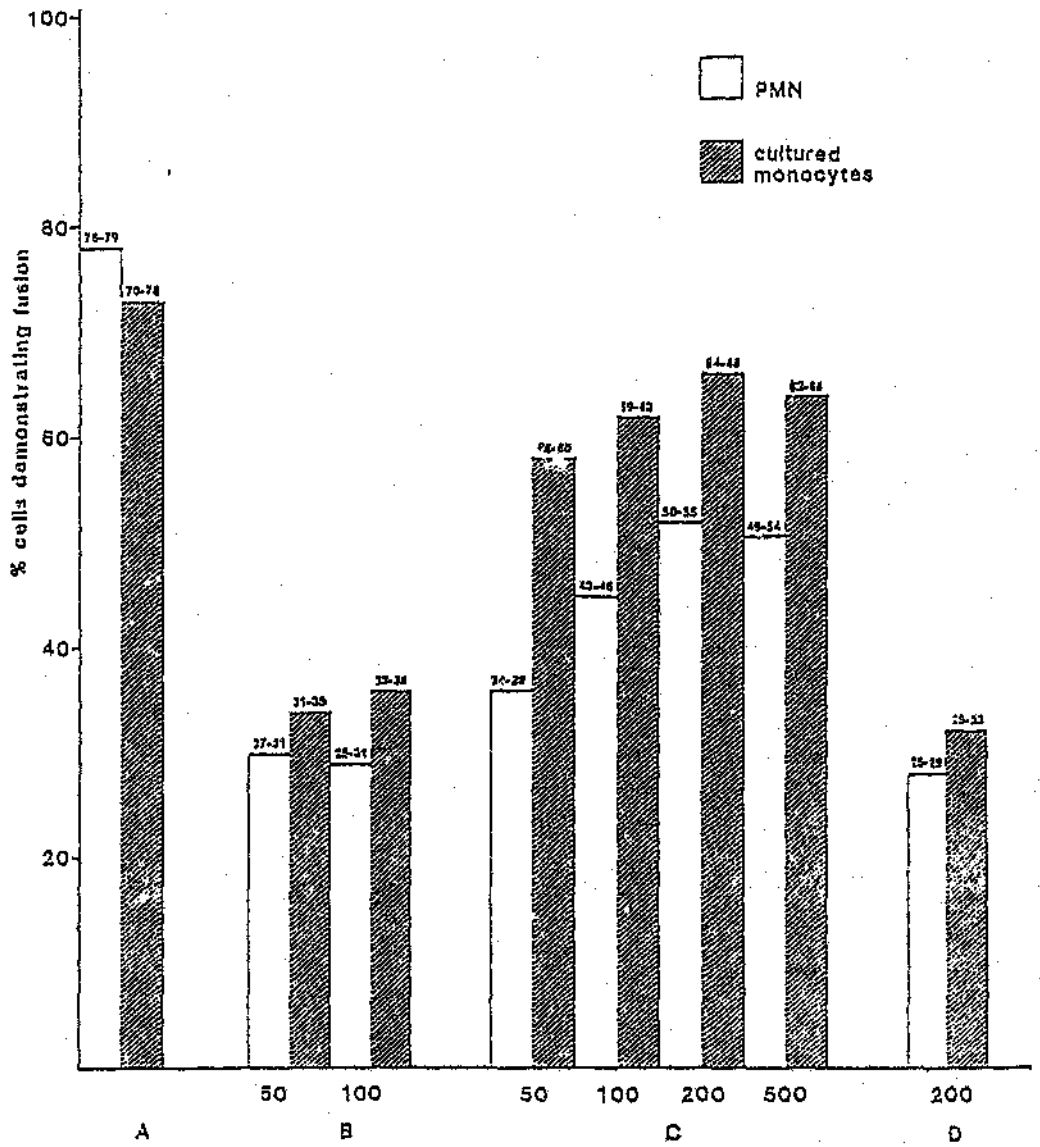


FIGURE 18. Phagosome-lysosome fusion in PMN cells and cultured monocytes in the presence of:
(A) Medium; (B) Varying concentrations (ug/ml) of the 25kDa fraction; (C) 25kDa fraction + varying concentrations of IFN-gamma (units/ml); (D) 25kDa fraction + 200U/ml of IFN-alpha.

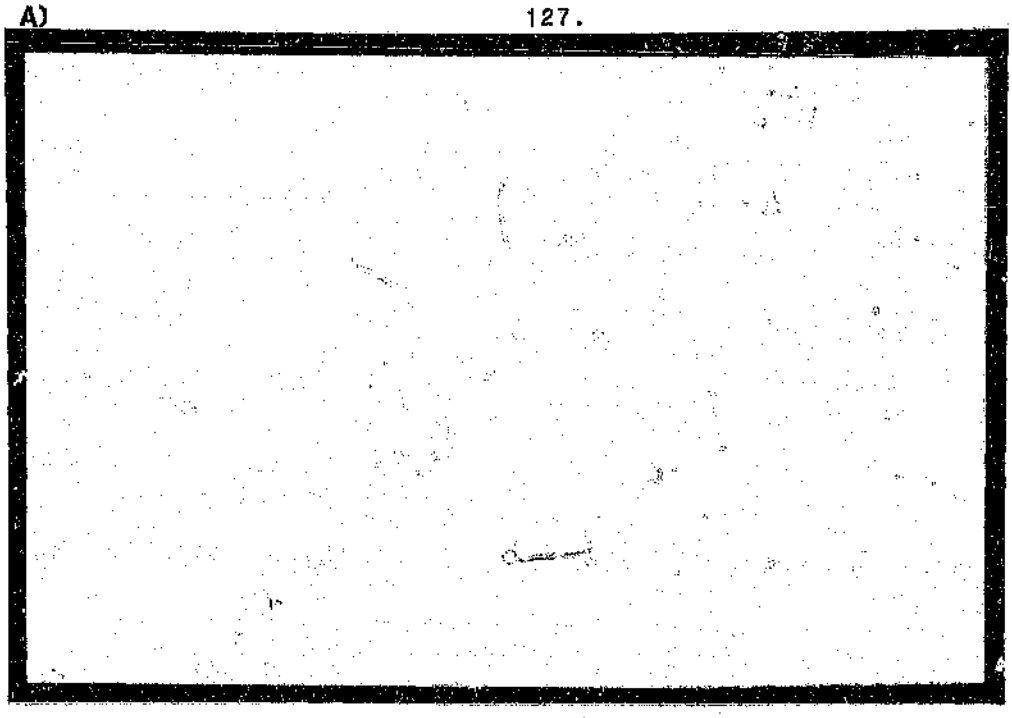
Figure 19 (A & B) demonstrates yeast particles which have been ingested by PMN cells not incubated with the 25kDa mycobacterial fraction. The photograph of cells seen under UV light (Figure 19B) shows fluorescent particles (having taken up acridine orange) indicating successful phagosome-lysosome fusion.

Figure 20 (A & B) demonstrates yeast particles which have been ingested by PMN cells incubated in the presence of the 25kDa fraction. Figure 20B, the same section as Figure 20A observed under UV light, shows dark patches where the yeast particles occur in the previous photograph. This is an indication that phagosome-lysosome fusion has not occurred.

5.7. LYSOSYME PRODUCTION BY PHAGOCYTES: EFFECT OF THE 25 Da MYCOBACTERIAL FRACTION AND IF.. GAMMA

When lysosyme production was assessed in the presence of several concentrations of the 25kDa mycobacterial fraction, a dose related reduction was observed for both PMN cells

A)



B)

FIGURE 19. PMN cells ingesting yeast particles under (A) white light and (B) U.V. light. Fluorescent yeast particles indicate that phagosome-lysosome fusion has occurred.

