# Mycobacterial inositol phosphoglycans and their effects on macrophages

Kia Joo Puan

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Department of Bacteriology
Royal Free and University College London Medical School

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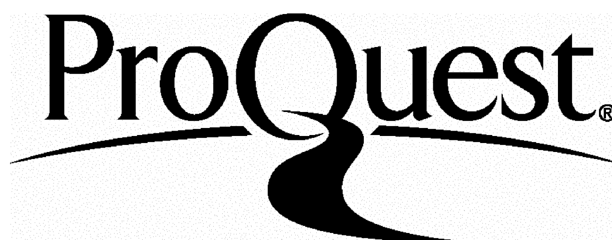

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# Abstract

Ligand binding of a number of growth factors and cytokines can result in the release of mediators known as inositol phosphoglycans (IPG) catalyzed by a glycosyl phosphatidylinositol specific phospholipase D. Two distinct sub-fractions of IPG derived from *M. vaccae* were able to mimic metabolic effects of mammalian IPG such as activating pyruvate dehydrogenase phosphatase and stimulating lipogenesis in adipocytes. In contrast, the IPG isolated from *M. tuberculosis* H37Rv were inhibitory in the mammalian assay systems, leading to the speculation that they might exert pathogenic roles in infected cells. Therefore, the effects of the IPG from *M. vaccae* and *M. tuberculosis* were evaluated on the functions of a murine macrophage cell line, J774A.1. Mycobacterial IPG of both fractions were not able to stimulate nitrite production in J774A.1 cells. However, they can synergise with IFN-$\gamma$ to produce high levels of nitrite. Western blotting analysis of the J774A.1 cell lysates treated with mycobacterial IPG plus IFN-$\gamma$ showed that the nitrite produced was attributed to the induction of inducible nitric oxide synthase protein. Unlike lipopolysaccharides (LPS), mycobacterial IPG-induced nitrite production was not inhibited by polymyxin B. However, LPS and mycobacterial IPG-induced nitrite production were inhibited by the L-arginine analogue, $N^G$-monomethyl-L-arginine (L-NMMA) but not by its inert enantiomer, $N^G$-monomethyl-D-arginine (D-NMMA). As IPG are released by GPI-PLD, an enzyme assay system using [$^3$H] myristate-labelled membrane-form variant surface glycoprotein (mfVSG) was set up to monitor the purification of GPI-PLD. It was found mannosylated lipoarabinomannan (ManLAM) inhibits GPI-PLD, raising the possibility that in mammalian cells the mycobacterial LAM inhibits release of endogenous IPG, while the

mycobacterium substitutes its own IPG, resulting in subtly disordered regulation of macrophage function.

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## Abbreviations

| | |
|---|---|
| AraLAM | arabinosylated lipoarabinomannan |
| BCG | bacillus Calmette Guérin |
| CD | clusters of differentiation |
| D-NMMA | $N^G$-monomethyl-D-arginine |
| DMEM | Dulbecco's modified eagle medium |
| EDTA | ethylenediaminetetraacetic acid |
| EGTA | ethylene glyco-bis[b-aminoethyl ether]-N,N,N',N'-tetraacetic acid |
| EU | endotoxin unit |
| FAD | flavin-adenine dinucleotide |
| FMN | flavin mononucleotide |
| GM-CSF | granulocyte macrophage colony stimulating factor |
| GPI | glycosylphosphatidylinositol |
| GPI-PLC | glycosylphosphatidylinositol phospholipase C |
| GPI-PLD | glycosylphosphatidylinositol phospholipase D |
| HEPES | N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid |
| HPLC | high performance liquid chromatography |
| IFN-$\gamma$ | interferon gamma |
| IL | interleukin |

| | |
|---|---|
| iNOS | inducible nitric oxide synthase |
| IPG | Inositol phosphoglycan |
| L-NMMA | $N^G$-monomethyl-L-arginine |
| LAM | lipoarabinomannan |
| LPS | lipopolysaccharide |
| ManLAM | mannosylated lipoarabinomannan |
| mf | membrane formed |
| NADH | nicotinamide-adenine dinucleotide (reduced) |
| NADPH | nicotinamide-adenine dinucleotide phosphate (reduced) |
| NF-κB | nuclear factor-kB |
| NO | nitric oxide |
| NP-40 | nonidet P-40 |
| PAMP | pathogen-associated molecular pattern |
| PBS | phosphate buffered saline |
| PDGF | platelet derived growth factor |
| RsLPS | Rhodobacter sphaeroides LPS |
| SDS-PAGE | sodium dodecyl sulphate polyacrylamide gel electrophoresis |
| TBS | tris-buffered saline |
| TEMED | N,N,N',N'-tetramethylethylenediamine |
| TNF-α | tumour necrosis factor alpha |
| VSG | variant surface glycoprotein |

# General Introduction

In the seventeenth century, the White Death killed millions of people in Europe. The causative agent of this disease is a bacterium, *Mycobacterium tuberculosis* (also known as tubercle bacillus), and the disease it causes is tuberculosis which remains one of the leading causes of human mortality (Murray, *et al.*, 1990, Bloom and Murray, 1992). It has been estimated that approximately two billion people are infected worldwide resulting in 3 million deaths per year. Despite the availability of a modern treatment, the incidence of tuberculosis continues to escalate contributing to a serious health problem in both developed and developing countries. This is largely attributed to the AIDS epidemic (Snider and Roper, 1992) as well as the emergence of multi-drug resistant strains of the tubercle bacillus (WHO, 1997).

The transmission of tuberculosis is by inhalation. In the lung, the bacilli are engulfed by pulmonary alveolar macrophages which line the surface of the alveoli. After phagocytosis, the bacilli can persist for a long time establishing a chronic infection. Hence, understanding of this pathogen-phagocyte interaction is central to determination of the outcome of the infection. Although macrophages are equipped with a host of antimicrobial agents, unactivated macrophages are unable to regulate the growth of *M. tuberculosis* . There is evidence to suggest that *M. tuberculosis* may use various strategies to evade killing by macrophages and to orchestrate an immune response towards its own advantage from within the macrophage. The ability to do this may be due to the potential of the pathogen to interfere with host cell signalling pathways, thereby influencing effectors and affectors of the immune system. Examples of these strategies include selective use of macrophage receptors (Schlesinger, *et al.*, 1990, Schlesinger, 1993), endosome trafficking modification by the pathogen (Xu, *et al.*, 1994), alteration of cellular signalling pathways (Knutson, *et al.*, 1998, Malik, *et al.*, 2000), aberrant receptors expression of the host cells (Saha, *et al.*, 1994,

Mohagheghpour, *et al.*, 1997), a defensive cell wall (Rastogi and David, 1988, McNeil and Brennan, 1991, Draper, 1998) and enzyme secretion (Deshpande, *et al.*, 1993, Harth, *et al.*, 1994).

## Receptor-mediated uptake of mycobacteria

The primary rôle of the macrophage in host defence is to engulf foreign matter and digest it in its highly developed lysosomal system. To perform this, the macrophages are equipped with a range of surface receptors which are capable of binding to self or non-self ligands. The self ligands include antibodies, complement components and surfactants which bind to the surface of the foreign matter forming a bridge between the bacterium and the receptors of the macrophage. The non-self ligands are more diverse and for mycobacteria they are predominantly carbohydrate in nature.

One way in which mycobacteria enter macrophages is through their ability to bind to complement receptors (Schlesinger, *et al.*, 1990, Schlesinger, 1996). C3 is the major complement component found in plasma and C3 deposition on the surface of the bacteria leads to the conversion to its active form C3b. The cleavage of the C3 to form C3b exposes the thioester bond which binds either with carbohydrate or amine on the surface of the bacteria. However, the relevant antigens on the mycobacteria have not been identified. These opsonised bacteria are recognised and internalised via specific complement receptors which should lead to a respiratory burst. As pathogenic mycobacteria lack sensitivity to complement-mediated lysis (Lammas, *et al.*, 1997), they are able to survive in the blood resulting in haematogenous spread of *M. tuberculosis.* Complement receptor CR3-mediated uptake of

mycobacteria does not cause activation of respiratory burst. Therefore, CR3 usage by pathogenic mycobacteria would partly suggest why they are not killed by macrophages. It has been reported recently that complement receptor-mediated phagocytosis of *M. tuberculosis* inhibits $Ca^{2+}$ signalling which results in reduced phagosome-lysosome fusion and intracellular mycobacterial survival (Malik, *et al.*, 2000).

There are numerous ways by which mycobacteria gain acess to macrophages One of which is through their ability to bind surfactants (as reviewed in Eggleton and Reid, 1999). These are a group of proteins known as collectins produced in the distal airspace of the lung. Surfactant A has been shown to bind to *M. tuberculosis* which results in enhanced phagocytosis by human macrophages (Gaynor, *et al.*, 1995). It has been suggested that surfactant A binds to LAM and the lipomannan on the surface of *M. bovis* BCG (Sidobre, *et al.*, 2000). Furthermore, high levels of surfactant A has been reported in HIV-infected individuals (Downing, *et al.*, 1995). In contrast to surfactant A, surfactant D is found to reduce the phagocytosis of the mycobacteria (Downing, *et al.*, 1995). The functional differences between these two surfactants on the effect of phagocytosis require further investigation.

Many other uptake mechanisms are involved and will be discussed in detailed in Chapter 3. They include mannose binding protein, Fc receptor, mannan binding lectin and mannan binding protein.

## Avoidance of destruction by pathogenic mycobacteria

The killing of bacteria by macrophages is attributed to the ability of macrophages to activate the antimicrobial mechanisms. These include phagosome-lysosome fusion, acidification of the phagosome, release of reactive oxygen intermediates and reactive nitrogen intermediates which culminates in the destruction of the pathogens. Perturbation of one these processes may result in a failure to eliminate the bacteria and lead to the intracellular survival of the bacteria.

It has been claimed that vacuoles containing live *M. tuberculosis* do not fuse with lysosomes (Hart, *et al.*, 1972, Hart and Armstrong, 1974, Hart, 1979). Although the underlying mechanism is not known, it remains one of the most tantalizing questions in the understanding of pathogenesis of tuberculosis. Acidification of the phagosome by macrophages is a prerequisite for the optimal microbicidal activity. It has been found that *Mycobacterium avium*-containing vacuoles lack acidification as compared to vacuoles containing inert IgG beads (Sturgill-Koszycki, *et al.*, 1994). The lack of acidification was later found to be attributed to failure of those vacuoles to retain proton-adenosine triphosphatase (ATPase) complexes. Other studies using immunoelectron microscopy have also shown that vacuoles containing mycobacteria interact selectively with early endosomes to acquire $Fe^{2+}$ bound to transferrin (Clemens and Horwitz, 1995). Recently, it been found that these vacuoles are permeable to macromolecules located in the cytosol (Teitelbaum, *et al.*, 1999). The mechanism underlying this phenomenon remains to be elucidated.

Lipoarabinomannan (LAM) is produced in large quantities by mycobacteria (Hunter, *et al.*, 1986). This glycolipid is a large molecule which consists of a large mannose core and multiple branched arabinose side chains. The entire complex is held on the surface of the cell wall via a phosphatidylinositol anchor (Hunter and Brennan, 1990). Previous studies have shown that LAM from *M. tuberculosis* inhibits macrophage functions (Chan, *et al.*, 1993) such as scavenging of oxygen free radicals, inhibition of protein kinase C (PKC) activity and the blocking of the expression of IFN-γ-inducible genes. These findings may support the observations that infected macrophages respond poorly to IFN-γ-induced expression of histocompatibility complex class (MHC) II molecules and intracellular killing (Knutson, *et al.*, 1998). It is also suggested that LAM inserts into the host cell membrane and may influence the cell signalling pathways (Ilangumaran, *et al.*, 1995). In a recent report, LAM has been found to inhibit mitogen activated protein kinase (MAPK) in human macrophages (Knutson, *et al.*, 1998). Since MAPK activation is involved in a broad range of cellular processes, inhibition of MAPK will have a profound effect on macrophage activation. LAM has been shown to activate a SH2-(Src homology)-containing-tyrosine-phosphatase-1 (SHP-1) which causes the dephosphorylation of MAPK, leading to its inhibition.

## Apoptosis and its rôle in mycobacterial infections

Recent reports from several laboratories have shown that apoptosis is triggered in monocytes, macrophages as well as in alveolar macrophages following mycobacterial infection (as reviewed in Fratazzi, *et al.*, 1999, Kornfeld, *et al.*, 1999). Keane *et al.*(1997) showed that primary alveolar

macrophages undergo apoptosis following infection with either *M. tuberculosis* H37Rv or H37Ra (attentuated mycobacterial strain). Although macrophage apoptosis was observed in both strains, H37Ra was more potent than H37Rv. Macrophage apoptosis was dependent on TNF-$\alpha$ since apoptosis was blocked by inhibition of endogenous TNF-$\alpha$ and apoptosis was enhanced by the addition of exogenous TNF-$\alpha$. The authors suggested that mycobacterial infection may sensitise the macrophage to TNF-$\alpha$-mediated apoptosis. Apoptosis is also observed in monocyte-derived macrophages infected with H37Rv, as reported by Placido *et al.* (1997) or H37Ra (Klingler*, et al.*, 1997). Since apoptosis of macrophages is achieved with live bacteria and not with heat-killed bacteria, it is likely that the apoptotic signal is generated from within rather than the result of activating the cell surface by the bacteria.

Apoptosis has also been identified in murine macrophages infected with *M. tuberculosis*. Rojas *et al.* (1997) tested two murine macrophage cell lines derived from B10R and B10S mouse strains which are genetically identical except at the *Bcg/Nramp* 1 gene locus (congenic mouse strains) in which B10S has a mutation at the Bcg/Nramp gene resulting in a different phenotype. The results showed that B10R cells were more prone to undergo apoptosis than B10S cells following infection with *M. tuberculosis* H37Rv. In contrast to alveolar macrophages, H37Ra did not trigger apoptosis in B10R cells suggesting that the degree to which *M. tuberculosis*-infected macrophages undergo apoptosis may be related to the virulence of the mycobacteria. The discrepancy between B10R and alveolar macrophages may reflect the cell type used and the inappropriate use of B10 cells as models for early infection events *in vivo* where alveolar macrophages may be a more representative model system. This report also showed a rôle for nitric oxide and inducible nitric oxide synthase (iNOS) in B10 cell apoptosis

using an iNOS inhibitor (aminoguanidine) and a nitric oxide donor (nitroprusside). B10R showed higher levels of nitric oxide and iNOS suggesting that nitric oxide may regulate mycobacterial-induced apoptosis. It is likely that there are other as yet unknown levels at which this phenomenon may be acting. It is possible that pathogenic mycobacteria may synthesize preformed factors which regulate macrophage apoptosis in favour of the pathogen.