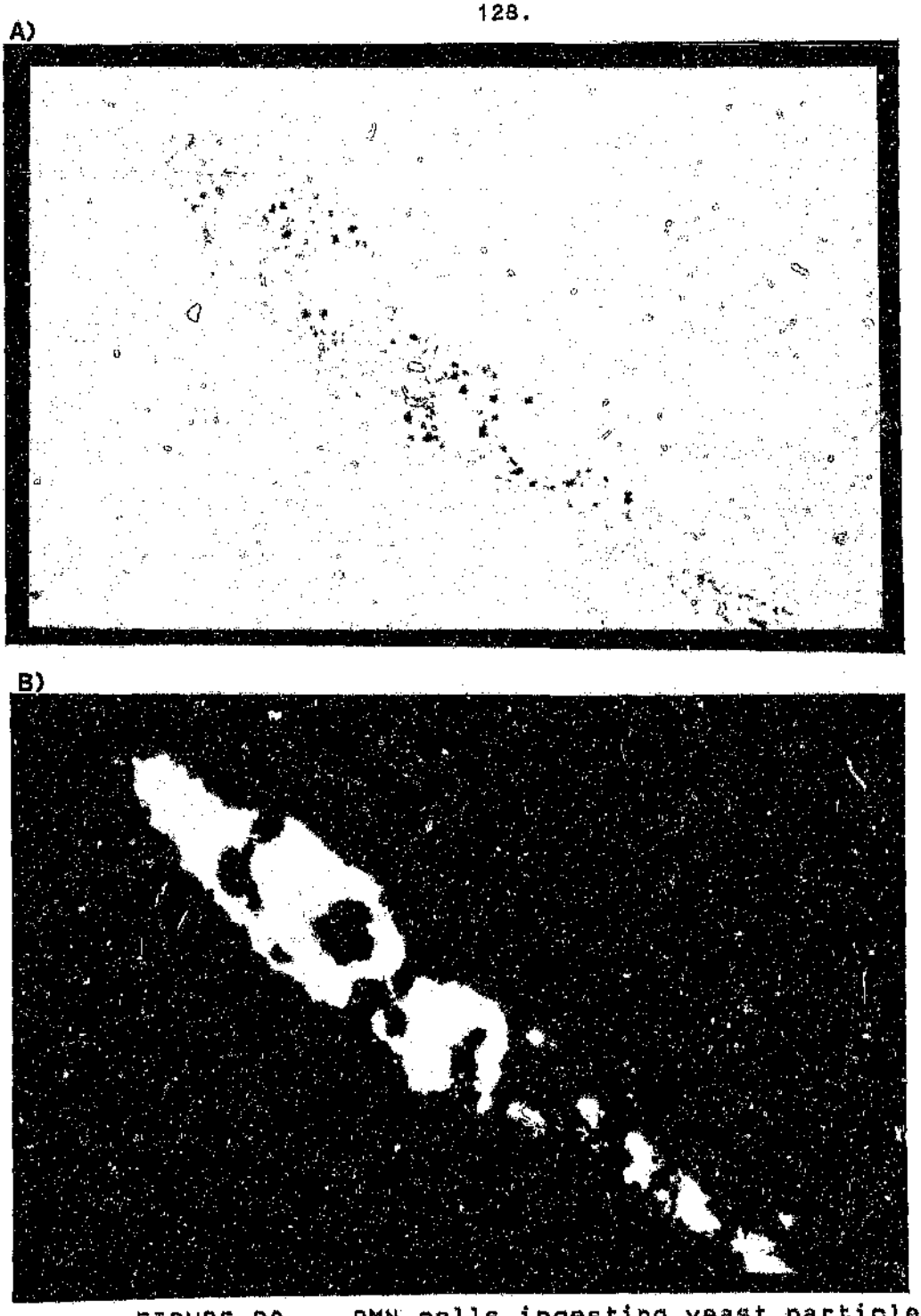


FIGURE 20. PMN cells ingesting yeast particles in the presence of the 25kDa mycobacterial fraction under (A) white light and (B) U.V. light. Dark patches seen in (B) in the same position as yeast particles seen in (A) indicate that no phagosome-lysosome fusion has occurred.

(Figure 21A) and cultured monocytes (Figure 21B). Maximal inhibition was seen with 50ug/ml of the 25kDa mycobacterial fraction. Higher concentrations did not reduce lysosyme production any further.

When IFN-gamma was included in the cultures containing 50ug/ml of the 25kDa mycobacterial fraction, lysosyme production was partially restored for both PMN cells (Figure 22A) and cultured monocytes (Figure 22B). The maximal restorative ability was observed at a IFN-gamma concentration of 200U/ml. INF-gamma on its own had no effect on the cells ability to produce lysosyme (results not shown).

5.8. NBT REDUCTION BY PMN CELLS IN THE PRESENCE OF THE 25kDa MYCOBACTERIAL FRACTION

When NBT reduction induced by endotoxin-activated serum (EAS) was assessed in the presence of the 25kDa mycobacterial fraction, a significant decrease in the number of cells with positive staining was observed (Table 5). When IFN-gamma at a concentration of 200U/ml was added to the cells together with the 25kDa mycobacterial fraction, NBT reduction was partially restored (Table 5).

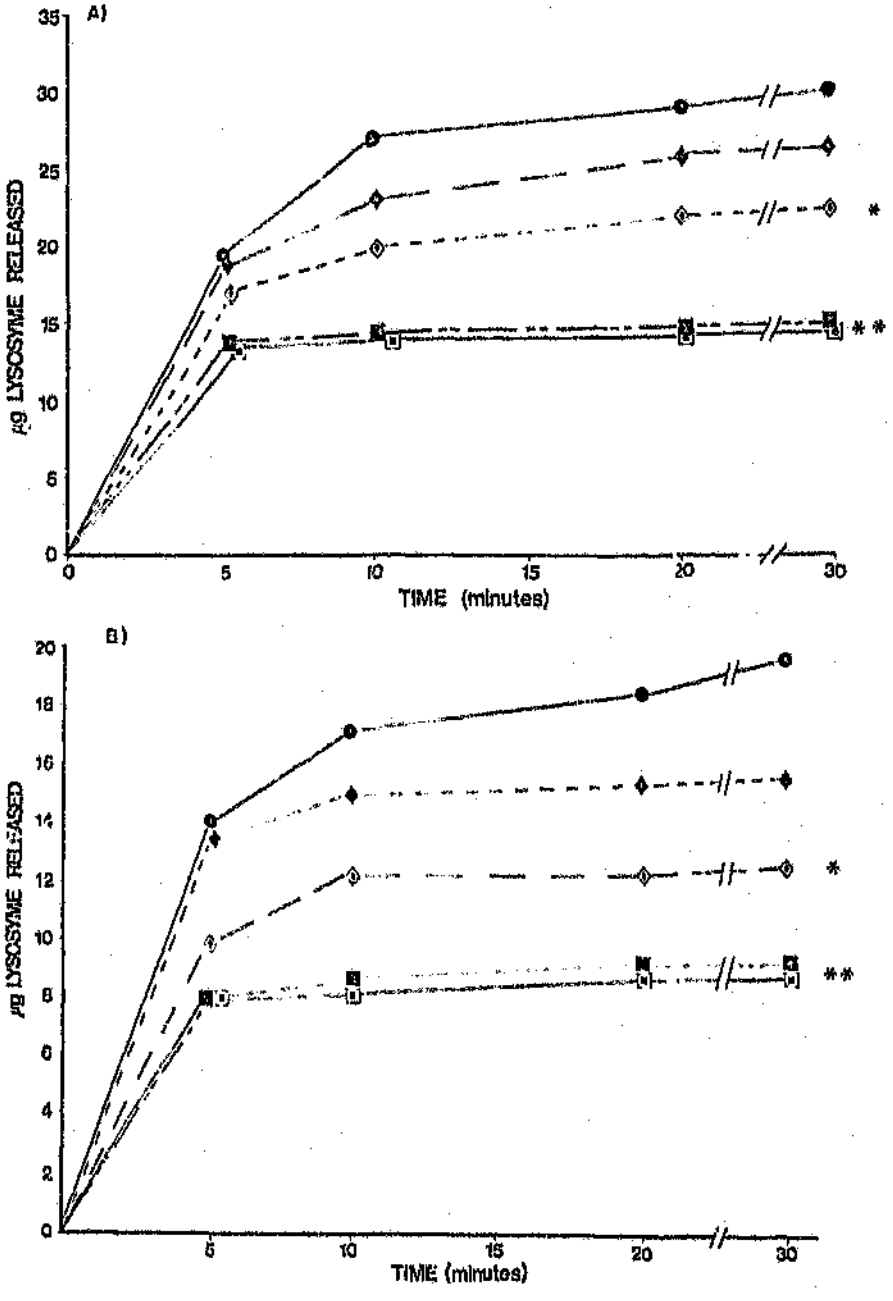


FIGURE 21. Lysosyme release by PMN cells (A) and cultured monocytes (B) in the presence of medium (●) or increasing concentrations of the 35kDa mycobacterial fraction:- (◆) 10ug/ml; (◈) 25ug/ml; (■) 50ug/ml; (▣) 100ug/ml
* p < .01 ** p< .001

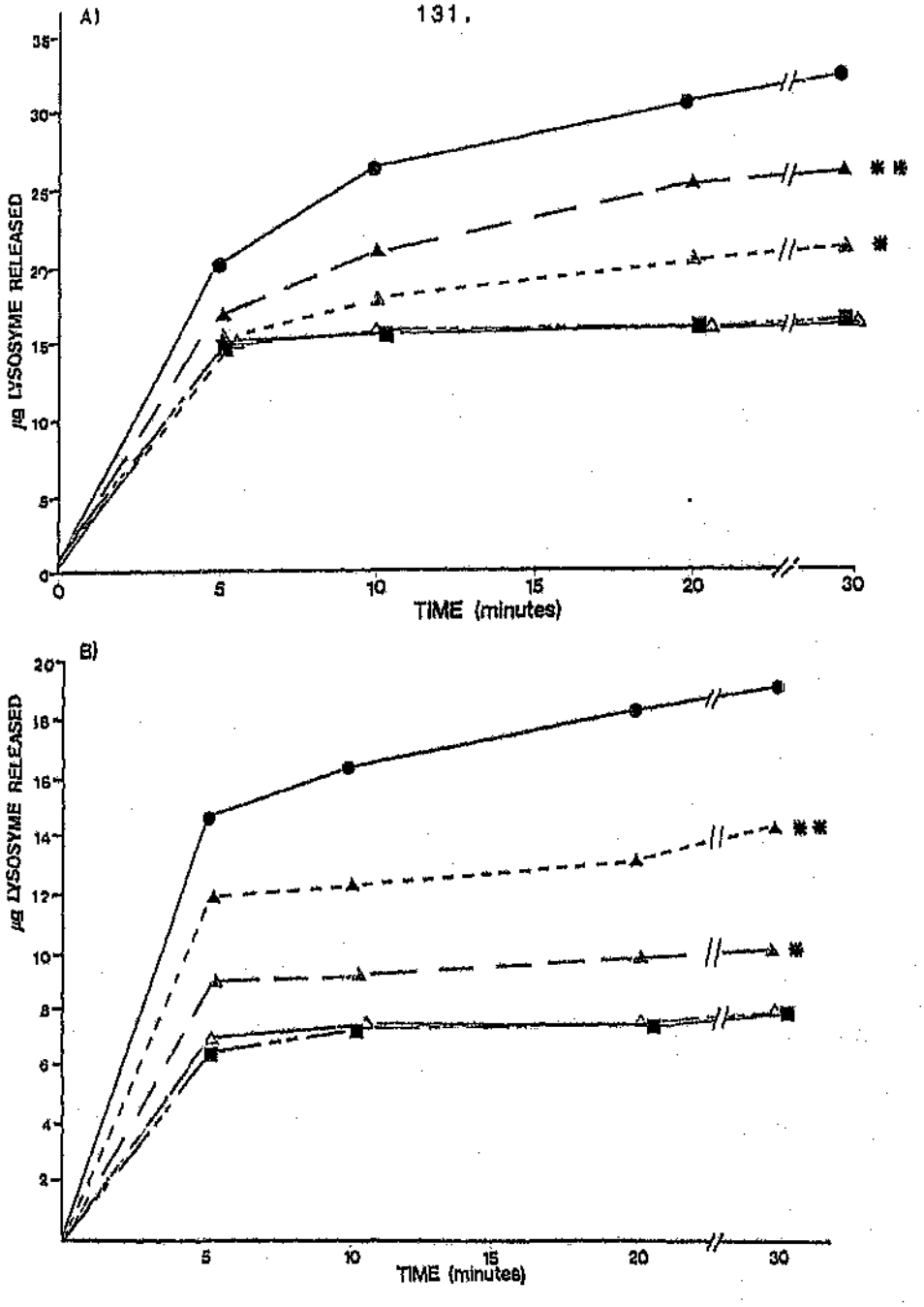


FIGURE 22. Effect of IFN-gamma on lysosyme release by PMN cells (A) and cultured monocytes (B). Cells incubated with medium (●); 25kDa mycobacterial fraction alone (■); 25kDa mycobacterial fraction (50ug/ml) and increasing concentrations of IFN-gamma (U/ml) 50 (△); 100 (▲); 200 (▲).
*P < .01 ** P< .001

TABLE 5. Effect of the 25kDa Mycobacterial Fraction on PMN NBT Reduction.

Cell System	Number of Cells Demonstrating NBT Reduction [a]
PMN cells (resting)	9 ± 1
PMN cells + EAS	58 ± 7
PMN cells + EAS + 25kDa fraction	26 ± 5[b]
PMN cells + EAS + IFN-gamma	61 ± 6
PMN cells + EAS + 25kDa fraction + IFN-gamma	40 ± 3[c]

[a] - Mean ± SD of 3 experiments

[b] - $P < .001$

[c] - $P < .01$

5.9. EFFECT OF THE 25kDa MYCOBACTERIAL FRACTION ON HYDROGEN PEROXIDE PRODUCTION

The effect of the 25kDa mycobacterial fraction on the ability of phagocytes to produce the oxygen-derived reactive species H_2O_2 is shown in Figure 23. At a variety of concentrations the 25kDa mycobacterial fraction significantly reduced H_2O_2 production in PMN cells and monocytes (Figure 23B). IFN-gamma, at a concentration of 200U/ml, was capable of partially restoring the inhibition produced by the 25kDa mycobacterial fraction (Figure 23C). IFN-alpha had no such restorative ability (Figure 23D).

5.10. HEXOSE MONOPHOSPHATE SHUNT (HMPS) ACTIVITY IN PHAGOCYTES INCUBATED WITH THE 25kDa MYCOBACTERIAL FRACTION AND IFN-GAMMA AND/OR IFN-ALPHA

Phagocytic cells incubated with Bakers' yeast particles in the presence of fresh autologous serum demonstrated a burst of HMPS activity (Table 6 - mean ± SD of 3 experiments). However, in the presence of the 25kDa mycobacterial fraction a significant decrease in HMPS activity was observed. This reduction of HMPS activity due to the 25kDa mycobacterial fraction was partially, although significantly, restored by IFN-gamma at a concentration of 200U/ml. IFN-alpha (200U/ml), however, could not restore the HMPS activity. IFN-gamma had no effect on the HMPS activity of resting phagocytes. This would indicate that IFN-gamma reverses the inhibitory effects of the 25kDa fraction and does not directly stimulate HMPS activity.

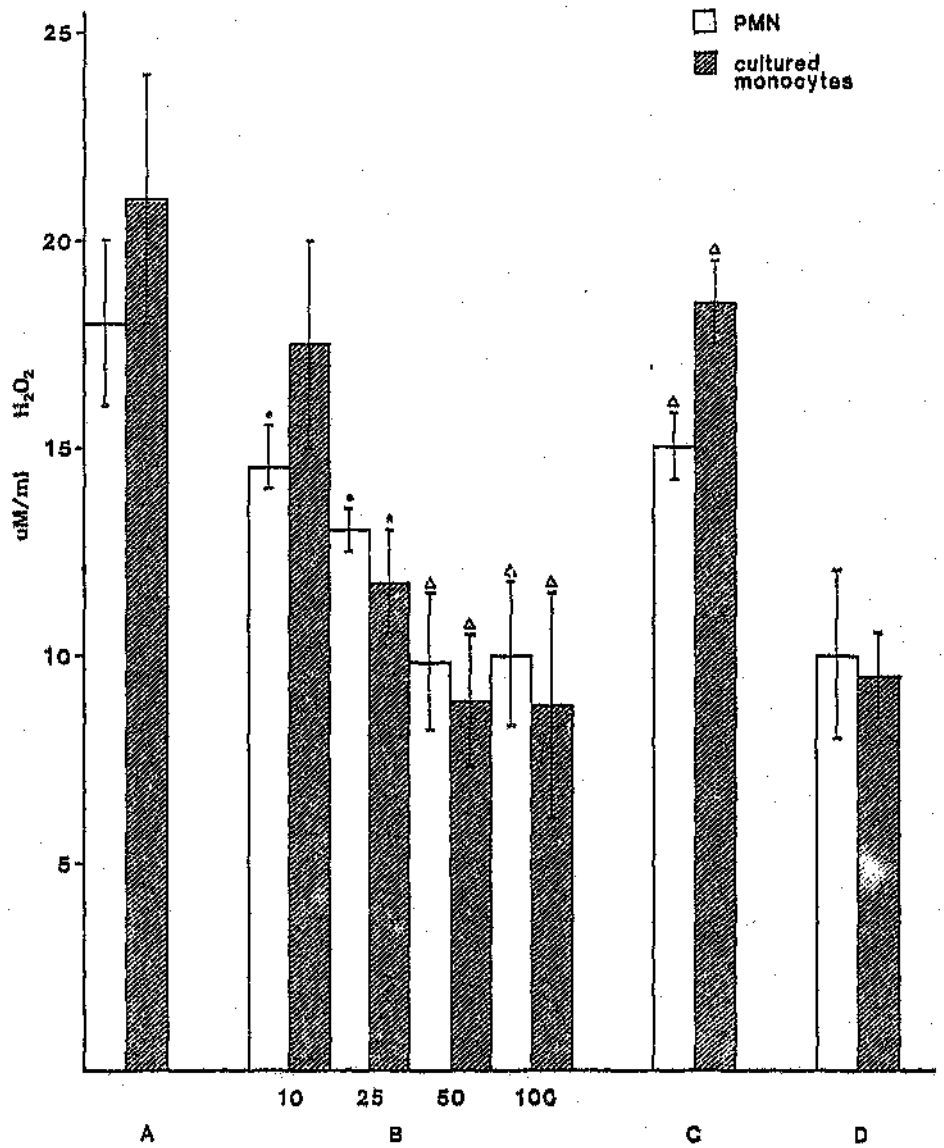


FIGURE 23. H_2O_2 production by PMN cells and cultured monocytes incubated with: (A) Medium; (B) various doses (ug/ml) of the 25kDa mycobacterial fraction; (C) 50ug/ml of the 25kDa mycobacterial fraction with IFN-gamma (200U/ml); (D) 50ug/ml of the 25kDa mycobacterial fraction with IFN-alpha (200U/ml). Mean ± SD of 3 experiments. *P < .01 Δ P< .001

TABLE 6. Effect of the 25kDa Mycobacterial Fraction and IFN-gamma on Phagocyte HMPS activity.

Activated Cells Incubated with:	Mean c.p.m. ± SD of 3 experiments	
	PMN Cells	Cultured Monocytes
Medium	24 792 ± 2 098	14 715 ± 554
IFN-gamma	24 121 ± 3 764	15 540 ± 664
25kDa Fraction [a]	10 074 ± 1 160	7 186 ± 1 920
25kDa Fraction + IFN-gamma [b]	16 167 ± 1 652	12 727 ± 994
25kDa Fraction + IFN-alpha	11 070 ± 1 480	6 064 ± 1 489

[a] $P < .001$

[b] $P < .005$

5.11. MEASUREMENT OF GLYCOLYTIC PATHWAY IN PMN CELLS AND CULTURED MONOCYTES

The level of lactate produced by PMN cells and monocytes (Figure 24) during the glycolytic pathway was significantly reduced by the presence of 50ug/ml of the 25kDa mycobacterial fraction in the system. The glycolytic pathway was significantly affected by the addition of the 25kDa mycobacterial fraction. Higher concentrations of the 25kDa fraction had no additional effect on glycolysis (results not shown).