TNF-α is the major initiator of apoptotic signalling in mycobacteria-infected macrophages. Studies have shown that intracellular infection of macrohages by *M. tuberculosis* can sensitise certain cell types to TNF-α-mediated death (Filley and Rook, 1991, Filley, *et al.*, 1992) . It is unknown if this sensitisation process occurs as a result of differential TNF-receptor 1 (TNFR1) expression or some downstream mechanism. TNFR1 mediates signalling pathways by differential association with various intracellular adaptor proteins (Liu, *et al.*, 1996, Lenardo, *et al.*, 1999, Wallach, *et al.*, 1999). It has been shown that the activation of TNFR1 results in the downstream activation of a NF-κB transcriptional factor which causes NF-κB to be translocated into the nucleus (Liu, *et al.*, 1996). Several reports have shown that nuclear translocation of NF-κB protects against TNF-α-mediated apoptosis, thus suggesting a mechanism of regulating apoptosis (Beg and Baltimore, 1996, Wang, *et al.*, 1996).

Apoptosis in mycobacteria-infected macrophage can also occur via other signalling pathways other than TNFR1. The P2X purinergic receptor family are plasma membrane ligand-gated ion channels activated by ATP. The macrophage expresses P2X7 whose activation causes apoptosis (North, 1996). Lammas *et al.* (1997) reported P2X7 was up-regulated in monocyte-derived macrophages following infection with *M. tuberculosis*, and exposure

to extracellular ATP resulted in apoptosis of these infected cells. A previous study by Molloy *et al.* (1994) has also shown apoptosis in BCG infected monocyte-derived macrophages following treatment with ATP. Interestingly, both studies showed macrophage apoptosis was accompanied by the intracellular killing of mycobacteria. In contrast to ATP, mycobacterial killing was not observed in those cells treated with $H_2O_2$ which causes cellular lysis. A rôle for Fas-mediated apoptosis in macrophages infected with mycobacteria has recently been proposed: Odd *et al.* (1998) reported a reduction in mycobacterial viability when infected macrophages were stimulated with recombinant soluble Fas ligand.

Although the evidence for macrophage apoptosis in response to tuberculosis is strong, it is less clear what rôle it plays in the pathology of the disease. It is possible that macrophage apoptosis benefits the bacillus by depriving the host of alveolar macrophages without producing local inflammation, which would be detrimental to bacterial survival. In this way, the induction of a specific T cell response is delayed. On the contrary, macrophage apoptosis may be beneficial to the host. This is because apoptosis of *M. tuberculosis*-infected macrophages results in the release of intracellular mycobacteria leading to their ingestion by incoming activated macrophages recruited to the site of the infection and thus leading to containment of the infection. Furthermore, the attenuated mycobacterial strains (H37Ra and BCG) are more potent inducers of apoptosis than virulent strains such as H37Rv and wild type *M. bovis* (Keane, *et al.*, 2000). This is consistent with a report showing that H37Rv evades macrophage apoptosis by the release of soluble TNF-receptor2 which results in the sequestration of the effects of TNF-α (Balcewicz-Sablinska, *et al.*, 1998). Thus, the virulence of mycobacteria appears to be, in part at least, related to which macrophage apoptosis is induced, because macrophages infected

with less virulent strains of *M. tuberculosis* result in more apoptosis. Whilst it is not clear how *M. tuberculosis* perturbs the apoptotic pathways, it is possible that the pathogen produces soluble factors which directly or indirectly interfere with macrophage's signalling pathways controlling apoptosis.

## Immunopathology of tuberculosis

The hallmark of tuberculosis is the ability of the bacillus to induce an immune response which ultimately leads to tissue damage (as reviewed in Rook and Hernandez-Pando, 1996). The transmission is initiated through the inhalation of nuclei droplets, each contains no more than three bacilli. Once in the alveolus, the bacilli gain entry into alveolar macrophages via surface receptors which include the macrophage mannose receptor (MMR), CR1 and CR3 complement receptors. In most cases, the ingested bacillus is often destroyed. Whether the bacillus is ultimately destroyed depends on the inherent microbicidal activity of the alevolar macrophage as well as the virulence of the ingested bacillus.

*M. tuberculosis*-infected macrophages produce a plethora of cytokines, including IL-1, IL-6, IL-10, TNF-α, and transforming growth factor beta (TGF-β) (Valone, *et al.*, 1988, Toossi, *et al.*, 1991, Barnes, *et al.*, 1992). These cytokines have the capacity to exert a profound immunoregulatory effects and mediates many of the clinical manifestations. For instance, IL-1 is an endogenous pyrogen which causes fever that is characteristics of tuberculosis. IL-6 drives immunoglobulin production by activated plasma cells. This cytokine has also been shown to induce antigen-specific T cell

suppression. TNF-α synergises with IFN-γ to induce production of reactive nitrogen intermediates and is essential for granuloma formation (Kindler, *et al.*, 1989). Paradoxically, TNF-α may also cause a number of immunopathological effects such as fever, weight loss, and tissue necrosis seen in tuberculosis. *M. tuberculosis* releases a factor(s) that increases the sensitivity of murine fibroblast cell line to the toxicity of TNF-α (Filley and Rook, 1991, Filley, *et al.*, 1992). It has been suggested that this factor is non-proteineous in nature since its effect was unaffected by heat treatment. TNF-α toxicity was also observed in human non-phagocytic cells following infection with *M. tuberculosis* (Filley, *et al.*, 1992). IL-10 inhibits cytokine production by macrophages and lymphocytes, and TGF-β suppresses T cell functions and macrophage effector functions.

In addition to being cytokine-producing cells, macrophages are antigen-presenting cells in the host response to *M. tuberculosis.* Mycobacterial antigens are processed and expressed in association with major histocompatibility complex (MHC) class II or class I molecules which are recognised by $CD4^+$ and $CD8^+$ αβ T cells respectively. $CD4^+$ Th1 cells are a source of IL-2 and IFN-γ which contribute to the enhancement of macrophage microbicidal activity. In contrast, CD4+ Th2 cells produce high levels of IL-4 in lepromatous leprosy associated with a high bacillary burden. IL-4 deactivates macrophages, block T cell proliferation by down-regulating IL-2 receptor expression, and therefore may inhibit the immune response to *M. tuberculosis.* It has also been demonstrated that mycobacterial lesions evoked by Th1 and Th2 responses are sensitive to the TNF-α mediated toxicity i.e. necrosis (Hernandez-Pando and Rook, 1994). In contrast, the inflammatory site induced by a Th1 response does become necrotic. The author suggested that the presence of a Th2 component contributes to disease. There is evidence that IgE and IgG4 antibody levels

are elevated in tuberculosis and both these antibodies are dependent on IL-4 which is a Th2 type cytokine (Rook and Hernandez-Pando, 1996). This is supported by a recent finding showing an increase in Th2-type cytokine (IL-4 and IL-13) mRNA levels in patients with tuberculosis. (Seah, *et al.*, 2000).

There is accumulating evidence that $\gamma\delta$ T cells play a rôle in the initial response to *M. tuberculosis* infection. These $\gamma\delta$ T cells accumulate in mycobacterial lesions and exhibit strong reactivity toward mycobacterial antigens (Kabelitz, *et al.*, 1991). A small non-peptide mycobacterial antigen has been identified from crude *M. tuberculosis* extracts based on its ability to induce proliferation of $V\gamma9^+$ $V\delta2^+$ T cells from healthy donors (Constant, *et al.*, 1994).

If the alveolar macrophage fails to destroy the bacilli, the bacilli multiply and eventually kill the macrophage. The bacilli released from the macrophage would attract monocytes, lymphocytes and polymorphonuclear neutrophils, none of which are capable of killing the bacteria efficiently. The newly arrived macrophages from the bloodstream readily ingest the released bacilli but are unable to destroy the bacilli because they have not been activated. As the infection progresses, macrophages and bacilli accumulate to form a granulomatous focus. It is believed that the process of granuloma formation serves as a mean of containing the pathogens, impeding their growth and preventing dissemination. Although the tubercle bacillus may survive in the centre of the lesion, it cannot multiply because of its anoxic condition, reduced pH, and the presence of inhibitory fatty acids.

In delayed-type hypersensitivity, the antigen-specific T cells are evoked to destroy non-activated macrophages which contain active bacilli. If the bacilli are few, the granulomatous lesions may regress. If the bacilli are numerous,

the lesions will enlarge to form tubercles with caseous centre. As the infection progresses, these lesions become fibrotic. As the infected macrophages are killed within the lesions, more macrophages are recruited to the infection focus mediated by cell-mediated immunity. This results in large areas of necrotic lesions, each surrounded by a layer of epithelioid cells and multi-nucleated giant cells. With a strong cell-mediated immunity, the infection may be arrested and the granulomas heal forming fibrous and calcified lesions. If the cell-mediated immunity is inadequate, viable *M. tuberculosis* within the macrophages may escape from the granuloma. This results in the rapid spread of the infection to regional hilar lymph nodes. The granuloma which previously contains the bacilli begin to soften and liquefy, providing a rich and oxygenated environment for the growth of extracellular mycobacteria. The lymph nodes become enlarged and rupture, releasing liquified necrotic material and causing bronchopnuemonia.

It is has been shown that granulomatous lesions are found chiefly in the lung, liver, and spleen of patients with disseminated disease. It has been shown that these lesions contain cytokines and growth factors such as epidermal growth factor (EGF), fibroblast growth factor, and transforming growth factor-$\alpha$ (Wyler, *et al.*, 1981, Ellingsworth, 1990). Moreover, granuloma and liquified tissue are excellent growth media for mycobacteria (Dannenberg, 1989). *M. tuberculosis* and *M. avium* express an EGF receptor capable of binding to recombinant human EGF (Bermudez, *et al.*, 1996). It was also observed that *M. tuberculosis* and *M. avium* grow faster in the presence of EGF. The N-terminal protein sequence of the purified *M. avium* EGF-binding protein showed significant homology to a group A streptococcal glyceraldehyde-3-phosphate dehydrogenase (GAPDH). In streptococci, GAPDH was shown to bind plasmin (Lottenberg, *et al.*, 1992), fibronectin, lysozyme, and cytoskeletal proteins (Pancholi and Fischetti, 1992). In

eukarytotic cells, GAPDH is a key glycolytic enzyme and is found to be associated with membrane and cytoskeletal structures (Arnold and Pette, 1968). There have been reports that proteins with similar structure are found on the membrane of both prokaryotic and eukaryotic cells and are involved in functions independent of their catalytic property (Carswell, *et al.*, 1975, Goudot-Crozel, *et al.*, 1989). The presence of EGF in granulomatous lesions and EGF-receptors on the surface of pathogenic mycobacteria suggests several hypotheses:- Firstly, EGF may act as a growth signal for *M. tuberculosis* at an appropriate time and location during an infection. Secondly, the mycobacterial EGF-receptors may provide a mechanism by which EGF is sequestered, thus impeding the maturation of granuloma formation to the benefit of the pathogen.

The host-pathogen interaction is highly complex as it involves at least three levels of cellular signalling:- intracellular signalling within the bacteria, intracellular signalling within the macrophage and extracellular signalling between the macrophage and the pathogen. Hence, the study of second messengers in mycobacteria may provide a key to the understanding of bacterial growth, host cell signalling mechanism and host cell-pathogen interactions.

**Aims of this project**

Cytokines such as IL-2 (Gaulton, *et al.*, 1988, Eardley and Koshland, 1991), transforming growth factor-β1 (Vivien, *et al.*, 1993, Vivien, *et al.*, 1994), and growth factors such as nerve growth factor (Chan, *et al.*, 1989, Represa, *et al.*, 1991) have been demonstrated to release inositolphosphoglycans (IPGs) from mammalian cells. The precise mechanism by which IPGs are released is not clear. There is circumstantial evidence in mammalian cells showing that IPGs are released from the hydrolysis of free-glycosylphosphatidylinositol (protein-free GPI) by a specific enzyme known as glycosyl phosphatidylinositol phospholipase D (GPI-PLD). A rôle for IPG as a second messenger has recently been proposed (as reviewed in Rademacher, *et al.*, 1994). IPG isolated from rat liver induces proliferation of a EGF receptor-transfected fibroblast cell line. Preliminary results have shown that IPG isolated from *M. vaccae* and *M. tuberculosis* are stimulatory to this cell line (Rook, *et al.*, 1999 Personal communication). This has led to the suggestion that mycobacterial IPG may mimic the mammalian ones in mediating cellular signalling pathways. In macrophages, this may result in the regulation of signalling pathways in favour of the pathogen.

Filley *et al.* (1991) showed that an L929 murine fibroblast cell line infected with *M. tuberculosis* was more sensitive to TNF-α-mediated cytotoxicity as compared to those uninfected fibroblast cell line following exposure to exogenous TNF-α. It is not clear how *M. tuberculosis* infection leads to an increased sensitvity of the cell to TNF-α toxicity. The same effect could be obtained using mycobacterial sonicate. Therefore, it can be inferred that the effect is due to mycobacterial factor(s). These factors are probably non-proteinaceous in nature since the effect was not abrogated by prior heat treatment. Hence, these factors are likely to be lipid or carbohydrate in

nature. This study is an attempt at exploring the possibility of mycobacterial carbohydrate acting as an interferring agent in cellular signalling.

The aims of this project are to:

1) further characterise and validate whether IPGs are present in mycobacteria by applying extraction procedure used for mammalian cells and testing fractions for IPG activity;

2) compare the IPGs isolated from a pathogenic mycobacterium (*M. tuberculosis*) with those from a non-pathogenic mycobacterium (*M. vaccae*) using functional assays to evaluate their a) metabolic stimulatory activity: pyruvate dehydrogenase phosphatase activation and lipogenesis assay. b) stimulatory effects on nitric oxide, IL-1$\beta$ and TNF-$\alpha$ production from macrophages;

3) investigate whether IPGs are involved in virulence by comparing IPG preparations of a virulent strain from a non-virulent strain of *M. tuberculosis*; and

4) elucidate the effects of mycobacterial IPG on macrophage functions by inhibiting GPI-PLD activity. Inhibition of the macrophage GPI-PLD activity would prevent the generation of endogenous IPG. Thus, the effect of mycobacterial IPG could be independently evaluated. GPI-PLD inhibition could be achieved using either blocking antibodies or specific inhibitors. The latter was not used because of their toxicity and potential secondary effects on mammalian cells. Thus, the following approaches were proposed:-

i) to devise a simple assay to measure GPI-PLD activity in order to monitor the purification of GPI-PLD from serum. This involved the isolation of radiolabelled membrane-form variant surface glycoprotein (mfVSG) from *Trypanosoma brucei* as a biological source of substrate for GPI-PLD;

ii) to purify GPI-PLD from human serum using ammonium sulphate precipitation and high performance liquid chromatography;

iii) to raise blocking antibodies to endogenous GPI-PLD by immunising an animal with the purified or partially purified GPI-PLD.