5.12. ANTIGEN AND MITOGEN PRESENTATION BY MACROPHAGES PRE-TREATED WITH THE 25kDa MYCOBACTERIAL FRACTION

The ability of macrophages, incubated with the 25kDa fraction, to present both mitogens and antigens was assessed by means of proliferation assays. Lymphocyte proliferation in response to the mitogens PHA, Con A and PWM, and to varying concentrations of the PPD antigen (1, 5 and 10ug/ml) was significantly reduced (Table 7). Table 7 demonstrates that the 25kDa mycobacterial fraction interferes with the macrophages ability to correctly present both antigen and various mitogens to the lymphocyte. The effect of the 25kDa fraction on macrophage presentation of PPD is maximal at a concentration of between 6 - 10ug/ml.

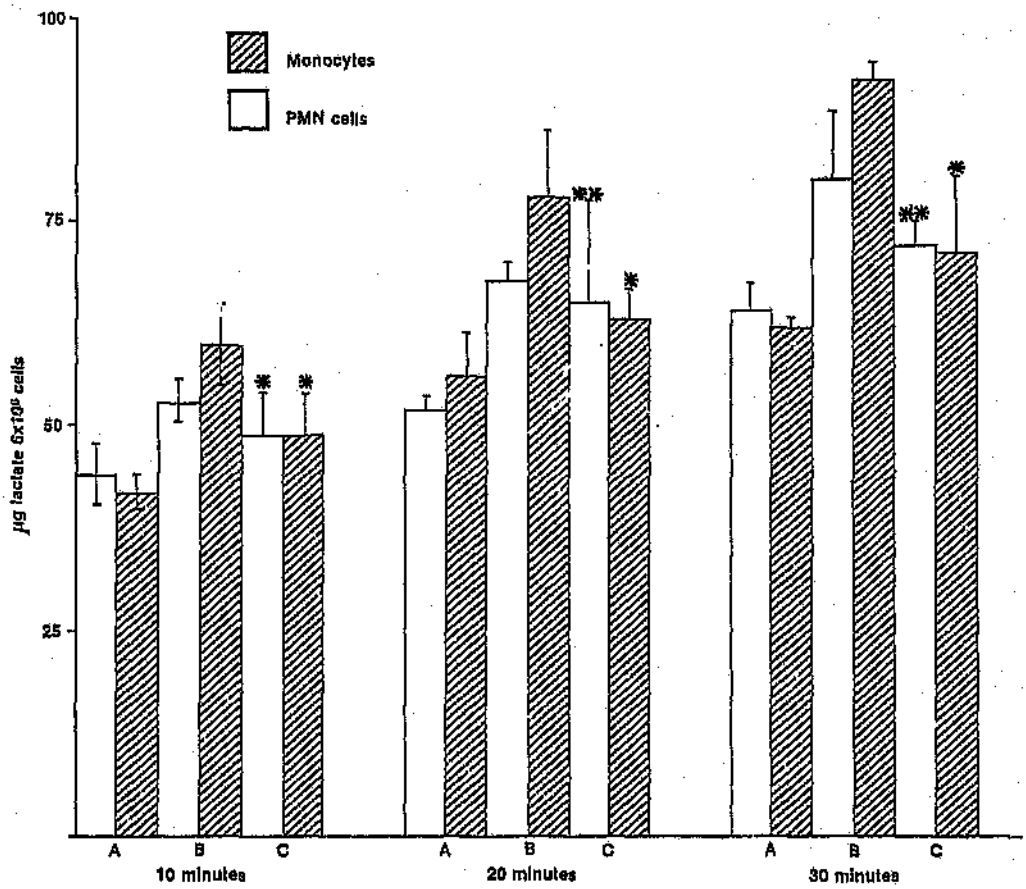


FIGURE 24. Lactate production by PMN cells and monocytes in the presence of: Serum (A); EAS (B); EAS + 50ug/ml 25kDa mycobacterial fraction (C)
* - P <.01
** - P <.05

TABLE 7. The Effect of the 25kDa Mycobacterial Fraction on Lymphocyte Proliferation to Mitogen and Antigen.

	Mean c.p.m. ± SE % Suppression in parenthesis	
Cell Systems	Untreated Macrophages	Macrophages pre-treated with the 25kDa Fraction
Control	453 ± 85	406 ± 90 (10)
PHA	52 415 ± 6 340	35 242 ± 4 495 (33)[a]
Con A	17 013 ± 4 228	10 719 ± 1 744 (37)[a]
PWM	27 479 ± 5 422	19 530 ± 3 901 (29)[a]
Control	1 167 ± 194	1 098 ± 172 (6)
PPD 1ug/ml	13 369 ± 3 920	9 687 ± 3 465 (27)[b]
PPD 5ug/ml	20 010 ± 2 435	15 229 ± 280 (24)[b]
PPD 10ug/ml	27 211 ± 4 812	22 030 ± 4 194 (19)[b]

[a] – P <.001

[b] – P <.01

5.13. DR EXPRESSION BY MACROPHAGES TREATED WITH THE 25kDa MYCOBACTERIAL FRACTION

Macrophages in culture spontanously express a low level of DR or Class II antigen on their surface (Figure 25A). Incubating the cells with yeast, LPS and IFN-gamma results in a marked increase in DR expression (Figure 25B, C & D unhatched respectively). Addition of 50ug/ml of the 25kDa mycobacterial fraction together with each of the DR inducing agents resulted in a significant reduction of DR expression (Figure 25B, C & D hatched). The spontaneous expression of DR on resting cells, however was unaffected by the addition of the 25kDa fraction (Figure 25A). Increasing the concentration of the 25kDa mycobacterial fraction further still showed no effect (results not shown).

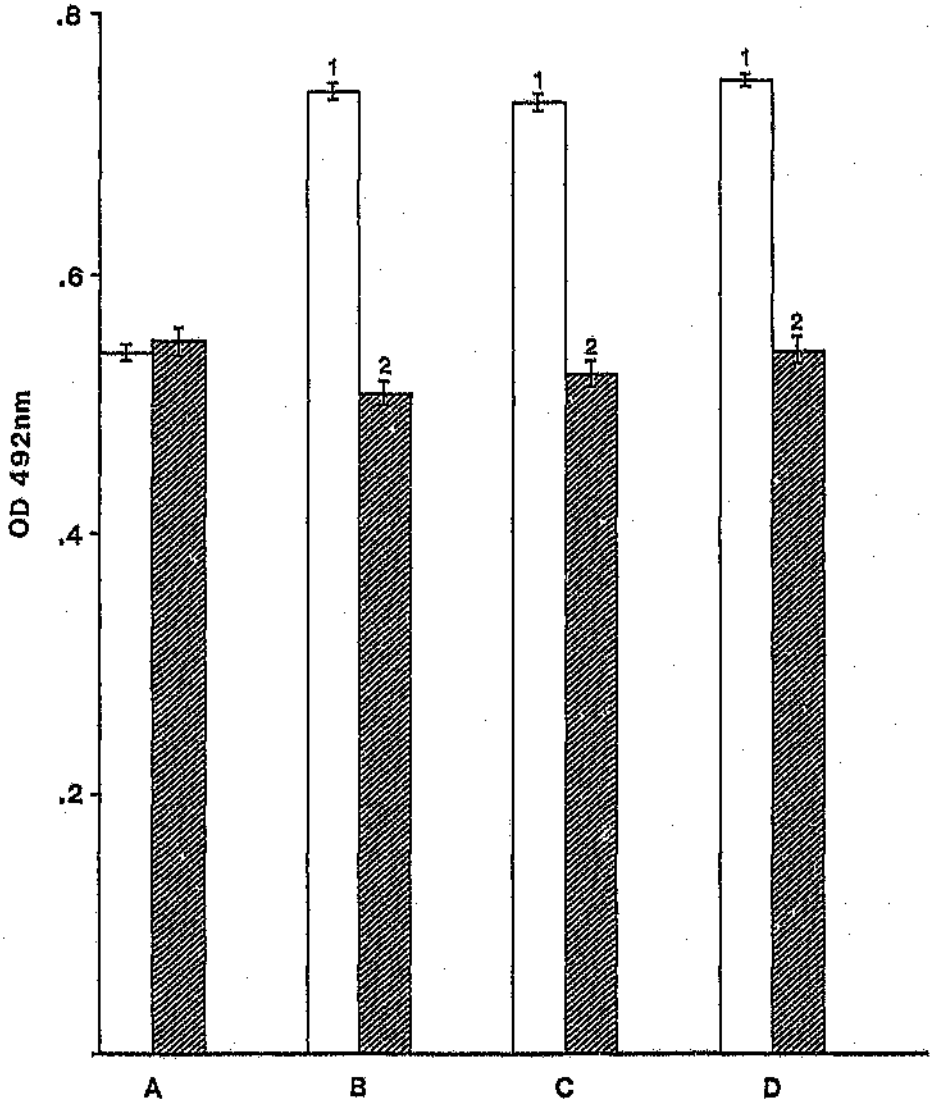


FIGURE 25. Effect of the 25kDa mycobacterial fraction on the induction of DR expression on: (A) Resting cells; macrophages stimulated with (B) Yeast; (C) LPS; (D) IFN-gamma

1. indicates a significant increase in DR expression (P <.001)

2. indicates a significant decrease in DR expression due to the 25kDa fraction (P <.001)

CHAPTER 6
DISCUSSION

It has been well documented that the majority of mycobacteria, including *M.tuberculosis*, reside within macrophages in tissue (Youmans, 1979; Nathan *et al.*, 1980). The survival of these organisms has been attributed to a variety of escape mechanisms employed by *M.tuberculosis* organisms. Such mechanisms may include inhibition of the production or neutralisation of O_2 metabolites (Kusunose *et al.*, 1976; Jackett *et al.*, 1978a; Chan *et al.*, 1989); inhibition of phagosome-lysosome fusion (Armstrong & D'Arcy Hart, 1971); degranulation of lysosomes (Armstrong *et al.*, 1975; Densen & Mandell, 1980; Katoh, 1981); disruption of the phagosomal membrane (Myrvik *et al.*, 1984) and interference with macrophage activation (Pabst *et al.*, 1988).

Previous work from this laboratory has demonstrated that sonic extracts from *M.tuberculosis* could be separated into several fractions by column chromatography with protein peaks ranging in molecular weight from >200kDa to as little as 14kDa. Of these a protein peak of Mr 25kDa appeared to be important in inhibiting the intracellular killing ability of phagocytic cells (Wadee *et al.*, 1987), while other fractions were recognised by a specific T cell clone (Wadee *et al.*, 1989). In an attempt to confirm the finding that the 25kDa fraction could inhibit intracellular killing of *M.tuberculosis* and to further define the role played by the 25kDa fraction, the present study undertook to separate *M.tuberculosis* extracts using standard column chromatography technology.

For standardisation, proteins of known molecular weights were separated through the column (Figure 12) and served as reference markers. The protein elution profile of *M.tuberculosis* extracts off the Sephacryl S.200 columns is shown in Figure 13. The major protein peaks obtained were arbitrarily defined A-I. The protein elution profile obtained in the present study concurred with that obtained in the previous study (Wadee *et al*, 1987) with Fraction H being identified as the 25kDa fraction. Preliminary studies with this fraction indicated that the active components present were carbohydrates and lipids. These findings are not presented here as they have been documentated previously (Wadee *et al*., 1987) and it is not within the scope of this dissertation to examine the chemical nature of the 25kDa fraction; but rather to examine its effects on phagocyte function.

For these studies phagocytic cells comprising both PMN cells and macrophages were employed. Initial experiments were undertaken to confirm the purity of each of the two phagocyte populations. The results presented in Figures 14 and 15, and in experiments which documented the percentage of OKM1 positive cells, clearly demonstrate the macrophage-like properties of the peripheral blood monocytes maintained in culture. The cells are identified by the monoclonal antibody OKM1; are esterase positive (Figure 14) and are actively phagocytic (Figure 15).

The purity of the population of PMN cells used was assessed by counting the cells in Turk's white cell counting fluid and by a differential count on a Sysmex K1000 cell counter (Figure 16) and were shown to be over 95% pure. Having obtained a purified population of macrophages and PMN cells, the present study undertook to examine the effect of the 25kDa fraction on various aspects of intracellular degradation of micro-organisms mediated by phagocytic cells

Initial studies demonstrated that the ingestion of yeast particles by phagocytes was unaffected by the presence of the 25kDa fraction (Table 2). These results confirm previous reports indicating that *M.tuberculosis* does not affect the phagocytic ability of PMN cells and macrophages. In this regard other workers have demonstrated that macrophages infected with mycobacteria were capable of ingesting adequate numbers of killed, attenuated or viable organisms (Armstrong & D'Arcy Hart, 1971). Furthermore, there is little evidence to suggest that mycobacteria may possess anti-phagocytic properties on their surface (Myrvik *et al.*, 1984).

Yeast cells have been shown to enter phagocytic cells by attachment to specific receptors (MFR; β-D-glucan and C3 receptors) (Riches *et al.*, 1983). Since the results of the present study clearly demonstrate the inability of the 25kDa fraction to inhibit phagocytosis, it would therefore seem likely that this molecule does not inhibit binding of yeast particles to relevant receptors. In addition, it has been suggested that

the C3 receptor, among others, is also utilised by *M.tuberculosis* to enter into the cells (Schlesinger *et al.*, 1990). Even though this pathway of entry into macrophages is common to mycobacteria and yeast the evidence obtained in this study excluded the possibility of a competitive entry between the two organisms. More importantly since two other mechanisms of entry are employed by yeast particles and the fact that mycobacteria do not appear to utilise the MFR and β-D-glucan receptors, the credibility of non-interference with phagocytosis is confirmed. Other workers have suggested an interference of phagocytosis by mycobacteria other than *M.tuberculosis*. Cree & Beck (1986) demonstrated that PMN cells and macrophages that had previously phagocytosed *M.leprae* ingested fewer zymosan particles on subsequent challenge. These authors suggest that the ability of phagocytes exposed to *M.leprae* to respond to a second phagocytic stimulus might be impaired by changes in the numbers of binding sites on the cell surface. Their investigations, however, also showed that *M.tuberculosis* did not significantly affect the phagocytic ability of the PMN cells and monocytes. Collectively such findings suggest that evasion of the bactericidal activity of phagocytes by *M.tuberculosis* is independent of phagocytosis.

To examine the effects of the mycobacterial component on the intracellular killing ability of phagocytes, initial experiments were previously undertaken whereby crude unfractionated mycobacterial extracts were used together with phagocytes. Such experiments demonstrated that crude mycobacterial extracts were

capable of inhibiting the intracellular killing ability of both PMN cells and cultured macrophages. In addition, these initial experiments demonstrated that of all the arbitrarily defined mycobacterial fractions obtained following column separation, only the 25kDa fraction was capable of inhibiting the intracellular killing of *S.aureus* organisms (Wadee *et al.*, 1987).

The present study was therefore specifically aimed at assessing the mechanism(s) by which the 25kDa mycobacterial fraction interferred with phagocyte defence mechanisms. The results shown in Figure 17B and Table 3 indicate that the 25kDa fraction was capable of significantly ($P < .001$) inhibiting the intracellular killing of *S.aureus* by both PMN cells and cultured macrophages. Inhibition of the intracellular killing ability of phagocytes was maximal at a final concentration of 50ug/ml of the mycobacterial fraction (Figure 17B). The use of higher concentrations of the 25kDa fraction did not further enhance the inhibition of intracellular killing ability of phagocytes (Figure 17B). In order to exclude the possibility that inhibition of intracellular killing was due to a cytotoxic effect of the 25kDa fraction, cell viability was assessed at the termination of the experiment and was found to be greater than 95%.

It has been well documented that IFN-gamma is important in activating macrophage and PMN cell antimicrobial activity (Walker & Lowrie, 1981; Crowle & May, 1981; Nathan *et al.*, 1983; Morrison *et al.*, 1989; Van Furth, 1990). Since other studies have also shown that IFN-gamma can reverse the

inhibition of intracellular degradation by phagocytes caused by various micro-organisms (Byrne *et al.*, 1986; Morrison *et al.*, 1989), it was of interest to examine whether rIFN-gamma could affect the inhibition of intracellular degradation caused by the mycobacterial extract. IFN-gamma, but not IFN-alpha, was shown to be able to partially restore the inhibition caused by the 25kDa fraction (Figure 17C and D respectively).

Although several studies have demonstrated that the intracellular killing of many organisms shuch as *M.fortuitum* (Geertsma *et al.*, 1990), *B.abortus* (Canning & Roth, 1989) and *C.albicans* (Perussia *et al.*, 1987) is significantly increased by the presence of IFN-gamma, other groups have shown some, or no, effect by IFN-gamma (Rook *et al.*, 1986(b); Toba *et al.*, 1989; Shiratsuchi *et al.*, 1990). A group from Case Western Reserve University showed that IFN-gamma had no effect on the intracellular multiplication of *M.avium* (Toba *et al.*, 1989) but in later studies the same group showed a variable effect, depending on the strain of *M.avium* used in the experiments (Shiratsuchi *et al.*, 1990). It also appears that differences between sources of macrophages affects t e outcome of whether IFN-gamma activates the cell or not (Shiratsuchi *et al.*, 1990). To this end experiments have demonstrated that IFN-gamma caused marked inhibition of the growth of *M.tuberculosis* organisms in murine peritoneal macrophages (Rook *et al.*, 1986(a)) and bone marrow-derived macrophages in culture (Flesch & Kaufmann, 1987), whereas only weak inhibition of growth of *M.tuberculosis* was seen in human macrophages (Rook *et al.*, 1986a).

This in part reflects the controversy of the exact role of IFN-gamma in activating macrophage mycobactericidal activity. Differences between strains of mycobacteria appears to be a major component in determining the role of IFN-gamma. Such a conclusion is exemplified by the work of Flesch & Kaufmann (1989), who demonstrated a differential susceptibility to IFN-gamma activated macrophages among different strains of *M.tuberculosis*. The results of this study clearly indicate that phagocytes activated with IFN-gamma have an enhanced killing ability for *M.tuberculosis*. It is realistic to assume that these studies are relevant to an *in vivo* situation since *M.tuberculosis* organisms were obtained from the cultures of clinical isolates.

The mechanisms involved in the intracellular killing and degradation of foreign organisms by phagocytes include the fusion of lysosomes to the phagosome containing the ingested organism; the release of lysosomal enzymes into the phagosome and the activation of the oxidative respiratory burst with the subsequent production of reactive oxygen metabolites such as O_2^- and H_2O_2 (Lowrie, 1983). In order to release hydrolytic enzymes from the lysosomes into the phagosomes containing the invading organisms, fusion of the two organelles must occur. Table 4 and Figure 18B demonstrate that in the presence of the 25kDa fraction, fusion of phagosomes containing ingested yeast particles to the lysosomes is inhibited. This effect was optimal at a concentration of 50ug/ml (Figure 18).