This work should further characterise and validate the rôle of IPG derived from pathogenic mycobacteria in host-pathogen interaction.

# Chapter One

## Isolation and Purification of Mycobacterial Inositol Phosphoglycans

## 1.1 The nature of glycosylphosphatidylinositols

Glycosylphosphatidylinositols (GPIs) are lipids found covalently linked to many membrane proteins attached to the outer leaflet of the cell membrane (as reviewed in Low and Saltiel, 1988, Low, 1989, Gerold, *et al.*, 1996). Since the elucidation of the first complete structure of the GPI-anchor of the variant surface glycoprotein (VSG) from *Trypanosoma brucei* by Ferguson (Ferguson, *et al.*, 1988) and the rat brain Thy-1 by Homans (Homans, *et al.*, 1988), a large number of differently modified GPI-anchors have been characterised. They comprise a large family of membrane proteins of diverse functions which include cell surface receptors (e.g. CD14 and the folate receptor), complement regulatory factors (e.g. CD55 and CD59), cell adhesion molecules (e.g. **l**ymphocyte **f**unction **a**ntigen (LFA)-3), ectoenzymes (e.g. alkaline phosphatase and 5'-nucleotidase) and the lymphocyte differentiation antigens (e.g. Thy-1). However, the functional relevance of these lipids other than providing an alternative mechanism of anchoring proteins remain obscure. The core structure of all GPI-anchors characterised thus far consists of ethanolamine-phosphate-6,Manα1,2-Manα1,6Manα1,4-GlcNα1,6-inositol-phosphate (Fig 1.1) indicative of a high degree of conservation throughout evolution. Moreover, there is increasing evidence that GPI may play a rôle in cell signalling (Romero, 1991, Rademacher, *et al.*, 1994, Gerold, *et al.*, 1996, Varela-Nieto, *et al.*, 1996, Jones and Varela-Nieto, 1998).

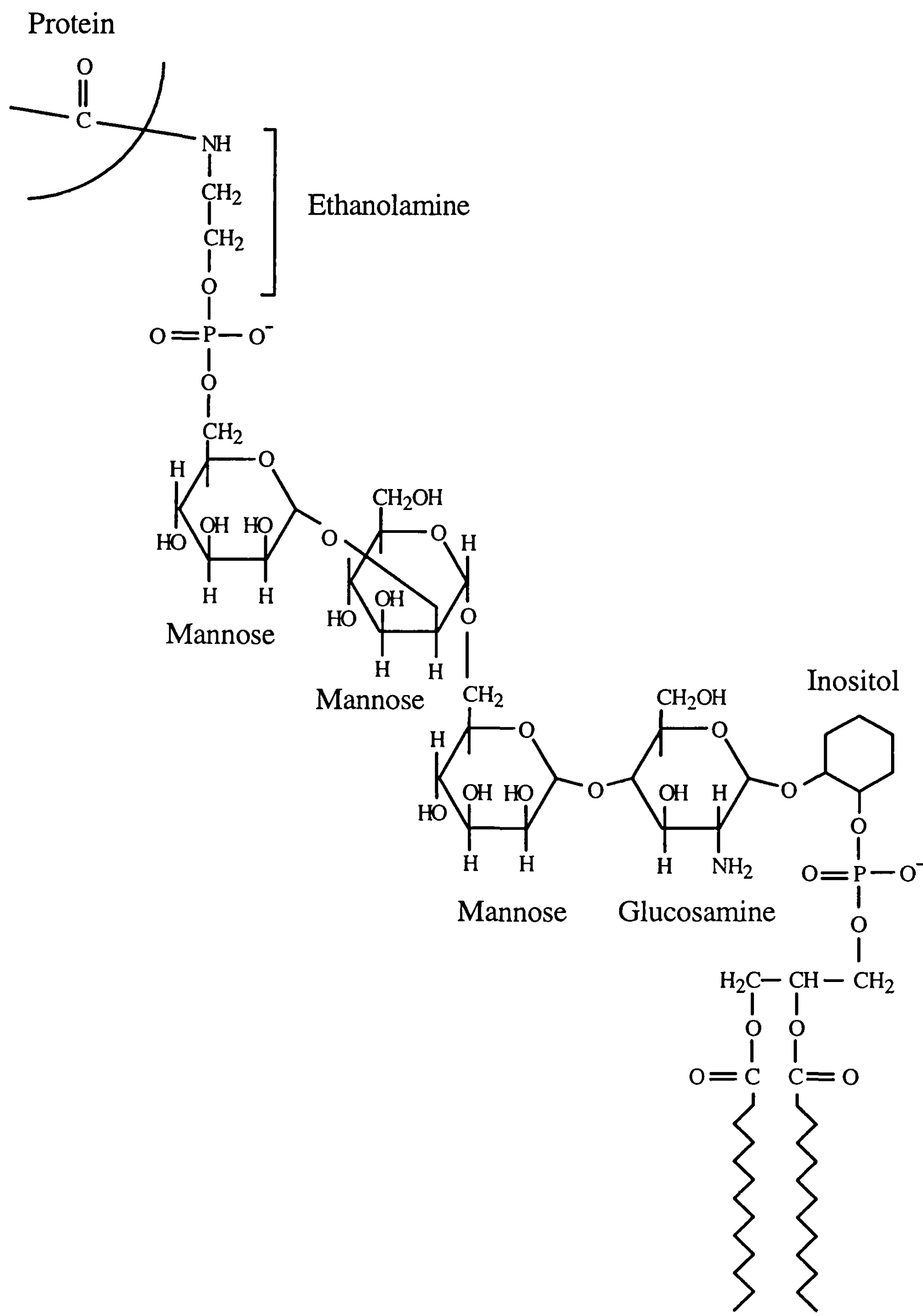



**Fig. 1.1. The minimal structure of a GPI-anchor.**

## 1.2 Cellular signalling via GPI-anchored proteins

### Auxiliary transmembrane receptor-mediated signalling

The majority of surface proteins such as the cytokine receptors and adhesion molecules have an extracellular ligand-binding domain, one or several transmembrane domains, which span the plasma membrane, and an intracellular domain. Signalling is mediated by protein tyrosine kinases or trimeric G-proteins associated with the intracellular domain. GPI lacks the intracellular domain and therefore cannot associate with cytoplasmic signalling molecules. However, there is cumulative evidence which indicates they retain the ability to participate in cell signalling. One way by which GPI can signal is by direct association with an auxiliary transmembrane protein receptor. For instance, the **c**iliary **n**eu**t**rophic **f**actor (CNTF) is a GPI-anchored protein which binds to the transmembrane signalling component subunit, gp130. Other examples include the leukocyte receptors CD14, CD16B and CD87 (also known as urokinase plasminogen activator receptor, uPAR) which form non-covalent complexes with **c**omplement **r**eceptor type 3 (CR3), a heterodimeric transmembrane β2-integrin (CD11b/CD18) (Petty and Todd III, 1996). These complexes are based on the interaction of the lectin site in the CR3 with the glycan moiety of the GPI-anchor. It is possible that the signalling capacity of CD14, CD16B and CD87 are dependent via this mechanism on the intracellular domains of CR3.

**GPI-microdomain-mediated signalling**

Another way by which GPI-anchors can participate in cell signalling is attributed to the very nature of the membrane in which the GPI-anchored proteins reside. GPI-anchor proteins are localised in a membrane microdomain enriched in glycosphingolipids and cholesterol (thus the name, GPI-complexes) which are normally devoid of transmembrane proteins. It is this membrane specialisation which confers the poor solubility of the GPI-anchored proteins to certain type of detergents, such as Triton X-100, Nonidet P-40 and deoxycholate, at low temperatures. This is in contrast with the good solubility of transmembrane proteins under the same conditions. It is this poor solubility of the GPI-anchored proteins which has led to the description as **d**etergent-**i**nsoluble **g**lycosphingolipid-enriched domains or DIGs (Brown and Rose, 1992, Fiedler, *et al.*, 1993). Whether or not GPI-complexes correspond to the composition obtained after detergent solubilisation of the native membrane remains contentious (Hooper, 1998). It is conceivable that solubilisation at low temperatures may remove some components present *in vivo*. Alternatively, the rather small originals microdomains may coalesce artificially to form larger patches. However, experiments in which artificial liposomes were used and incorporation of fluorescent-labelled GPI-proteins into native membranes indicate that the GPI microdomains are a reality and not detergent artefacts. Moreover, GPI-complexes can be obtained by mechanical membrane disintegration in the absence of any detergents.

## Caveola-mediated signalling

Similar to GPI microdomains, are specialised organelles known as caveolae which are flask-shaped invaginations found in many mammalian cells (as reviewed in Anderson, 1993b, Anderson, 1993a, Parton and Simons, 1995, Parton, 1996). They were described originally by Yamada using electron microscopy (Yamada, 1955) and were found to be particularly abundant in adipocytes, smooth muscle and endothelial cells. Caveolae or plasmalemmal vesicles were first identified as endocytic compartments which appeared to be involved in the transport of molecules across cells by the process of transcytosis. As this process is less likely to occur in smooth muscle and skeletal muscle, other studies have suggested that caveolae may be involved in the internalisation of macromolecules (Tran, *et al.*, 1987, Keller, *et al.*, 1992). Studies on receptor-dependent folate transport (Rothberg, *et al.*, 1990) have led to the hypothesis that caveolae may be involved in the uptake of small molecules and ions across the cell membrane by a process known as potocytosis (Anderson, 1993c, Anderson, 1993a). Unlike clathrin-coated pits, which are adapted for the uptake of large molecules, caveolae are designed to concentrate and perhaps temporarily store small molecules and ions at the cell surface. These can be delivered into the cells for intracellular or intercellular signalling. One feature of caveolae is that they contain a rich source of densely packed GPI-anchored membrane proteins. Studies using immunogold electron microscopy have shown that each caveola can contain different species of GPI-anchored membrane proteins (Ying, *et al.*, 1992). Another attribute of caveolae is the presence of a membrane coat, caveolin, on the cytoplasmic surface of the membrane. Caveolin (also known as **v**esicular **i**ntegral membrane **p**rotein, VIP-21) is a 21-kilodalton integral membrane protein that was originally

isolated based on its low density and poor solubility in detergent (Rothberg, *et al.*, 1992). Antibodies directed against the GPI-linked protein have been shown to co-precipitate with other src-family protein tyrosine kinases implicated in signal transduction. Moreover, studies from many different laboratories have shown that antibodies directed against different GPI-anchored membrane proteins can elicit a signal response in some cells. The responses are functionally diverse and they include: activation of tyrosine kinases, an increase in cytoplasmic $Ca^{2+}$ and inositol phosphate level, and proliferation. It is noteworthy that most of these effects were elicited through the ligation of GPI-anchored proteins using antibodies. Unless verified using natural ligands, these effects may not reflect what is likely to occur *in vivo.* Nonetheless, cellular responses can be elicited after ligation of CD14 with lipopolysaccharide (LPS), CD16B with immunoglobulin-coated immune complexes or **u**rokinase **p**lasminogen **a**ctivator **r**eceptor (uPAR) with its cognate ligand, **u**rokinase **p**lasminogen **a**ctivator (uPA).

**Inositol phosphoglycan-mediated signalling**

While it is clear that all the mechanisms outlined above demonstrate that GPI-anchored proteins may function in cellular signalling, they themselves are not mediators of the responses. There is accumulating evidence that **i**nositol **p**hospho**g**lycans (IPGs) are produced from the hydrolysis of a protein-free GPI following insulin stimulation. These IPGs can interact directly with insulin-sensitive enzymes such as pyruvate dehydrogenase phosphatase and cyclic adenosine monophosphate (cAMP)-dependent phosphodiesterase. Recently, hormones as diverse as interleukin-2 (Gaulton, *et al.*, 1988, Eardley and Koshland, 1991), transforming growth

factor-β1 (Vivien, *et al.*, 1993, Vivien, *et al.*, 1994), nerve growth factor (Chan, *et al.*, 1989, Repressa, *et al.*, 1991) and thyroid-stimulating hormone have been found to stimulate the release of IPG from cells.

The first of these studies came from Larner and colleagues who showed that the inhibition of cAMP-dependent protein kinase by insulin was mediated by a family of heat and acid stable molecules (Larner, *et al.*, 1974). These compounds were further purified by gel chromatography and shown to inhibit adenylate cyclase (Larner, *et al.*, 1979, Malchoff, *et al.*, 1987) and activate mitochondrial pyruvate dehydrogenase (Jarett and Seals, 1979). In 1986, Saltiel and Cuatrecasas reported the isolation of two similar but distinct mediators from rat liver following stimulation with insulin. Both mediators display a bimodal response to cAMP phosphodiesterase, had a molecular weight of about 1,400, and were resistant to acid treatment. Phosphodiesterase-stimulating activity was attentuated by periodate treatment indicative of the presence of carbohydrate residues. However, the loss of phosphodiesterase-stimulating after nitrous acid deamination suggested that they both contained non-N-acetylated glucosamine (GlcN) linked to an inositol ring via a glycosidic bond. As both mediators could be separated by ion-exchange chromatography and high-voltage electrophoresis it was concluded that they were likely to contain different net negative charges. Later, Saltiel and colleagues showed that the mediators could be metabolically labelled with [$^3$H]-glucosamine and [$^3$H]-inositol in a murine myocyte $BC_3HI$ cell line (Saltiel, *et al.*, 1987). The production of these mediators was stimulated by insulin and was attributed to the hydrolysis of a phosphatidylinositol glycan glycolipid on the cell membrane by a phosphatidylinositol specific phospholipase C. Moreover, the stimulation of insulin resulted in a rapid generation of a distinct species of [$^3$H] myristate-labelled diacylglycerol which can be distinguished from arachidonate-

containing diacylglycerol derived from the hydrolysis of phosphoinositides. The effects of insulin on the $BC_3HI$ cells to produce the IPG mediators and diacylglycerol can be reproduced by treating glycolipids purified from the plasma membrane with *Staphylococcus aureus* phosphatidylinositol phospholipase C. Further analysis by Mato (1987) and Larner (1988) has led to the identification of two different IPGs: IPG A-type and IPG P-type. IPG A-type where A indicates its ability to inhibit cAMP-dependent protein kinase (also known as protein kinase **A** or PKA), contains a mixture of myo- and chiro-inositol, glucosamine and phosphate groups (Mato, *et al.*, 1987). In contrast, IPG P-type where P indicates its ability to activate **p**yruvate dehydrogenase phosphatase, contains D-chiro-inositol, galactosamine and mannose (Larner, *et al.*, 1988).