The photograph in Figure 20B shows a non-fuorescent yeast particle within a phogosome, indicating an inhibition of phagosome-lysosome fusion.

Although the mechanism(s) by which phagosome-lysosome fusion is inhibited has not been clearly identified, the possibility exists that this process may involve a dysfunction of the phagosome or lysosomal membrane or both (Goren *et al.*, 1976). The present study, and several others, have shown an inhibition of phagosome-lysosome fusion by mycobacterial organisms. Armstrong & D'Arcy Hart (1971) showed an inhibition of phagosome-lysosome fusion by intact virulent *M.tuberculosis* organisms; Gordon *et al.* (1980) recognised that the addition of ammonia can inhibit phagosome- lysosome fusion and that the content of ammonia in culture filtrates of *M.tuberculosis* organisms is sufficient to account for this effect. Other studies have indicated that polyanionic substances such as suramin (D'Arcy Hart & Young, 1975) and poly-d-glutamic acid (Draper *et al.*, 1979) may result in an inhibition of phagosome-lysosome fusion.

On the basis of these studies Goren *et al.* (1976) examined the effects of a polyanionic substance from *M.tuberculosis* on phagosome-lysosome fusion and demonstrated similar effects to those seen with suramin and poly-d-glutamic acid. However later work done by Goren *et al.* (1987a & b) questions the validity of these studies. They suggest that acridine orange is not a good marker to use as an indicator of phagosome-lysosome fusion because it is capable of traversing biological membranes and because it is trapped by polyanionic substances. These

polyanionic substances, they suggest, concentrate in lysosomes as a 'gelatinous sluggishly moving hydrocolloid that ionically traps the cationic acridine orange." The ability of the acridine orange to traverse that membranes does not appear to be a serious consideration in the present studies since the phagocytosis of viable yeast resulted in the prompt delivery of the marker to the yeast containing phagosomes. Furthermore, no fluorescence of the cytoplasm of the cell was observed (Figure 20). In addition, Goren *et al.* (1987a & b) have suggested that the artifactual ionic entrapment may only apply when polyanionic substance[illegible] acterial sulphatides, are used in studies of phago[illegible]sion using acridine orange. Therefore substa[illegible] mycobacterial fraction which are not anionic [illegible]agosome-lysosome fusion by mechanisms other than a simple [illegible]rapment of the dye.

Furthermore Lowrie & Andrew (1988) expressed doubt about the theory that the phagosome-lysosome fusion is simply an artifact of the test system. They suggest that the artifacts introduced by the entrapment theory brough forward by Goren *et al.* (1987 a & b) contributes only to an overestimation of the inhibition, but does not threaten the reality of the phenomenon that mycobacteria do inhibit phagosome-lysosome fusion. This lends credibility to the results of the present study indicating the phagosome-lysosome fusion is inhibited by the 25kDa mycobacterial fraction and that this may be one of the mechanisms whereby mycobacteria would elude intracellular degradation by phagocytes.

In order to assess the effects of IFN-gamma on the fusion process and the inhibition thereof by the 25kDa mycobacterial fraction these studies were extended to include experiments in which phagosome-lysosome fusion in cultures were examined in the presence of either IFN-gamma or IFN-alpha. The results (Figure 18C & D) clearly indicate that IFN-gamma (Figure 18C), but not IFN-alpha (Figure 18d), partially restores the inhibition of phagosome-lysosome fusion caused by the mycobacterial component. Restoration was never complete even when higher concentrations of IFN-gamma were used. When IFN-gamma was used in control cultures the phagosome-lysosome fusion process was not improved to any significant degree. The ability of IFN-gamma to reverse inhibition of phagosome-lysosome fusion adds further proof that the entrapment theory of Goren *et al.* (1987a & b) does not apply to this system since the fraction is still present, but the dye is better able to the phagosome.

The results of the present study provide further evidence to indicate that the defect in bactericidal ability could, in part, be ascribed to an inhibition of phagosome-lysosome fusion since IFN-gamma reverses both the intracellular killing ability of phagocytes and the inhibition of phagosome-lysosome fusion.

An effective antimicrobial mechanism used by phagocytes to destroy invading organisms is mediated by the release of lysosyme which is present in high concentrations in specific granules in the cell cytoplasm (Gordon *et al.*, 1974).

Furthermore the release of lysosyme from these granules has been shown to be raised upon phagocyte activation (Kowanko & Ferrante, 1987; Lowrie & Andrew, 1988). Although lysosyme has been demonstrated to be capable of digesting the basal layers of the *M.tuberculosis* cell walls *in vitro* (Takeya, *et al.*, 1963) it is not effective in eliminating invading mycobacterial organisms. The present study therefore undertook to examine whether the 25kDa fraction had any effect on the release of lysosyme by the phagocytes in addition to its effects on phagosome-lysosome fusion. Such studies indicate that the 25kDa mycobacterial fraction significantly ($P < .001$) reduced lysosyme release from activated phagocytes (Figure 21). The effect was dose dependent and could be observed 5 minutes after cell activation. The observation that this fraction depressed lysosyme levels in activated leukocyte supernatants is in keeping with the findings of Ridley *et al.* (1985) who showed that lysosyme synthesis ceased in macrophages which had ingested *M.leprae*. The results of the present study and those reported by others (Ridley, 1985; Lowrie & Andrew, 1988) suggest the likelihood that mycobacterial organisms, once ingested and degraded intracellulary, could release a fraction similar to that described herein. This component could then either inhibit the further release of lysosyme; neutralise any stored active lysosyme or could in the long term inhibit the *de novo* synthesis of lysosyme by the phagocyte. Even though such concepts need to be documented, the finding that the amount of lysosyme present in the culture supernatant was reduced as early as 5 minutes suggests that the fraction either neutralises

lysosyme or inhibits its release. In support of such a concept is the evidence provided by Lowrie & Andrew (1988) who demonstrated a similar inhibition of lysosomal enzymes by mycobacterial cell wall components.

Studies examining the inhibition of lysoyme release by the mycobacterial component also undertook to examine the role played by IFN-gamma in reversing such effects. The results (Figure 22) clearly demonstrate the IFN-gamma significantly ($P < .001$) reverses the inhibition caused by the 25kDa mycobacterial fraction. The effect was dose dependent and maximal at a concentration of 200U/ml. When higher concentrations of IFN-gamma were used no further restoration of lysosyme production was observed.

Although IFN-gamma has been shown to increase lysosyme release from both neutrophils (Kowanko & Ferrante, 1987) and mononuclear phagocytes (Lewis *et al.*, 1990), these effects could be shown only after 1 hour or a 6 hour incubation period respectively. The experimental conditions in the current investigation utilised shorter incubation periods of 30 minutes. It is therefore unlikely the IFN-gamma could affect the early release of lysosyme and its effects in these experiments are therefore due to a reversal of the inhibitory effects of the 25kDa mycobacterial fraction and not a direct stimulatory effect on the lysosyme release by the cells. Furthermore, in the present study, IFN-gamma did not have any effect on resting phagocytes suggesting that this lymphokine may act as a primer of phagocyte

lysosyme release rather than a direct activator of macrophage lysosyme production. Regardless of the mechanism of phagocyte activation, the results presented herein clearly point to IFN-gamma as a potentiator of intracellular killing mechanisms especially in the potential role it may play in counteracting mycobacterial escape mechanisms.

The oxidative intracellular killing apparatus of phagocytes also plays an important role in the antimicrobial activity of these cells. This is substantiated by the fact that patients with chronic granulomatous disease, who have a deficiency of one of the enzymes involved in the oxidative respiratory burst of the phagocytes are incapable of killing a number of bacterial organisms (Baboir, 1978b). Many other studies have shown that the oxidative metabolites are important for the intracellular killing ability of phagocytes (McRipley & Sharra, 1967; Klebanoff & Hamon 1975; Haidaris & Bonventre, 1982; Nathan *et al.* 1983). Since mycobacteria survive intracellularly, it was possible that the micro-organisms had an effect on the oxidative process of the phagocytes. The results of the present study indicates that the 25kDa mycobacterial fraction inhibits H_2O_2 production by both PMN cells and cultured monocytes (Figure 23B) ($P < .001$) and affects the NBT reduction by PMN cells (an assay which measures superoxide production) (Table 5) ($P < .001$). This inhibition is dose dependent and maximal at a concentration of 50ug/ml of the 25kDa mycobacterial fraction. This is not due to a direct cytotoxic effect on the leukocyte since cell viability after incubation with the 25kDa mycobacterial fraction for 2 hours was consistantly greater than 95%.

Many studies have provided indirect evidence that H_2O_2 may be an important mycobactericidal factor. Mitchison *et al.* (1963) showed that there was a correlation between the susceptibility to H_2O_2 and virulence of *M.tuberculosis* organisms. Jackett *et al.* (1978a), in addition, showed that H_2O_2 produced in macrophages is tuberculocidal and that this effect was enhanced by the low pH within the cells. However, although virulent strains of *M.tuberculosis* were shown to be resistant to H_2O_2 (and therefore possibly able to cause disease) an attenuated avirulent laboratory strain was also shown to be resistant to H_2O_2. The ability of *M.tuberculosis* to resist destruction can therefore not be attributed solely to its resistance to H_2O_2. It is possible that the conclusions drawn by these authors provide credence to the present study which demonstrates an inhibition of H_2O_2 and O_2^- production by the 25kDa mycobacterial fraction, thereby protecting virulent organisms from destruction.

In their studies Jackett *et al.* (1973) also indicate that they could not demonstrate an effective O_2^- dependent killing mechanism from *M.tuberculosis*. Later studies by Pabst *et al.* (1988) indicate that a sulphide from *M.tuberculosis* was capable of inhibiting the production of O_2^- by macrophages stimulated with PMA. May & Spagnuolo (1987) also demonstrated that mycobacterial culture filtrate, PPD and cell wall arabinogalactan significantly failed to stimulate O_2^- production. Together with the present findings, these studies suggest that once phagocytes have degraded mycobacteria,

inhibitory molecules such as the one described herein and the mycobacterial arabinogalactan, could be released to prevent further production of O_2^-.

An alternative explanation for the inability of O_2^- to kill or degrade *M.tuberculosis* organisms could be that the organism inactivates the molecule, thereby protecting itself. Chan *et al.* (1989) demonstrated that *M.leprae* contains a phenolic glycolipid (PGl) which scavenges O_2^-. It has also been suggested that superoxide dismutase (SOD), present in the mycobacteria, inactivates the O_2^- which is produced (Kusunose *et al.*, 1976). Although the 25kDa mycobacterial fraction could be acting in a similar manner, the fact that it interferes with several other systems involved in intracellular killing makes this unlikely.

Activation of phagocytes by IFN-gamma results in an enhanced production of oxygen radicals (Pick, 1986; Cassatella *et al.*, 1988; Bellstab & Schaffner, 1989) and an enhanced ability of the cell to kill invading organisms (Walker & Lowrie, 1981; Haidaris & Bonventre, 1982; Nathan *et al.*, 1983). To such ends Walker & Lowrie (1981) demonstrated that mouse peritoneal macrophages activated with lymphokines had an increased ability to kill *M.microti*. In addition they also demonstrated that the cells had an increased ability to produce H_2O_2 and that this raised H_2O_2 production was responsible for the increased killing of the organism. In the present study the addition of IFN-gamma, together with the 25kDa mycobacterial

fraction resulted in a significantly increased production of H_2O_2 ($P < .001$) and O_2^- ($P < .01$) as compared to cultures with the 25kDa mycobacterial fraction alone (Figure 23C and Table 5, respectively). Such findings are not surprising since several studies have provided evidence in favour of IFN-gamma acting as a primer of phagocyte function and in particular to that of H_2O_2 and O_2^- production. These include studies in which H_2O_2 production by PMN cells in response to a variety of stimuli was enhanced in the presence of rIFN-gamma (Berton *et al.*, 1986; Cassatella *et al.*, 1988). The present studies also suggest that IFN-gamma acts as a primer of intracellular killing mechanisms since this lymphokine had no stimulatory effect on its own but was capable of partially restoring the functions inhibited by the 25kDa mycobacterial fraction (Table 5).

Activation of an NADPH oxidase system results in the production of superoxide and H_2O_2 (Babior, 1978a). This ultimately transfers an electron from NADPH to molecular oxygen. During this process the NADPH is oxidised to NADP and it is the availability of this latter co-enzyme which controls the rate of oxidation of glucose-6-phosphate via the HMPS (Babior, 1978a). Analysis of the HMPS pathway is therefore another means of assessing the respiratory burst of the cell. In accordance with the results obtained for the NBT assay the HMPS was also significantly reduced ($P < .001$) in the presence of the 25kDa mycobacterial fraction at a concentration of 50ug/ml (Table 6). IFN-gamma (200U/ml), but not IFN-alpha, partially reversed the

effect of the 25kDa mycobacterial fraction. Wilson *et al.* (1986) indicated that lipids, sphinganine and sphingosine inhibit the oxidative burst in human neutrophils. This was thought to be mediated by inhibiting the activation of the NADPH oxidase system. Previous studies from this laboratory (Wadee *et al.*, 1987) have suggested that this may indeed be the case. In these studies, the 25kDa mycobacterial fraction was identified to be comprised of protein, carbohydrate and lipid. Chemically modifying the fraction indicated that the important constituents were the carbohydrate and lipid moieties. Results not presented here indicate that these components were crucial to the inhibitory effect of the 25kDa mycobacterial fraction, since removal of these constituents resulted in a reversal of their inhibition of HMPS activity to control levels. It is therefore possible that the lipid and carbohydrate components present in the 25kDa mycobacterial fraction may act in a similar manner to that described by Wilson *et al.* (1986) in inhibiting the activation of the NADPH oxidase system. This remains a possibility that requires further evidence for documentation.

Previous reports have indicated that glycolysis is the main source of the energy required for phagocytosis (Sbarra & Karnovsky, 1959; Cohn & Morse, 1960; Cline, 1966). Adenosine triphosphate (ATP), which is utilised to phosphorylate glucose during glycolysis, is also the source of energy required by the cells for phagocytosis. It is during cleavage of this molecule that large amounts of energy are released (Karnovsky, 1962).

Although phagocytosis of yeast particles remains unaffected by the 25kDa mycobacterial component it was still of interest to determine whether this factor had any effect on the glycolytic activity of phagocytes since stored ATP might provide sufficient energy to maintain phagocytosis for a short period of time. Another reason for examining this metobolic pathway is the fact that the glucose-6-phosphate required by the HMPS is provided for by glycolysis (Baboir, 1978a). In this system, glucose is phosphorylated to glucose-6-phosphate which now enters the HMPS. The present study has demostrated that the 25kDa mycobacterial component significantly suppressed HMPS activity (Table 6). Even though this component has been shown to interfere with the production of superoxide anion and the oxidation of NADPH to NADP as assessed by NBT reduction, it does not exclude the possibility that HMPS activity may be inhibited due to a reduced supply of energy and glucose-6-phosphate resulting from an inhibition of the glycolytic pathway. The results presented in Figure 25 clearly demonstrate that the mycobacterial fraction significantly affected the glycolytic pathway of activated phagocytes. It is possible though that the stored ATP within cells is sufficient to provide the energy for phagocytosis. These findings also point to the fact that the inhibitory effect of the mycobacterial fraction is in the disruption of intracellular killing systems rather than the phagocytic potential of these cells.

The present studies have indicated that *M.tuberculosis* organisms have the ability to evade intracellular degradation by preventing or inhibiting a variety of bactericidal mechanisms. Since intracellular degradation is neccessary for macrophages to be able to present antigen to lymphocytes, the present study undertook to examine the role, if any, of the mycobacterial fraction on the presentation of antigens by macrophages. These studies were performed by the use of two experimental systems. The first system comprised an investigation into the proliferative response of lymphocytes to mitogens and an antigen, in the presence of monocytes pre-treated with the 25kDa mycobacterial fraction. The results indicate a significant suppression of lymphocyte blastogenesis to the mitogens PHA, Con A and PWM and to the antigen PPD (Table 7). The results of the present study suggest therefore that in addition to inhibiting the intracellular killing ability of phagocytes, the 25kDa mycobacterial fraction has the ability of interfering with antigen presentation by macrophages as well. It is possible that this is a direct consequence of poor antigen processing, as evidenced by an inhibition of the intracelluar degradative mechanisms and due to a reduced proliferation because of inhibition of the glycolytic pathway. This could add to its effects and lend more credibility to the mycobacterial component as an immunosuppressive agent. The present study did not explore these avenues any further as it was beyond the scope of this dissertation. Nevertheless, these findings agree in part with those presented by Ellner & Daniel (1979) and by Kleinherz *et al.* (1981) who demonstrated that a mycobacterial component, arabinomannan, was capable of suppressing macrophage

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dependent, antigen-induced activation of human lymphocytes. They suggest that there is an activation of suppressor macrophages which downregulate the immune response by a negative feedback mechanism. Whether the 25kDa mycobacterial fraction acts in a similar manner or whether it prevents the macrophage from processing and presenting antigen has not yet been determined. However, the results of the present study suggest that the 25kDa mycobacterial fraction may be acting at the level of inhibiting the processing and presentation of antigen by macrophages rather than a direct activation of suppressor macrophages (Table 7). This is confirmed by further experiments which indicate that there is a reduced expression of Class II antigens on macrophages incubated with the mycobacterial component. In a second series of experiments, the expression of the HLA Class II antigens in response to macrophage activation was assessed. In these studies, adherent monocytes were activated in the presence of the 25kDa mycobacterial fraction. The expression of DR antigens was induced by incubating cells with LPS, Bakers' yeast or IFN-gamma. Other systems contained these activators in the presence of the 25kDa fraction. Results obtained from a modified cellular ELISA used to determine DR expression (Figure 26) demonstrate a marked inhibition of Class II antigen expression by the 25kDa fraction. Class II expression of resting macrophages was unaffected (Figure 26) just as the expression of such antigens was not affected by the 25kDa fraction if it were added to prestimulated cultures (results not shown). These findings suggest that mycobacteria have the ability of interfering with the expression of the Class II molecules if present prior to or at the time of macrophage

activation. However since the 25kDa mycobacterial fraction does not affect pre-activated cells, the present studies suggest an additional role for this mycobacterial component in inhibiting the activation of resting macrophages. This is important for the pathogenesis of tuberculosis. If the initial number of infecting organisms is small, the macrophage activated by cytokines, such as IFN-gamma, is able to present the degraded antigen together with Class II antigens to lymphocytes and so activate the CMI response. However, if the infective dose is high, the degraded material which is released could prevent further activation of the other macrophages and so cause immune suppression and consequently tuberculosis. Whether the inhibition of Class II expression is mediated by an interference in the actual synthesis of the Class II antigen or by an abrogation of transport to the cell surface is at present unclear. This aspect of the effect of the 25kDa mycobacterial fraction obviously requires further study.