A complete structural determination of IPG has been hampered by the scarcity of the material. Nevertheless, in collaboration with Rademacher and colleagues has led to the partial characterisation of the IPG from bovine liver (Rademacher, 1999). Studies using differential solvent extraction and chromatography have shown that the structure of IPG is similar to that described by Mato. Preliminary data have shown that the partial structure of the IPG resembles the parasite-derived GPI-anchors (*Trypanosoma brucei* VSG) rather than the mammalian-derived GPI-anchors (Rademacher, *et al.*, 1994). It is found that the IPG contains $\alpha$-galactose residues and the $\alpha$-galactose-containing GPI-anchors derived from *T. brucei* VSG has insulin-mimetic activity (Ferguson, *et al.*, 1988). These have led to the suggestion that the IPGs which are involved in signal transduction are not the biosynthetic precursors of the GPI-anchored proteins.

## 1.3 Innate immunity in Limulidae

Ever since the proposal of the *Theory of Host Immunity* by the Russian embryologist, Elie Metchnikoff, the quest for the understanding of the innate immune system remain a challenge to immunologists. The primary rôle of the innate immune system is to combat infection. Much of what we understand about the innate immune system has come from the study of invertebrates. It is well known that bacterial components can elicit a variety of responses in invertebrates including haemolymph coagulation, melanin formation, phagocytosis and killing of the invading microorganisms. One of the best invertebrate model is the horseshoe crab, *Limulus.* Like many invertebrates, horseshoe crab has developed a unique system to detect microbial antigens such as LPS, peptidoglycans. and β-(1,3)-glucan. Since both vertebrates and invertebrates can respond to these substances, it is likely that they may have evolved from a common recognition system.

In *Limulus*, the haemolymph which contains haemocytes (also called amoebocytes) is extremely sensitive to bacterial LPS. A minute amount of LPS is sufficient to activate the haemocyte and induce cell adhesion, aggregation, followed by coagulation factors degranulation, lectins and antimicrobials. Therefore, it is very useful for the detection and quantification of traces of LPS in clinical applications (Reinhold and Fine, 1971, Yin, *et al.*, 1972, Tanaka and Iwanaga, 1993). Unlike the haemocytes of insects or other invertebrates, a single type of haemocyte is found in the haemolymph (Toh, *et al.*, 1991). Found in each haemocyte are two type of secondary granules: large and small granules (Toh, *et al.*, 1991). Upon exposure to a Gram-negative bacterium, the haemocyte detects LPS on the surface and releases the contents of the granules by exocytosis. The contents released

include two zymogens or biosensors, factor C (Muta, *et al.*, 1993) and factor G (Muta, *et al.*, 1995), which are autocatalytically activated, by LPS and β-(1,3)-glucan respectively. The activation can initiate a coagulation cascade, resulting in the conversion of coagulogen to an insoluble coagulin gel. These are later followed by the immobilisation and killing of the bacterium (as reviewed in Muta and Iwanaga, 1996a, Muta and Iwanaga, 1996b). The coagulation reaction of *Limulus* haemolymph can be divided into two pathways: LPS-mediated and the β-(1,3)-glucan-mediated coagulation reactions as illustrated in Fig. 1.2 (as reviewed in Iwanaga, 1993).

**Coagulation mediated by lipopolysaccharide**

LPS-mediated coagulation operates via three serine protease zymogens (factor C, factor B, and proclotting enzyme) and a clottable protein, coagulogen. In the presence of LPS, factor C is autocatalytically activated to an active form, factor $\overline{C}$. The active factor $\overline{C}$ activates the zymogen factor B to factor $\overline{B}$, which then activates the proclotting enzyme to clotting enzyme. The resulting clotting enzyme converts coagulogen to a insoluble coagulin gel. Purified factor C is a mixture of a 123 kilodalton single-chain form and a two-chain form composed of a heavy (H) chain and a light (L) chain. Both forms can be activated by LPS. Factor C is also activated by lipid A (the core structure of LPS) and phospholipids such as phosphatidylinositol, phosphatidylserine and cardiolipin. The amino acid sequence of factor C shows that the H chain which binds to LPS, contains a five "sushi" domain (epidermal growth factor (EGF)-like domain) and a C-type lectin like domain (Muta, *et al.*, 1991). The sushi, also known as short consensus repeat (SCR), was named because of the similarity between the domain structure and the

traditional Japanese cuisine, sushi. As these sushi domains are also found in the mammalian complement factors, the finding of such domains in invertebrates is totally unprecedented.

**Coagulation mediated by β-(1,3)-glucan**

The discovery of β-(1,3)-glucan-mediated coagulation arose from the study of patients with fungal infection and patients undergoing haemodialysis with cellulosic dialyser (Pearson, *et al.*, 1984). It was found that the plasma from these patients give a positive reaction in the *Limulus* test despite the absence of LPS. These pseudopositive reactions were later shown by Iwanaga and others to have been caused by the presence of β-(1,3)-glucans in the patients' plasma (Kakinuma, *et al.*, 1981, Morita, *et al.*, 1981). This β-(1,3)-glucan-sensitive protease zymogen was isolated and named as factor G (Muta, *et al.*, 1995).

Factor G is directly autocatalytically activated by β-(1,3)-glucan to an active form, factor $\overline{G}$ as shown in Fig. 1.2. The active form activates proclotting enzyme to clotting enzyme resulting in the coagulin gel formation. Factor G is a heterodimer composed of two subunits: α and β, associated with a non-covalent bond (Muta, *et al.*, 1995). It has been suggested that binding of β-(1,3)-glucans by the α subunit exposes the activation site of β subunit which is normally masked in its inactive state. The active β subunit activates other β subunits. Purified factor G is also activated by other glucans containing β-(1,3) linkages from different origins, but it is not activated by LPS (Muta, *et al.*, 1995). Whilst the most effective activators are linear β-(1,3)-glucans,

shorter oligosaccharides containing two to seven glucose units do not activate factor G.

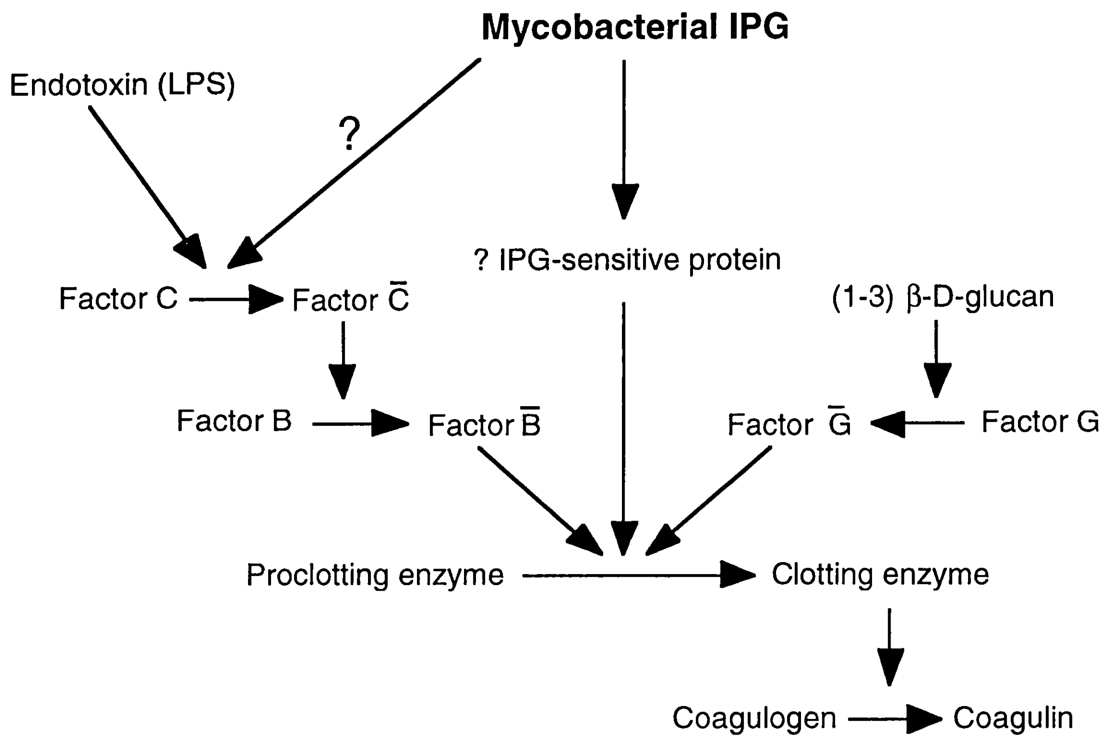



**Fig. 1.5. Activation of *Limulus* coagulation cascade by mycobacterial IPG.** LPS-sensitive factor C which carries the lectin-like domain may be activated by mycobacterial IPG, which results in haemolymph coagulation. Alternatively, mycobacterial IPG may activate a uncharacterised IPG-sensitive factor leading to the activation of the proclotting enzyme.

## 1.4 Objectives of this chapter

The objectives of this chapter were to:

1) determine whether IPGs are present in *M. vaccae* R877R, *M. tuberculosis* H37Rv and *M. tuberculosis* H37Ra. The approach is to isolate and purify mycobacterial IPGs based on the method for the isolation of mammalian IPGs (Nestler, *et al.*, 1991). Kunjara *et al.* (1995) have shown that P-type and A-type from rat liver activate pyruvate dehydrogenase phosphatase and stimulate lipogenesis in adipocytes respectively. In collaboration with Prof. T. W. Rademacher, the purified mycobacterial IPGs were evaluated in parallel with mammalian IPGs on their ability to activate bovine heart pyruvate dehydrogenase phosphatase and stimulate lipogenesis in rat adipocytes.

2) prove that the activities of IPG were not due to contaminating endotoxin using the *Limulus* ameobocyte test for endotoxin. Polymyxin B is a cationic polypeptide produced by *Bacillus polymyxa.* Previous studies have shown that polymyxin B can antagonise the effect of LPS on a number of biological assays. Thus, in some experiments mycobacterial IPGs were pretreated with polymyxin B.

3) compare the IPGs isolated from a non-pathogenic mycobacterium *(M. vaccae*) from a pathogenic mycobacterium (*M. tuberculosis*)

4) compare the IPGs isolated from a virulent strain of *M. tuberculosis* H37Rv from an avirulent strain of *M. tuberculosis* H37Ra

## 1.5 Preparation of Sauton's medium

### Materials

All reagents were purchased from BDH Laboratories Supplies, Poole, England, AnalaR® grade unless stated otherwise. The catalogue numbers are indicated in parentheses. The following recipe was used to prepare one litre of Sauton's medium.

| | |
|---|---|
| Glycerol, $CH_2OH.CHOHCH_2OH$ = 92.09 (101186M) | 30 ml |
| L-Asparagine, anhydrous, F.W. = 132.1 (Sigma A-0884) | 6.0 g |
| Citric acid, $C(OH)(COOH)(CH_2.COOH)_2.H_20$ = 210.14 (10081) | 2.0 g |
| Dipotassium hydrogen orthophosphate, anhydrous, GPR, $K_2HPO_4$ = 174.18 (29619) | 1.5 g |
| Magnesium sulphate, $MgSO_4$. $7H_20$ = 246.47 (10151) | 0.25 g |
| Ammonium ferric citrate, brown, GPR (27163) | 0.05 g |
| Agar, high gel strength, (Sigma. A-6924) | 12 g |

Ammonia solution, concentrated, GPR, About 35% (w/w) $NH_3$, specific gravity is 0.880. $NH_3$ = 17.03 (27141)

D(+)-glucose, $C_6H_{12}O_6$ = 180.16 (101174Y) prepared as a 40% (w/v) solution filtered-sterile with a 0.2 μm Acrodisc® filter.

### *Bacterial strains*

*M. vaccae*, R877R NCTC 11659 was obtained from the Department of Bacteriology, Windeyer Institute of Medical Sciences, University College London Medical School.

*M. tuberculosis* H37Rv was kindly provided by Prof. Douglas Young from the Imperial College School of Medicine, London, whilst *M. tuberculosis* H37Ra was provided by Patricia Brooks from the National Institute of Medical Research, London.

### Methods

The recipe was adapted from that of Boyden and Sorkin (1955). Glycerol was added to a flat-bottomed flask which contained 200 ml deionised water. This was placed on a hotplate at 100°C. The following ingredients were added: L-asparagine; citric acid; dipotassium orthophosphate; magnesium sulphate and ammonium ferric citrate. The resulting solution was made up to a volume of 1 litre with deionised water and allowed to cool to room temperature. The solution was adjusted to pH 6.2 using neat ammonia solution followed by the addition of agar. The solution was sterilized by autoclaving at 121°C under a pressure of 15 lb for 15 min. The solution was later equilibrated in a water-bath at about 56°C before adding 25 ml of filter-sterilised 40%(w/v) D-glucose solution. The contents were swirled gently and placed in the bath for 5 min before dispensing 30 ml into each of 100 ml containers.

## 1.6 Isolation of mycobacterial IPG

### Materials

All glass apparatus was soaked in 4 M nitric acid for a minimum of 10 h, washed with copious amount of double distilled water and finally rinsed several times with deionised water.

Formic acid, 98/100%. GPR, H.COOH = 46.03 (BDH 28430).

β-mercaptoethanol, d = 1.114 g/ml. $C_2HOS$ = 78.13 (Sigma I-7522).

EDTA, disodium salt, dihydrate, C10H14N2O8.2H2O = 372.2 (Sigma E-5134)

Activated charcoal, acid washed with phosphoric and sulfuric acids (Sigma C-5510).

*Hydrochloric acid*, HCl, 5 M volumetric, 182.30 g/L at 20°C, AnalaR® grade (BDH 190665R).

*Boiling solution:* prepared as a solution containing 50 mM formic acid, 1 mM b-mercaptoethanol and 1 mM EDTA, disodium salt.

*Activated Charcoal:* prepared by washing three times with deionised water. During each the charcoal was centrifuged at 18,400 x g for 30 min to remove small particulates. It was then baked in an oven to dryness at 120°C and stored at room temperature until use.

*Anion exchange resin:* AG 1-X8 20-50 mesh, hydroxide form, analytical grade and 8% cross-linked resins (catalogue no. 140-1422, Bio-Rad Laboratories, Hercules, CA).

*10%(v/v) Ammonium hydroxide:* prepared from a concentrated, AnalaR® grade ammonia solution in deionised water.

*50 mM HCl*, pH 1.3: prepared from a 5 M volumetric, AnalaR® grade HCl stock. Diluted 1 in 5 with deionised water to 1 M HCl working stock using volumetric flasks and further diluted 1 in 20 with deionised water to 50 mM. The pH was adjusted to 1.3 with 1 M HCl.

*10 mM HCl*, pH 2.0: prepared from 50 mM HCl in deionised water and pH adjusted to pH 2.0 with 1 M HCl.

*1 mM HCl*, pH 3.0: prepared from 10 mM HCl in deionised water and pH adjusted to pH 3.0 with 1 M HCl.

## Method

### *Conversion of AG 1X-8 hydroxide to formate form*

The resins were suspended with deionised water and the resulting slurry was transferred into a beaker and allowed to stand for 5 min at room temperature to allow binding to the resins to occur. The supernatant containing the smaller particles and free hydroxide ions was discarded. This procedure was repeated once. The slurry was transferred into a liquid chromatography column and ample time was given to allow the resins to pack into the column. The excess water was removed by allowing it to pass through the column, leaving just enough water to cover the top of the resin bed. The pH of the effluent was determined by using pH paper. To convert

resins to their formate form, 1 M formic acid was poured gently down the side of the column, and monitored by measuring the pH of the effluent. Five bed volumes of 1 M formic acid were required to completely convert the resins to the formate form. After conversion, the resins were washed with 4–5 bed volumes of deionised water until a stable pH was obtained. The converted resins were stored as a slurry in water at 4°C.

### *Cultivation of mycobacterial strains*

*M. tuberculosis* H37Rv and H37Ra were grown on solid Sauton's medium at 36°C for about 6–8 weeks. A period of 4–5 months was needed to grow up 35 slopes of bacteria which were equivalent to about 20 g bacteria wet weight. *M. vaccae* R877R was grown on the same medium at 32°C for about 3–4 weeks. Unlike *M. tuberculosis*, *M. vaccae* needed a period of 3–4 weeks to grow up 40 slopes of bacteria which were equivalent to about 20 g bacteria wet weight. Special precautions were taken to harvest the bacteria in a Class I safety cabinet equipped with a containment level 3 facility.

### *Isolation of mycobacterial IPG*

The isolation of mycobacterial IPG was based on that described by Nestler (1991) for the isolation of mammalian IPG. The following procedures were performed to isolate mycobacterial IPG from approximately 22 g of mycobacteria wet weight.

Confluent mycobacterial growth was scraped from Sauton's medium with a sterile metal spatula and transferred to a preweighed 500 ml Duran® bottle containing about 50 glass beads and 40 ml of boiling solution. The bottle was weighed again and the wet weight of the mycobacteria was determined by subtraction. The suspension was made up to a volume of 66 ml with boiling solution (3 ml boiling solution per gram of wet weight mycobacteria) and boiled for 5 min. After boiling, the solution was allowed to cool on ice for 15 min. The suspension was sonicated for 30 min using a 100-Watt model VibraCell™ (Sonics and Materials, Inc., Danbury, Connecticut, U.S.A.). The sonicate was centrifuged at 29,500 x g for 90 min at 4°C using a Beckman J2-21M/E Ultracentrifuge (Rotor JA-20, Beckman Instruments Inc, Palo Alto) rotor. The supernatant was mixed with activated charcoal (10 mg/ml of supernatant) and incubated at 4°C for 10 min. The charcoal was removed by centrifugation at 29,500 x g for 30 min at 4°C. The resulting solution was diluted with 10 volumes of deionised water and the pH was adjusted to 6.0 with 10% ammonium hydroxide and gently shaken overnight in a 5-litre conical flask which contained 50 ml of AG 1-X8 (20-50 mesh) formate resin at room temperature. The resin was poured into a liquid chromatography column (catalogue no. 6-4755, Supelco Inc., Bellefonte, PA) and washed with 100 ml (2 bed volumes) of deionised water followed by 100 ml of 1 mM HCl, pH 3.0. The column was eluted with 250 ml (5 bed volumes) 10 mM HCl, pH 2.0 to obtain the P-type IPG, and then 250 ml 50 mM HCl, pH 1.3 to obtain the A-type IPG. Both fractions were adjusted to pH 4.0 with 10% ammonium hydroxide and dried to appropriate volume in a Büchi rotary evaporator at 37°C. In some cases, the concentrates were freeze-dried in a preweighed Universal container and weighed after freeze-drying to obtain the amount of IPG isolated. The IPG were redissolved in 400 µl sterile deionised water and aliquoted into smaller volumes, freeze-dried and stored at -20°C.

## 1.7 Limulus amoebocyte lysate test for LPS

### Materials

E-TOXATE® kit was purchased from Sigma Chemical Co. St Louis, MO which comprises:

1. E-TOXATE® Working Solution: reconstituted in the Endotoxin-Free water provided, and gently swirled to dissolve the lysate. Repeated freeze-thaw cycles will decrease sensitivity of the lysate, therefore they were avoided.
2. Endotoxin Standard (*E. coli* 0.55:B5 lipopolysaccharide) standardised against USP Reference Standard Endotoxin. A 4000 EU/ml Endotoxin Standard Stock Solution was prepared in Endotoxin-Free Water.
3. Endotoxin-Free water

Culture tubes, 10 x 75 mm glass: autoclaved at 121°C and heated in an oven at 175°C for about 3 h.

**Methods**

The protocol was provided by the manufacturer was followed. The Endotoxin Standard Stock Solution was mixed and diluted as indicated below:

| Tube No. | Endotoxin | Endotoxin-free Water (ml) | Final conc. (EU/ml) |
|---|---|---|---|
| 1 | 0.2 ml Endotoxin Standard Stock Soln | 1.8 | 400 |
| 2 | 0.2 ml from Tube No.1 | 1.8 | 40 |
| 3 | 0.2 ml from Tube No.2 | 1.8 | 4 |
| 4 | 0.3 ml from Tube No.3 | 2.1 | 0.5 |
| 5 | 1.0 ml from Tube No.4 | 1.0 | 0.25 |
| 6 | 1.0 ml from Tube No.5 | 1.0 | 0.125 |
| 7 | 1.0 ml from Tube No.6 | 1.0 | 0.06 |
| 8 | 1.0 ml from Tube No.7 | 1.0 | 0.03 |
| 9 | 1.0 ml from Tube No.8 | 1.0 | 0.015 |

| Tube | | Sample | Endotoxin-Free Water | E-TOXATE® Endotoxin Standard Dilution | Working Solution |
|---|---|---|---|---|---|
| A | Test for endotoxin in sample | 0.1 ml | — | — | |
| B | Test for E-TOXATE® inhibitor in sample | 0.1 ml | — | 0.01 ml of 4 EU/ml | 0.1 ml |
| C | Negative control | — | 0.1 ml | — | 0.1 ml |
| D | Standard | — | — | 0.1 ml of 0.5 EU/ml | 0.1 ml |
| E | Standard | — | — | 0.1 ml of 0.25 EU/ml | 0.1 ml |
| F | Standard | — | — | 0.1 ml of 0.125 EU/ml | 0.1 ml |
| G | Standard | — | — | 0.1 ml of 0.06 EU/ml | 0.1 ml |
| H | Standard | — | — | 0.1 ml of 0.03 EU/ml | 0.1 ml |
| I | Standard | — | — | 0.1 ml of 0.015 EU/ml | 0.1 ml |

Nine tubes, A-I were prepared as shown above. E-TOXATE® Working Solution was added to each tube starting with tubes I then through the

highest endotoxin standard. The tubes were gently mixed, covered with Parafilm®, and incubated in a water bath for 1 h at 37°C. After incubation, the tubes were removed one at a time and inverted 180°. A positive test is the formation of a hard gel which permits complete inversion without disruption of the gel.

In some experiments, LPS and *M. vaccae* IPGs were pretreated with 25 μg/ml of polymyxin B at room temperature for 1 h prior to incubation with the *Limulus* lysate.

## 1.8 Results

### Differential effects of mycobacterial IPGs on the activation of pyruvate dehydrogenase phosphatase (PDH)

Fig. 1.3. shows that the rat liver, *M. vaccae* and the virulent strain of *M. tuberculosis* H37Rv all contained PDH phosphatase-stimulating activity in the P fraction. The P-type IPG in the rat liver was about 4 times as active as the P-type found in *M. vaccae* and *M. tuberculosis* H37Rv. The PDH phosphatase stimulating activity was not restricted to the P fractions but it also found in the A fraction of the rat liver and *M. vaccae.* The A fractions of the both strains of *M. tuberculosis*, the virulent strain H37Rv and the avirulent strain H37Ra contained PDH phosphatase inhibitory activity not found in the rat liver or *M. vaccae.*

### Differential effects of mycobacterial IPGs on the stimulation of lipogenesis in adipocytes

IPG A-type from the rat liver, *M. vaccae* and the avirulent strain of *M. tuberculosis* H37Ra stimulated lipogenesis in adipocytes. However, IPG-A type from the virulent strain of *M. tuberculosis* H37Rv was inhibitory (Fig. 1.4). The level of lipogenic IPG A-type activity in the rat liver was very similar to that of *M. vaccae* IPG A-type. In contrast, lipogenic activity was only found in the P fraction of the avirulent strain of *M. tuberculosis* H37Ra.

This assay was performed by Dr S. Kunjara

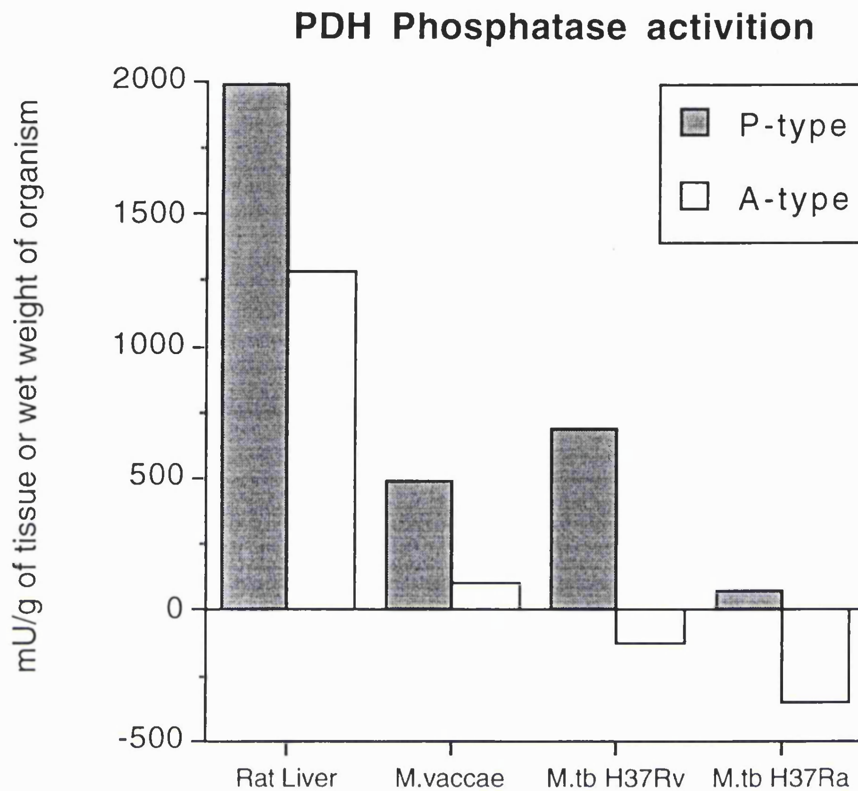



**Fig. 1.3. Pyruvate dehydrogenase (PDH) phosphatase activition by different sub-fractions of IPG.** Rat liver P- or A-type IPG, *M.vaccae* P- or A-type IPG, virulent strain of *M. tuberculosis* (M.tb) H37Rv P- or A-type IPG or an avirulent strain of *M. tuberculosis* H37Ra P- or A-type IPG was incubated with PDH phosphatase and pyruvate dehydrogenase complex (PDC) for 2 min. The activated PDH phosphatase was determined by measuring the rate of production of NADH catalysed by PDC. The values were expressed as milliunits per gram of tissue or wet weight organism, where one unit of PDH activity is the amount required to increase the basal rate by 50%. As the values were evaluated based on duplicate samples, SEM were not possible.

This assay was performed by Dr S. Kunjara

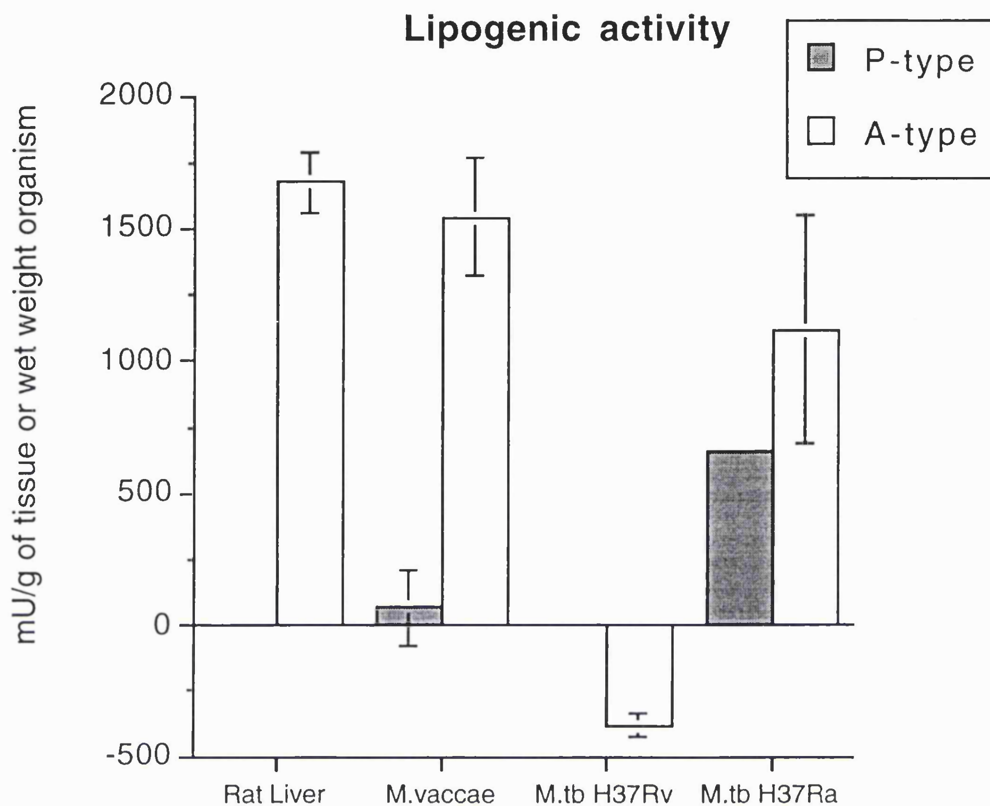



**FIg. 1.4. Lipogenic activity of different sub-fractions of IPG in adipocytes.** Adipocytes isolated from rat epididymal fat pads were incubated with rat liver P- or A-type, *M.vaccae* P- or A-type, *M. tuberculosis* (M.tb) H37Rv P- or A-type or *M. tuberculosis* H37Ra P- or A-type. The lipogenic activity of adipocytes was the measure of the rate of incorporation of 5 mM [U-$^{14}$C] glucose to fatty acids at 2 hr. The values were expressed as mean ± SEM triplicate samples. One unit lipogenic activity is the amount required to incorporate the basal level by 50%.