The interaction of mycobacteria with macrophages in relation to the expression of DR antigens by the latter has been the subject of investigation by several groups recently. An examination of peripheral blood from tuberculous patients demonstrated that the macrophage population expressing DR antigens in this group was considerably decreased in comparison to normal healthy controls (Tweardy *et al.*, 1984; Ellner, 1986). This was particularly true for patients who had a depressed response to PPD. Furthermore, when macrophages from these patients were grown in culture 24 hours, their expression of Class II antigens rose to

normal levels. Even though these authors have suggested that the increase in DR expression may be due to a maturation of cells in culture (Tweardy *et al.*, 1984), it is possible that the high concentration of the mycobacterial components present in the circulation could inhibit the macrophage's ability to synthesise or express DR on their surface and once cells are left in culture this inhibitory effect may be lost, thus allowing the cells to express DR. This is particularly relevant to the present study which demonstrates a significant reduction of Class II antigen expression in the presence of the 25kDa fraction. It is possible that the macrophages in the presence of mycobacteria and in particular the 25kDa mycobacterial fraction could present less antigen not only by inhibiting the various intracelluar processing mechanisms but also by inhibiting the expression of Class II antigens. Current immunological dogma states that activation of CD3+, CD4+ helper/inducer lymphocytes occurs by antigen presentation in the presence of Class II antigens. However antigen presentation to suppressor cells does not occur via Class II but rather by the expression of antigen in the presence of Class I antigens. In keeping with such thoughts, the studies of Tweardy *et al.* (1984) and Ellner (1986) clearly show the activation of suppressor cells by macrophages not expressing Class II antigens. This lends support to the possibility of reduced cellular immune responses due to mycobacterial components such as the 25kDa fraction.

Similar reports have demonstrated that other mycobacteria also have the ability of inhibiting HLA Class II expression. Kaye *et al.* (1986) showed the expression of Ia by mouse peritoneal macrophages was increased by IFN-gamma and that this expression was suppressed by *M.microti*. Similarly Collings *et al.* (1985) and Poulter *et al.* (1984) described a reduction of HLA-DR expression by cells from lesions of patients infected with *M.leprae*. Collectively, these results point to an additional mechanism adopted by mycobacteria to evade the host's immune system. By reducing HLA-DR expression the mycobacteria suppresses the hosts ability to activate a cell mediated immune response and to irradicate the invading organism. With regard to the 25kDa fraction described herein, the results suggest a model whereby mycobacteria upon ingestion and degradation release this factor which could in turn inhibit activation of any other macrophages in the surrounding area thus ensuring the sur .val of remaining mycobacteria which may subsequently be phagocytosed.

CONCLUSION

This study is in accordance with previous work done in this laboratory (Wadee *et al.*, 1987) and clearly demonstrates that a 25kDa mycobacterial fraction obtained by separating sonicated *M.tuberculosis* extracts by column chromatography inhibits the intracellular killing ability of both PMN cells and macrophages. To more specifically determine the mode of action of the mycobacterial fraction, its effects on various steps involved in the microbicidal process of the phagocytes were studied. Although the 25kDa mycobacterial fraction has no effect on the ability of these cells to phagocytose foreign material it significantly inhibits the ability of phagosomes (containing ingested yeast) to fuse with lysosomes and it also inhibits the release of lysosyme from the lysosomal granules.

In addition to the inhibition of possible enzymatic degradative processes, the 25kDa mycobacterial fraction also significantly inhibited the production of toxic oxygen radicals such as H_2O_2 and O_2 anion, probably by inhibiting one or more steps of the HMPS pathway. Glycolysis which provides the energy for the HMPS pathway was not significantly affected, indicating that the 25kDa mycobacterial fraction affected the oxidative systems of the cells rather that the energy providing pathways. Since the degradative mechanisms of the cell are so efficiently inhibited by the mycobacterial component, it was interesting to see whether the fraction affected the antigen presenting

function of macrophages. The 25kDa mycobacterial fraction was shown to significantly inhibit antigen and mitogen presentation to lymphocytes. Important components for antigen presentation by macrophages is intracellular degradation of the foreign organism and presentation of the resulting antigenic fragments in conjunction with DR to the lymphocytes. In addition to the inhibition of the intracellular degradative process the 25KDa mycobacterial fraction also inhibited the ability of activated macrophages to express DR on its surface.

These results provide some explanation for the pathogenesis due to infection with mycobacterial organisms, in particular *M.tuberculosis*. Once ingested, the organism could be degraded and the breakdown products could contain a 25kDa-like mycobacterial fraction which would then prevent further degradation of the ingested mycobacteria. These could then accumulate intracellularly and result in the pathology associated with tuberculosis.

The present study also ascertained that IFN-gamma but not IFN-alpha was able to partially reverse the inhibition caused by the 25kDa mycobacterial fraction in each of the systems which were affected. These studies would indicate that IFN-gamma can be an important therapeutic agent for the treatment of tuberculosis since it enhances the phagocytes ability to kill and degrade the invading *M.tuberculosis* organisms. The results also suggest that IFN-gamma may have clinical relevance in the treatment of mycobacterial diseases.

APPENDIX 1

Phosphate Buffered Saline (PBS) 0.15M (pH 7.2)

		1 000ml
NaCl	(Univar, South Africa)	8g
KCl	(Univar, South Africa)	0.2g
$Na_2HPO_4-2H_2O$	(BDH, Poole, England)	1.15g
KH_2PO_4	(BDH, Poole, England)	0.2g

Dissolve in 900ml distilled water.

Adjust the pH to 7.2.

Make up to 1 000ml with distilled water.

Sterilise by autoclaving.

APPENDIX 2

Ammonium Chloride NH_4Cl 0.83%

		1 000ml
NH_4Cl	(Merck, Darmstadt, Germany)	8.4g
$KHCO_3$	(Merck, Darmstadt, Germany)	1.09g
EDTA	(Merck Darmstadt, Germany)	37.2mg

Filter sterilise.

APPENDIX 3

Opsonised Bakers' Yeasts

2 ml of 6 x 10^6 yeasts/ml were centrifuged at 400g for 10 minutes.

Yeasts were resuspended in 2ml of fresh human serum and incubated at 37°C for 30 minutes.

The opsonized yeasts were kept on ice until needed.

APPENDIX 4

Endotoxin Activated Serum (EAS)

Fresh human serum was incubated with 5mg/ml of lipopolysaccharide (*E.coli* 0127:B8 - Difco Laboratories, Detroit, USA) at 37°C for 45 minutes.

Aliquots of the EAS were stored at -20°C until needed.

APPENDIX 5

Nitroblue Tetrazolium Reagent (NBT)

NBT reagent (Grade III - Sigma, St. Louis, MO, USA) was prepared by dissolving NBT in PBS at a concentration of 1mg/ml.

The solution was left at 56°C in order to dissolve the NBT crystals.

The solution was then centrifuged at 800g for 10 minutes to remove any undissolved dye particles and the supernatant was collected and stored in the dark at 4°C.

APPENDIX 6

Harris' Haematoxylin

Haematoxylin crystals	5g
Absolute alcohol	50ml
Potassium aluminiumsulphate	100g
Distilled water	1 000ml
Mercuric oxide	2.5g
Glacial acetic acid	40ml

Dissolve potassium aluminiumsulphate crystals in boiling distilled water.

Dissolve haematoxylin in alcohol.

Mix the two solutions and boil rapidly.

Remove from the heat and add mercuric oxide slowly.

Heat solution until it becomes dark purple.

Remove immediately and plunge flask into ice water.

Add acetic acid to cold solution.

Filter before use.

APPENDIX 7

Phenol Red

5mg of phenol red (Merck, Darmstadt, Germany) in 10ml of saline was dissolved by incubating at 37°C for 30 minutes.
Solution was filtered through a 0.45 micron filter before use.

APPENDIX 8

Acidified Instagel

1 000ml Instagel (Packard, Illinois, USA)
5.5ml 10M HCl

Acid was added slowly to the Instagel while stirring.
The solution was stirred until all the acid was dissolved.

APPENDIX 9

Glycolysis Assay Buffer

3.75g glycine
2ml 30M hydrazine hydrate

Make up to 100ml with distilled water.

APPENDIX 10

Sodium Carbonate Buffer 0.05M (pH 9.6) 1 000ml

Na_2CO_2	(BDH, Poole, England)	1.590g
$NaHCO_3$	(BDH, Poole, England)	2.930g

Make up to 900ml with distilled water.

Adjust pH to 9.6

Make up to 1 000ml with distilled water.

0.5% Bovine Serum Albumin (BSA)

Dissolve 0.5g BSA (Seravac Pentex Products, Illinois, USA) in 100ml sodium carbonate buffer.

Store at -20°C until needed.

APPENDIX 11

Peroxidase Substrate for cELISA

A) Substrate Buffer

Citric acid	(BDH, Poole, England)	1.029g/100ml
Na_2HPO_4	(BDH, Poole, England)	1.448g/100ml

Dissolve in distilled water.

Adjust to pH 5.0.

Make up fresh daily.

B) Substrate

Immediately prior to use add 15ul of cold 30% H_2O_2 (BDH, Poole, England) and one O-phenylenediamine tablet (Zymed Laboratories Inc., California, USA) per 12ml substrate buffer.

Keep the substrate solution entirely covered until used.

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