## Activation of the *Limulus* amoebocyte lysate by mycobacterial IPG

*Limulus* amoebocyte lysate incubated with LPS results in the formation of a hard gel but not with Endotoxin-Free water as shown in Table 1. The coagulation of the *Limulus* lysate in response to LPS was dose-dependent. The lowest concentration of LPS which resulted in the coagulation of the haemolysate was found to be 0.125 EU/ml (LPS at 12.5 pg/ml). Mycobacterial IPG of both P- and A-types from *M. vaccae* resulted in the gelation of *Limulus* lysate. To further determine whether the gelation of *Limulus* lysate was attributed to the presence of LPS, LPS or *M. vaccae* P- or A-type IPG was pre-treated with 25 µg/ml of polymyxin B. The results were striking, LPS pretreated with polymyxin B failed to cause gelation of the *Limulus* lysate. However, the gelation of *Limulus* lysate by *M. vaccae* P- and A-type IPGs was unaffected by the presence of polymyxin B.

| Sample | Formation of a hard gel | |
|---|---|---|
| | Without PB | With PB |
| Negative Control | — | N.D. |
| LPS 0.015 EU/ml | — | N.D. |
| LPS 0.03 EU/ml | — | N.D. |
| LPS 0.06 EU/ml | — | N.D. |
| LPS 0.125 EU/ml | — | N.D. |
| LPS 0.25 U/ml | + | — |
| LPS 0.5 U/ml | + | — |
| LPS 4 EU/ml | + | N.D. |
| LPS 40 EU/ml | + | N.D. |
| LPS 400 EU/ml | + | N.D. |
| *M.vaccae* P-type | + | + |
| *M.vaccae* A-type | + | + |

**Table 1. Limulus analysis of *M.vaccae* IPG.** 11 tubes were set up as described. The E-TOXATE® Working Solution containing the *Limulus* lysate was incubated with Endotoxin-Free water alone (Negative Control), LPS at 0.25 U/ml or 0.5 U/ml or *M. vaccae* P- or A-type IPG (1/100 each) at 37°C for 1 h. In some tubes, LPS and *M.vaccae* IPG were treated with 25 μg/ml of polymyxin B at room temperature for 1h prior to the exposure of the *Limulus* lysate. A positive result was interpreted as the formation of a hard gel which permits complete inversion of the tube to 180° without disruption of the gel (+). All other soft gels, turbidity, increase in viscosity, clear liquid, were considered negative (—). N.D., not determined, PB, polymyxin B. 10 endotoxin unit (EU) was equivalent to 1 ng of LPS.

## 1.9 Discussion

Mycobacterial IPGs, like mammalian IPGs can be isolated from the avirulent strain of *M. tuberculosis* H37Ra, the virulent strain of *M. tuberculosis* H37Rv and *M. vaccae* R877R using the AG 1X-8 anionic exchange column. Since the mycobacterial IPG co-purified precisely with the mammalian IPG at the same molarity of HCl, they are likely to share similar structures with the mammalian IPGs. This is exemplified by the comparable level of lipogenic activity of the *M. vaccae* A-type IPG and the rat liver A-type IPG. This was less marked in the PDH phosphatase assay, where the P-type IPG from the rat liver is about four times as active as the P-type IPG from *M. vaccae* and the virulent strain of *M. tuberculosis* H37Rv. Nonetheless, the fact that the level of PDH phosphatase-stimulating activity in *M. vaccae* P-type was the same as that of *M. tuberculosis* H37Rv P-type suggests that mycobacteria contain mediators that mimic the metabolic effects of mammalian IPG. Interestingly, the virulent strain of *M. tuberculosis* H37Rv differs from the avirulent strain of *M. tuberculosis* H37Ra in that the former contained no lipogenic activity in either the P- or the A-type. In contrast, the avirulent strain of *M. tuberculosis* H37Ra contained lipogenic activity in both P- and the A-types. Moreover, lipogenesis in adipocytes was inhibited by the A fraction of *M. tuberculosis* H37Rv which suggests that it may be toxic to adipocytes. This is reminiscent of the increase in the sensitivity of TNF-$\alpha$-mediated toxicity of a L929 murine fibroblast cell line, normal human fibroblasts and human endothelial cells following infection with the virulent strain of *M. tuberculosis* H37Rv (Filley and Rook, 1991, Filley, *et al.*, 1992).

The contamination of LPS has always been a major concern in the isolation of active components from many other micro-organisms, not just

mycobacteria. This is particularly important in the study of bacterial pathogenicity in macrophages because LPS is a powerful activator of macrophages. Therefore, the *Limulus* assay for endotoxin was employed to quantify the level of LPS in mycobacterial IPG preparations. Mycobacterial P- and A-type IPGs at 1:100 dilution resulted in the formation of a hard gel suggesting the presence of LPS. In contrast to LPS, activation of *Limulus* lysate by mycobacterial IPGs was not inhibited by polymyxin B. As already mentioned, there are two distinct pathways which lead to activation of the *Limulus* coagulation cascade. One involves the LPS-sensitive serine protease zymogen, factor C. Factor C carries a unique lectin-like domain which shares sequence similarity with C-type lectin. It is conceivable that the mammalian IPGs, like the mycobacterial IPGs, which are known to contain an inositol ring and several hexoses, can bind to the lectin-like domain necessary for the initiation of the coagulation cascade.

The alternate pathway of the activation cascade involves the zymogen, factor G. Unlike factor C, factor G is sensitive to (1-3) β-D-glucan predominantly found on the surface of fungi. Factor G is directly activated by (1-3) β-D-glucans and its active form activates the proclotting enzyme to clotting enzyme resulting in the coagulin gel formation. As short chain oligosaccharides containing two to six glucose residues do not activate factor G at all (Muta, 1996), it is unlikely that mycobacterial IPG will activate the coagulation cascade via the factor G pathway. To definitively determine whether mycobacterial IPG acts through factor C or G, LPS specific and β-D-glucan specific *Limulus* test kits will have to be used. According to Muta, mycobacterial IPG may activate another protein in the *Limulus* lysate that is yet to be identified which result in the activation of the coagulation cascade (Muta, 1996) as depicted in Fig.1.5.

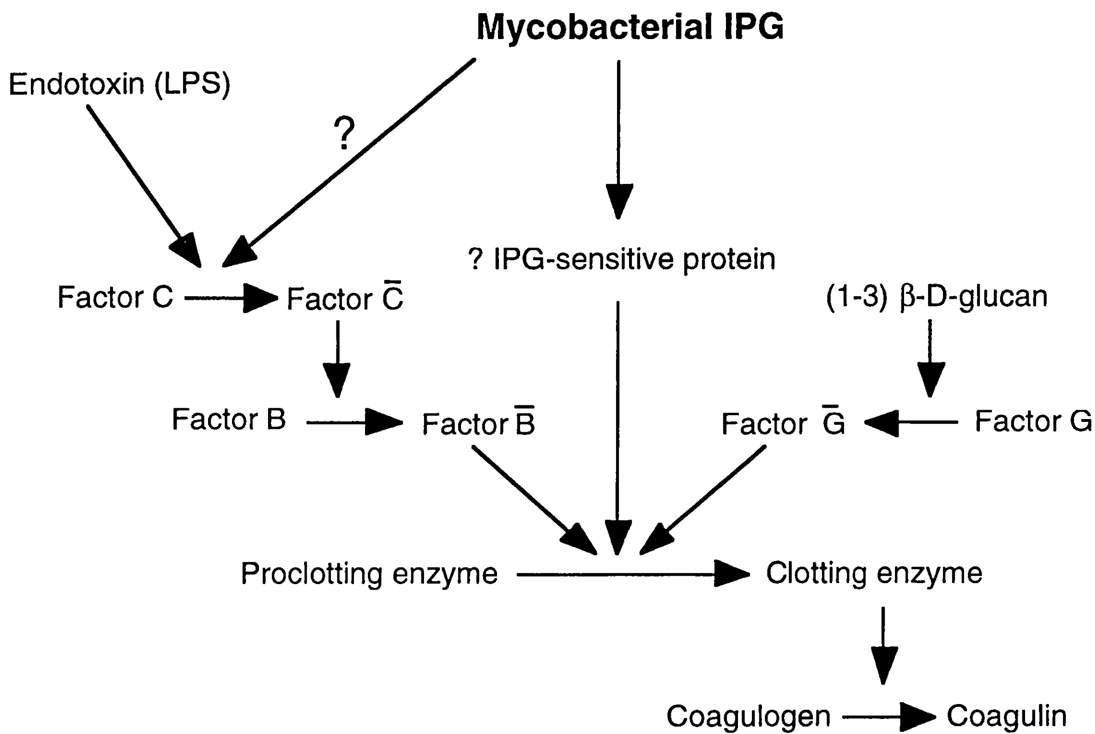



**Fig. 1.5. Activation of *Limulus* coagulation cascade by mycobacterial IPG.** LPS-sensitive factor C which carries the lectin-like domain may be activated by mycobacterial IPG, which results in haemolymph coagulation. Alternatively, mycobacterial IPG may activate a uncharacterised IPG-sensitive factor leading to the activation of the proclotting enzyme.

# Chapter Two

## Mycobacterial Inositol Phosphoglycans and their Effects on Macrophage Functions

## 2.1 The nature of the mycobacterial cell surface

### The mycobacterial cell wall

Few micro-organisms can replicate and survive inside macrophages as they contain an abundance of acidic vacuoles and hydrolytic enzymes. *M. tuberculosis* is an example of one such organism which has evolved to survive and multiply in this intracellular environment. The success of the tubercle bacillus lies partly in its formidable cell wall. Like many micro-organisms, the cell wall consists of a typical lipid bilayer which separates the cytosol from the outside environment. Anchored to the lipid bilayer is a massive peptidoglycan-arabinogalactan-mycolic acid complex. The peptidoglycan layer is made up of a highly cross-linked polymer composed of N-acetylglucosamine and N-glycolylmuramic acid linked by β(1–4) glycosidic bonds. Linked covalently to the peptidoglycan is another polymer, arabinogalactan, which consists of linear strands of D-arabinofuranose and D-galactofuranose and the arabinogalactan binds to mycolic acids which are high-molecular weight fatty acids ranging from 70 to 90 carbon atoms long. It is probably this high mycolate content that confers the acid-fastness, a characteristic of all mycobacteria.

### Lipoarabinomannan

Another *M. tuberculosis* cell wall component that have been the focus of research is lipoarabinomannan (LAM) (Chatterjee and Khoo, 1998). This

molecule is attached via two fatty acid chains, palmitic (C16:0) and tuberculostearic (C19) acids, into the cell membrane. The core of the LAM molecule consists of a linear α(1–6)-linked D-mannopyranose backbone to which are attached short chains of D-mannopyranose residues and D-arabinofuranose residues. This mannan core in turn is directly attached to position 6 of the myo-inositol of a phosphatidylinositol anchor (Hunter and Brennan, 1990). Thus, there is no covalent attachment with the mycobacterial cell wall. In LAM the nonreducing terminal of the mannan core is linked to a polymeric arabinofuranose chain known as arabinan. Since the phosphatidylinositol mannan core is embedded within the cell wall, the arabinan of LAM has been implicated in the immunopathogenesis of leprosy (Sibley, *et al.*, 1988) and tuberculosis (Moreno, *et al.*, 1989, Barnes, *et al.*, 1992).

Chatterjee *et al.* (Chatterjee, *et al.*, 1991) showed that arabinan of LAM from a rapidly growing species of *Mycobacterium* comprises two distinct types of nonreducing termini giving rise to linear $Ara_4$ and branched $Ara_6$ (the subscripts of Ara denotes the number of arabinose residues) upon digestion with endoarabinase. Since the arabinofuranoses terminate at the arabinan, this type of LAM is termed as arabinofuranosyl-terminated LAM (AraLAM). In contrast, the same two nonreducing termini of the arabinan from the virulent Erdman strain of *M. tuberculosis* was found to be extensively capped with residues of α-mannopyranose, hence its name mannopyranosyl-terminated LAM or ManLAM (Chatterjee, *et al.*, 1992a). The initial speculation that mannose-containing LAM was restricted to virulent strains of *M. tuberculosis* has not been supported by recent structural studies. It is now known that all *M. tuberculosis* strains examined, which include Erdman, H37Rv and H37Ra, as well as the *M. bovis* BCG vaccine strain contain ManLAM (Prinzis, *et al.*, 1993, Venisse, *et al.*, 1993, Khoo, *et al.*, 1995). It has been found that

the activity of the AraLAM may be attributed to the inositol phosphate capping, a feature not found on LAMs from *M. tuberculosis* or *M. leprae* (Khoo, *et al.*, 1995).

## Effect of lipoarabinomannan on macrophages

LAM is a major glycolipid found in all mycobacteria studied. Since its first characterisation by Hunter and Brennan (1990), it has been shown to exert a wide range of immunomodulatory functions (as reviewed in Chatterjee and Khoo, 1998). It is highly immunogenic and can stimulate macrophages to produce a wide array of cytokines such as TNF-$\alpha$ (Chatterjee, *et al.*, 1992b), GM-CSF, IL-1, IL-6 and IL-10 (Barnes, *et al.*, 1992). Earlier studies have shown that LAM can abrogate T cell activation (Kaplan, *et al.*, 1987) and inhibit IFN-$\gamma$ and LPS-induced macrophage activation (Sibley, *et al.*, 1988). As LAM from *M. tuberculosis* is a potent scavenger of oxygen free radicals and can inhibit protein kinase C activity (Chan, *et al.*, 1993), it is considered as a putative virulence factor.

There are two types of LAM, AraLAM and ManLAM. Both are differentially modified at the non-reducing termini of the arabinfuranosyl side chains and are derived from different species of mycobacteria (Khoo, *et al.*, 1995). Previous studies have shown that AraLAM from *M. tuberculosis* H37Ra is a potent inducer of TNF-$\alpha$ and NO in murine macrophages as compared to H37Rv ManLAM (Adams, *et al.*, 1993, Anthony, *et al.*, 1994). AraLAM is also an effective inducer of TNF-$\alpha$, GM-CSF, IL-1$\alpha$ and IL-1$\beta$, IL-6, IL-8 and IL-10 in human peripheral blood mononuclear cells (Barnes, *et al.*, 1992, Dahl, *et al.*, 1996), and TNF-$\alpha$ and IL-1$\beta$ in a THP-1 human monocytic cell line

(Zhang, *et al.*, 1993). Moreover, AraLAM but not ManLAM can induce early response genes such as *c-fos*, *KC* and *JE* in bone marrow-derived macrophages (Roach, *et al.*, 1994). Recently, AraLAM but not ManLAM, was found to induce IL-12 mRNA expression in a J774 macrophage cell line which suggests it drives a Th1 response (Yoshida and Koide, 1997). These results, suggest that ManLAM may exert its rôle as a virulence factor in the development of tuberculosis. However, this hypothesis was questioned when the original strain of *M. tuberculosis* H37Ra from which AraLAM was derived was found not to be *M. tuberculosis* H37Ra but a fast-growing *Mycobacterium* sp (Khoo, *et al.*, 1995).

## 2.2 Macrophage and *Mycobacterium tuberculosis:* The key to pathogenesis

### Intracellular fate of *M. tuberculosis*

Much of our understanding on the interaction of the *M. tuberculosis* phagosome and the macrophage lysosome came from the early work of D'Arcy Hart and co-workers (Armstrong and Hart, 1971, Armstrong and Hart, 1975). The lysosomes are packed with a range of hydrolytic enzymes which are capable of degrading bacterial cell walls leading to lysis. During phagocytosis, the bacterial phagosome fuses with the lysosome to form a phagolysosome, releasing their contents into the vacuole, thus initiating the intracellular killing and digestion of the non-pathogenic bacteria. In the case of *M. tuberculosis*, there are evidences showing that it can prevent phagolysosomal fusion (McDonough, *et al.*, 1993, Clemens and Horwitz,

1995, Clemens, 1996). McDonough *et al.* (1993) have shown that the inhibition of phagolysosomal fusion can be achieved only with live bacilli, not dead ones. Previous studies have demonstrated that the *Mycobacterium*-containing vacuoles is relatively inactive because it does not readily fuse with endosomal compartments containing colloidal markers and horseradish peroxidase. However, further studies have revealed that the vacuoles containing *M. avium* or *M. tuberculosis* (H37Rv and H37Ra) contain a late endosomal/lysosomal marker, **l**ysosomal-**a**ssociated **m**embrane **p**rotein-1 (LAMP-1) which suggests that the vacuoles interact with the host cell endosomal pathway (Sturgill-Koszycki, *et al.*, 1994, Xu, *et al.*, 1994, Russell, 1995).

Acidification of the intracellular compartment is mediated by the activity of a multicomponent enzyme complex known as the vesicular proton-ATPase complex. Techniques using immunoelectron microscopy and immunoblotting have found that the proton-ATPase complex is absence in the *Mycobacterium*-containing vacuoles. This is in direct contrast with other intracellular pathogens such as *Leishmania mexicana* in which the proton-ATPase on the membrane of the phagosome resulted in a reduction in local pH. It remains to be established whether this phenomenon is due to the inhibition of the phagosome with the proton-ATPase, or the rapid expulsion of the proton-ATPase from the phagosome. Whilst the inhibition of phagolysosomal fusion remain elusive, the net effect is the lack of acidification of the *M. tuberculosis*-containing vacuole, which is crucial for the survival of the organism within the macrophages (Crowle, *et al.*, 1991).

## 2.3 Microbicidal action of macrophages

### Oxygen-dependent mechanisms

Macrophages have a repertoire of antimicrobial mechanisms which can be broadly classified into two types: oxygen-dependent and oxygen-independent mechanisms (as reviewed in O'Brien, *et al.*, 1996). The former involves a rapid increase in the uptake of oxygen which is known as the respiratory burst. This is followed by the reduction of molecular oxygen to superoxide anion and hydrogen peroxide, which is catalysed by a multicomponent enzyme, NADPH oxidase. Further reaction between the superoxide anion and hydrogen peroxide can lead to the formation of highly reactive oxygen species such as singlet oxygen and hydroxyl radicals. Although these reactive species have been implicated in the killing of microbes, their rôles in the killing of *M. tuberculosis* are less convincing. There are some data from Chan *et al.* (1992) who demonstrated that a clone of a murine macrophage J774 cell line that was unable to produce a respiratory burst was capable of killing *M. tuberculosis.* Moreover, in an unpublished report by O'Brien *et al.* (1996), murine fibroblasts treated with IFN-$\gamma$ could inhibit the replication of *M. tuberculosis* without producing a detectable respiratory burst.

### *Nitric oxide*

Nitric oxide(NO) is produced from the oxidation of L-arginine to citrulline, and is catalysed by the enzyme **n**itric **o**xide **s**ynthase (NOS) in mammalian cells (as reviewed in Hibbs, *et al.*, 1987, Nathan, 1992). Hitherto, three isoforms of NOS have been found. The **c**onstitutive NOS (**c**NOS), **n**euronal NOS (**n**NOS or NOS1) and **e**ndothelial NOS (**e**NOS or NOS3) are calcium and calmodulin-dependent, whereas the **i**nducible NOS (iNOS or NOS2) (Stuehr, *et al.*, 1991, Xie, *et al.*, 1992, Nathan, 1997) has activity that does not depend on the elevation of intracellular calcium and calmodulin. The numbers after NOS denote the historical order in which they were first cloned. NO has been implicated in a wide variety of biological processes which include vasodilation, neurotransmission, cytotoxicity, tumorcidal and antimicrobial activity.

The NOSs have a bi-domain structure: an oxygenase domain within the amino-terminal half and a reductase domain within the carboxyl-terminal half. Complementary DNA analysis has shown that all three isoforms of NOS share 30-40% amino acid identity to cytochrome P-450 reductase, a member of the drug metabolising enzymes. In mammals, two monomers of NOS have to homodimerise to produce NO. The production of NO requires three co-substrates (L-arginine, NADPH and $O_2$) and five cofactors (FAD, FMN, calmodulin, tetrahydrobiopterin and haem). In comparison, NOS2 or iNOS in mouse macrophages produce the most NO (Xie and Nathan, 1994), orders-of-magnitude higher than the other isoforms. Unlike NOS1 and NOS3, NOS2 is expressed in response to inductive stimuli (Hibbs, *et al.*, 1992). Many cytokines and bacterial products, often acting in synergy, have been shown to stimulate the expression of NOS2. So far, IFN-γ has been the only

cytokine shown to be effective when treated alone (Stuehr and Marletta, 1987). IFN-γ alone is insufficient to generate high levels of NO in murine macrophages, although it can synergise with TNF-α to induce an increase of NOS2 expression resulting in a large amount of NO (Drapier, *et al.*, 1988). In addition to TNF-α, synergy can be also achieved through the combination of IFN-γ and LPS in which the latter can stimulate macrophages to produce TNF-α. The importance of IFN-γ, as a inducer of NOS2 as well as an antimycobacterial has been shown by the studies of IFN-γ-knock-out (Cooper, *et al.*, 1993, Flynn, *et al.*, 1993) and IFN-γ receptor-knock-out mice following infection with *M. tuberculosis* and *M. bovis* BCG respectively. These mice were not only unable to restrict the growth of the mycobacteria, they succumbed rapidly to the infection. The rôle of IFN-γ is further supported by reports that infants and children with a defect in the IFN-γ receptor are highly susceptible to BCG (Jouanguy, *et al.*, 1996) and other mycobacterial infections (Newport, *et al.*, 1996).

It is evident that the expression of NOS2, which leads to the production of NO, correlates with the protection against *M. tuberculosis*, *M. bovis* and *M. leprae* among other intracellular pathogens in murine macrophages. Denis (1991) has demonstrated that TNF-α and GM-CSF-activated human macrophages kill *M. avium* by the action of reactive nitrogen intermediates (RNI). In contrast, Bermudez *et al.* (1993) have reported that RNI are not released by human macrophages activated with TNF-α and GM-CSF, and RNI is not required for the killing of *M. avium*. As a result, these have caused controversy as many investigators have failed to demonstrate NO production in human macrophages. Several studies have shown that the inability of human macrophages to produce NO is attributable to the deficiency of the cofactor tetrahydrobiopterin, which is essential for the dimerisation of NOS2, and thus the production of NO (Weinberg, *et al.*, 1995). Despite the indirect

evidence from a number of laboratories, the scepticism persists even after Nathan, Ho and co-workers confirmed the positive findings (Nicholson, *et al.*, 1996). These author used immunocytochemistry to show that 11 of 11 patients with culture-positive pulmonary tuberculosis reacted with a monospecific antibody specific for human NOS2 in bronchoalveolar lavage fluid. Furthermore, all the NOS2-positive macrophages displayed diaphorase activity, which suggests the presence of a high-output pathway of NO. Thus, human macrophages may produce NO under certain conditions as in the case of pulmonary tuberculosis

**Oxygen-independent mechanisms**

The oxygen-independent systems include lysosomal enzymes, antimicrobial proteins and peptides. The lysosomes contain hydrolytic enzymes which can result in the digestion of micro-organisms under the right conditions. Previous studies by Kanai and Kondo (1970) have shown that lysosomes isolated from BCG-immunised mice contain substances that can kill *M. tuberculosis*. In addition to the hydrolytic enzymes, the lysosomes also contain a family of proteins and peptides which have antimicrobial activity (as reviewed in Spitznagel, 1990, Lehrer, *et al.*, 1993). Seven of these proteins have been described in human neutrophils. They include four **h**uman **n**eutrophil **p**eptides (HNP 1-4), also known as defensins; cathepsin G; a 37 kDa **c**ationic **a**ntimicrobial **p**rotein (CAP37), also known as azurocidin; and **b**acterial **p**ermeability **i**ncreasing factor (BPI), also known as CAP57. Although the majority of the proteins have shown to be active against a broad range of micro-organisms, their activity against *M. tuberculosis* remain to be elucidated. There are reports showing that

defensins are active against *M. avium* (Ogata, *et al.*, 1992) and *M. fortuitum* (Lehrer, *et al.*, 1993).

## 2.4 Tumour necrosis factor-α

Tumour necrosis factor (TNF)-α, also known as cachectin, is a pleiotropic cytokine produced primarily by activated macrophages (Mathews, 1978, Mathews, 1981) and T cells (Cuturi, *et al.*, 1985). Its original description was based on its ability to cause necrosis of methylchoanthrene-induced sarcoma in mice, and it was shown to selectively kill transformed and neoplastic cell lines (Carswell, *et al.*, 1975). It is now known to cause a wide range of biological activities mostly related to inflammatory and immunomodulatory functions (as reviewed in Vassalli, 1992, Fiers, 1993, Tracey and Cerami, 1993).

Human TNF-α is translated as a transmembrane protein of 26 kDa (Kriegler, *et al.*, 1988) which is cleaved by a metalloproteinase to give a 17 kDa soluble form of TNF-α (Gearing, *et al.*, 1994). In solution, only the trimer of TNF-α is biologically active (Wingfield, *et al.*, 1987). However, TNF-α can also exist as a type II transmembrane form since its carboxyl terminus is located extracellularly. This membrane-bound TNF-α retains its biological function and can be readily detected in macrophages stimulated with LPS. Macrophages with this membrane-bound TNF-α may contribute to local inflammatory responses without systemic release of toxic TNF-α (Luettig, *et al.*, 1989).

There is little doubt that in mice TNF-α plays a vital rôle in protection against *M. tuberculosis* infection. This is supported by studies on mice treated with a monoclonal antibody against TNF-α, which showed that mice lacking the TNF-α p55 receptor succumbed to *M. tuberculosis* infection (Flynn, *et al.*, 1995). Furthermore, Chan *et al.* (Chan, *et al.*, 1992) have shown that TNF-α can synergise with IFN-γ to stimulate NO production in mouse macrophages, and thence to the killing of *M. tuberculosis.* Conversely, macrophages treated *in vitro* with inhibitors of NOS such as aminoguanidine or N-monomethyl-L-arginine (L-NMMA), fail to produce NO or to kill *M. tuberculosis* (Chan, *et al.*, 1995). TNF-α has been implicated in *M. bovis*-induced granuloma formation, which is essential for the containment of *M. bovis* infection (Kindler, *et al.*, 1989) Others have demonstrated that *in vivo* iNOS may be critical for maintaining a persistent or latent state of *M. tuberculosis.* These experiments have recently been confirmed with iNOS transgenic mice (MacMicking, *et al.*, 1997). Thus, TNF-α in the mouse may be essential for protection against tuberculosis.

While TNF-α may play a protective rôle in the mouse, the relevance of TNF-α in humans in protection against tuberculosis is uncertain. There are at least two lines of evidence which suggest that TNF-α is protective. Firstly, Hirsch *et al.* (1992) have found that TNF-α can increase the antimycobacterial activity of human alveolar macrophages. Secondly, TNF-α has been shown to localise at the site of infection in patients with tuberculosis pleuritis who mount a protective response to the infection (Barnes, *et al.*, 1990). However, this is by no means conclusive because others have found that peripheral blood mononuclear cells from tuberculosis patients with cachexia and pyrexia, produce more TNF than tuberculosis patients without those manifestations (Cardranel, *et al.*, 1990). Moreover, studies by Rook and colleagues have demonstrated that local injection of TNF-α into an

inflammatory site previously treated with mycobacterial antigens can lead to a necrotic reaction (Al Attiyah, *et al.*, 1992a, Al Attiyah, *et al.*, 1992b). It later emerged that this necrosis of the inflammatory site was not due to the nature of the antigenic challenge but rather on the nature of the inflammatory response following immunisation (as reviewed in Rook and Al Attiyah, 1991). This is because similar necrosis can also be induced using Gram-negative bacteria or LPS as seen in a Shwartzman reaction (Shwartzman, 1937). There is increasing evidence that inflammatory lesions evoked by mixed Th1 and Th2 responses are susceptible to TNF-α-mediated necrosis (as reviewed in Hernandez-Pando and Rook, 1991). In contrast, the lesions that are elicited by "pure" Th1 responses are not affected by subsequent injection with TNF-α (Hernandez-Pando and Rook, 1994).

## 2.5 Interleukin-1

IL-1 consists of two distinct gene products. IL-1α and IL-1β (as reviewed in Dinarello, 1994, Dinarello, 1996). They both can bind to the same receptor to elicit a variety of responses which include inflammation, growth and haematopoiesis (Bagby, 1989, Fibbe and Falkenburg, 1990). Both IL-1α and IL-1β are produced by a variety of cell types, primarily by monocytes or macrophages in response to stimulation with LPS and other inflammatory agents (as reviewed in Cavaillon and Haeffner-Cavaillon, 1990). Neither IL-1α nor IL-1β contains the secretory signal sequence and thus their secretions do not occur through the endoplasmic reticulum (Rubertelli, *et al.*, 1990). Studies using immunochemistry have shown that they are both localise in the cytosol and are not released via the classical secretory pathway (Rubertelli, *et al.*, 1990). IL-1α and IL-1β are synthesised as 31 kDa

precursor forms (known as proIL-1α and proIL-1β respectively) which can be cleaved to mature forms of 17 kDa (Hazuda, *et al.*, 1991). Only the mature IL-1β is biologically active, however, both the pro and mature forms of IL-1α are active (Mosley, *et al.*, 1987).

In lipopolysaccharide(LPS)-stimulated macrophages, the proIL-1β is processed by interleukin-1β converting enzyme (ICE) to mature IL-1β (Black, *et al.*, 1989). ICE is a cysteine protease which cleaves at the aspartic-alanine (116-117) positions of proIL-1β (Black, *et al.*, 1989, Howard, *et al.*, 1991). This enzyme is synthesised principally in macrophages as an inactive proenzyme which autoprocesses to an active tetramer composed of 10 kDa and 20 kDa subunits (Walker, *et al.*, 1994, Wilson, *et al.*, 1994). The release of mature IL-1β is associated with the processing of proIL-1β by ICE because the release of IL-1β is reduced by ICE inhibitors (Thornberry, *et al.*, 1992, Miller, *et al.*, 1995). This is also in line with a report showing that macrophages from ICE-deficient mice do not release IL-1β in response to LPS (Kuida, *et al.*, 1995). Pro-IL-1β is not restricted to cleavage by ICE because other enzymes such as elastase and granzyme A have also been shown to cleave proIL-β to biologically active form.

Although the rôles of IL-1β in tuberculosis is unclear, there are two lines of evidence which suggest that IL-1β is implicated in the pathology of tuberculosis. Firstly, studies have shown that IL-1β is produced in macrophages stimulated with whole *M. tuberculosis*, and its components which include heat shock protein 70 (Retzlaff, *et al.*, 1994), lipoarabinomannan (Zhang, *et al.*, 1993, Dahl, *et al.*, 1996) and two mycobacterial proteins with molecular masses 20 kDa and 46 kDa (Wallis, *et al.*, 1990). Secondly, Law *et al.* (1996) have shown that IL-1β is released

spontaneously by bronchoalveolar cells in patients with pulmonary tuberculosis.

## 2.6 Objectives of this chapter

The objectives were to:

1) to investigate if mycobacterial IPGs stimulate nitric oxide production in a J774A.1 murine macrophage cell line in the presence or absence of exogenous recombinant murine IFN-γ.

2) prove that the IPG activity was not attributed to contaminating endotoxin and LAM using polymyxin B and anti-LAM monoclonal antibody respectively;

3) compare the nitrite produced in J774A.1 cells stimulated with IPGs isolated from a non-pathogenic mycobacterium (*M. vaccae*) and IPGs from a pathogenic mycobacterium (*M. tuberculosis*);

4) compare the levels of nitrite produced in J774A.1 cells stimulated with IPGs from a virulent strain of mycobacterium (H37Rv) and IPGs from an avirulent strain (H37Ra);

5) verify that if mycobacterial IPG-induced nitric oxide production in J774A,1 cells was the result of the expression of iNOS protein; and

6) assess the ability of mycobacterial IPGs to activate J774A.1 cells by quantifying the levels of TNF-α and IL-1β release in the media.

## 2.7 Maintenance of J774A.1

### Materials

All reagents were purchased from Life Technologies Ltd, Paisley, U.K. unless stated otherwise.

*Complete medium:* Dulbecco's Modified Eagle medium (DMEM) without sodium pyruvate (catalogue no. 41965) supplemented with 10 % foetal bovine serum, 2 mM L-glutamine (catalogue no. 25030), MEM non-essential amino acids (catalogue no.11140) and 100 U/ml of penicillin and 100 μg/ml streptomycin (catalogue no. 15070)

*Cell scraper, sterile:* purchased from Greiner Labortechnik Ltd, Gloucestershire, U.K., catalogue no. 541070.

*Nunc™ tissue culture flask:* 80 $cm^2$

J774A.1 was kindly provided by Prof. Douglas Young (Imperial College School of Medicine)

### Methods

The J774A.1 is a macrophage cell line originally derived from a female BALB/c mouse (Ralph and Nakoinz, 1975, Ralph, *et al.*, 1975, Ralph and Nakoinz, 1977). The cells are readily adherent, phagocytic and bind specifically to antibody-coated erythrocytes. The cell line was maintained in culture by growth in Dulbecco's Modified Eagle medium without sodium

pyruvate supplemented with 10 % foetal bovine serum, 2 mM L-glutamine, MEM non-essential amino acids, and 100 U/ml of penicillin and 100 µg/ml streptomycin, under 5 % $CO_2$ at 37°C. The cells were grown in 80 $cm^2$ Nunc™ tissue culture flasks to confluency after which the non-adherent cells and culture medium were removed and replaced with fresh culture medium. The adherent J774A.1 cells were collected by gently scraping the cells with a sterile cell scraper.

## 2.8 Stimulation of J774A.1 cells

### Materials

*IFN-γ:* recombinant murine IFN-γ with a specific activity of 1 x $10^7$ U/mg purchased from Pharmingen, San Diego, CA, catalogue no. 19301U, Lot # B607-05. In some cases, another lot of recombinant mouse IFN-γ (0.2-1 x $10^8$ U/mg) from the same supplier was used Lot #M024692. Prepared as a 50 µg/ml stock solution in 1 mg/ml BSA in PBS previously sterile-filtered and stored as aliquots at -80°C.

*LPS: Escherichia coli* serotype O127:B8 LPS purchased from Sigma Chemical Co., St. Louis, MO, catalogue no. L-4516. Prepared as a 20 µg/ml stock solution in DMEM and stored at 4°C.

*Mycobacterial IPGs:* were prepared as previously described.

*Mammalian IPGs:* were kindly provided by Sylvie Deborde and Dr Sirilaksana Kunjara, UCL Medical School, Department of Molecular Pathology.

*L-NMMA:* $N^G$-Monomethyl-L-arginine, monoacetate salt purchased from Calbiochen-Novabiochem, La Jolla, CA, catalogue no. 475886. Prepared as

a 0.1 M solution in sterile deionised water, aliquoted and stored at -20°C until use.

*D-NMMA:* $N^G$-Monomethyl-D-arginine, monoacetate salt purchased from Calbiochen-Novabiochem, La Jolla, CA, catalogue no. 475892. Prepared as a 0.1 M solution in sterile deionised water, aliquoted and stored at -20°C until use.

*ManLAM:* Mannose-capped lipoarabinomannan provided by Dr John Belisle (Colorado State University, Fort Collins, CO., through National Institutes of Health Contract NO1-AI-75320) was derived from *M. tuberculosis* H37Rv. H37Rv LAM contains 3.46 ng endotoxin per 1.0 mg LAM. Prepared as a 0.5 mg/ml stock solution in sterile water and stored at -20°C.

*AraLAM:* Arabinofuranosyl-terminated lipoarabinomannan also provided by Dr John Belisle was isolated from a rapid grower *Mycobacterium* sp. AraLAM contains 0.9 ng endotoxin per 1.0 mg of AraLAM as determined by the Limulus Amboecyte Assay. Prepared as a 0.5 mg/ml stock solution in sterile water and stored at -20°C.

### Methods

J774A.1 macrophages were incubated with rat liver IPG, mycobacterial IPG, LPS, ManLAM or AraLAM immediately after the addition of IFN-γ. Macrophages were incubated with only one of the activators. To verify whether mycobacterial IPG-induced NO production in J774A.1 cells was the result of the oxidation of L-arginine catalysed by nitric oxide synthase, nitrite levels were compared in culture medium containing active L-arginine analogue, L-NMMA and its inert enantiomer, D-NMMA. *M. tuberculosis*

H37Ra P-, A-type IPG or LPS was incubated simultaneously with IFN-γ with or without L-NMMA or D-NMMA.

## 2.9 Pretreatment of mycobacterial IPG and LPS with polymxin B

### Materials

*Polymyxin B:* Polymyxin B sulphate purchased from Sigma Chemical Co., St Louis, MO, catalogue no. P-1004. Prepared as a 10.8 mg/ml stock solution in DMEM and stored at -20°C.

### Methods

*M. tuberculosis* H37Ra P-type or A-type IPG, *M. tuberculosis* LAM or LPS was incubated with polymyxin B at a final concentration of 10 μg/ml for 1 h at room temperature. Equal amount of stimuli incubated under the same conditions with DMEM was used as controls. J774A.1 cells were incubated with 240 U/ml of IFN-γ and mixtures pretreated with or without polymyxin B for 48 h. The nitrite concentrations were determined by using the Griess reaction as described below.

## 2.11 Quantification of nitrite using the Griess reaction

### Materials

Sulfanilamide, $C_6H_8N_2O_2S$ = 172.2, was purchased from Sigma Chemical Co., St. Louis, MO, catalogue no. S-9251.

N-1-naphthyl ethylene diamine dihydrochloride, $C_{12}H_{14}N_2.2HCl$ = 259.2, was obtained from Sigma Chemical Co., St. Louis, MO, catalogue no. N-9125.

Orthophosphoric acid, $H_3PO_4$ = 98, minimum assay 85 %, AnalaR® grade, was purchased from BDH Laboratories Supplies, Poole England, catalogue no. 10173

*Griess reagent:* prepared as a 100 ml solution containing 1% (w/v) Sulfanilamide, 0.1% (w/v) N-1-naphthyl ethylene diamine dihydrochloride, 2.5% (v/v) Orthophosphoric acid. The solution was kept away from light at room temperature.

### Methods

The nitrite formed in the supernatant was assayed using the Griess reagent (Ding, *et al.*, 1988).and the nitrite formed is indicative of NO produced by the cells (Marletta, *et al.*, 1988). NO rapidly reacts with oxygen to yield a variety of nitrogen oxides which decomposes to nitrite in an aqueous solution *in vitro* (Miles, *et al.*, 1996). Culture supernatant (90 µl) was transferred to a 96-

well flat-bottomed Maxisorp™ plate. An equal volume of the Griess reagent was added to give a bright red colour. The plate was allowed to stand at room temperature for 10 min before reading the absorbance at 570 nm using a Dynatech MR5000 microtitre plate reader (Guernsey, Channel Islands). The nitrite concentrations were determined using sodium nitrite standards ranging from 1.56–100 μM diluted in Dulbecco's Modified Eagle medium without sodium pyruvate. The lowest limit of detection was determined and found to be 1.56 μM.

## 2.12 Western blotting of iNOS protein

### Materials

*Tris-buffered saline (TBS):* 25 mM Tris-HCl in 0.9% NaCl solution adjusted to pH 7.6 with 1 M NaOH.

*Lysis buffer:* 150 mM NaCl; 1 mM EDTA; 2 mM EGTA; 10 mM NaF and 1%(w/v) NP-40.

*Dithiothreitol:* a reducing agent purchased from Sigma Chemical Co., St. Louis, MO, catalogue no. D-9779. Prepared as a 1 M stock solution in cold sterile water, aliquoted and stored at -20°C.

*Protease inhibitors:*

*Leupeptin hydrochloride:* a thiol protease inhibitor purchased from Sigma Chemical Co., St. Louis, MO catalogue no. L-0649. Prepared as a 5 mg per ml stock solution in cold sterile water and stored as aliquots at -20°C.

*Aprotinin:* a serine protease inhibitor purchased from Sigma Chemical Co., St. Louis, MO, catalogue no. A-1153. Prepared as a 5 mg per ml stock solution in 0.01 M HEPES buffer (BDH 43601) and stored as aliquots at -20°C.

*Phenylmethylsulfonyl fluoride:* another serine protease inhibitor purchased from Sigma Chemical Co., St. Louis, MO, catalogue no. P-7626. Prepared as a 25 mg per ml stock solution in isopropanol (Sigma I-9516) and stored as aliquots at -20°C.

*Wash buffer:* 10 mM Tris pH 7.5, 100 mM NaCl and 0.1% (v/v) Tween 20.

*Blocking buffer:* 5%(w/v) non-fat milk in wash buffer.

*Primary antibody:* murine monoclonal anti-mouse iNOS IgG2a (Transduction Laboratories, Lexington, KY, U.S.A. catalogue no. N32020) prepared at 1 in 2,500 with blocking buffer.

*Secondary antibody:* polyclonal anti-mouse IgG conjugated with HRP (Transduction Laboratories, Lexington, KY, U.S.A. catalogue no. M15345) prepared at 1 in 2,500 with blocking buffer.

*Substrate:* Enhanced chemiluminescence, ECL™ RPN 2108 (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire).

*New RX Fuji Medical X-ray film:* purchased from Fuji Photo Film Co. Ltd, Tokyo catalogue no. 03E270.

*Development Folders:* purchased from Tropix, Bedford, MA, U.S.A. catalogue no. XF030.

*SDS-PAGE Molecular Weight Standards, High Range:* purchased from Bio-Rad Laboratories, Hercules, CA, catalogue no. 161-0303.

**Methods**

To ascertain whether the production of nitrite in J774A.1 macrophages was the result of the induction of the inducible nitric oxide synthase (iNOS) protein, macrophages were stimulated with *M. tuberculosis* H37Rv, *M. tuberculosis* H37Ra, *M. vaccae* IPG and LPS with or without IFN-$\gamma$ for 24 h. The nitrite accumulated in the medium was assayed by using the Griess reaction whereas the cell extracts were prepared and analysed by Western blotting (Wei, *et al.*, 1995). An equal amount of total protein from each samples was loaded into a 7 % sodium dodecyl sulphate-polyacrylamide gel and separated by electrophoresis. The separated proteins were blotted onto a nitrocellulose membrane and probed with a monoclonal antibody specific

for the carboxyl terminus of the iNOS proteins containing two NADPH binding sites (between amino acid 961 and 1144). The reaction was revealed by enhanced chemiluminescence and detected with an X-ray film. The film was scanned, converted into a bitmap and analysed using a Image Gauge version 3.01 densitometric software (Fuji Photo Film Co., Ltd.). The band intensities were later expressed as relative densitometric units and plotted graphically

***Preparation of cell lysates***

The medium was centrifuged at 150 x g and stored at -20°C until used for quantification of soluble TNF-α. The cells were quickly washed with 1 ml ice-cold TBS prior to the addition of 300 μl per well of lysis buffer containing 1 mM dithiothreitol, 50 μg per ml leupeptin, 50 μg per ml aprotinin and 50 μg per ml PMSF (added to the buffer just before use). The resulting suspension in the well was allowed to cool on ice to 0°C. The cells were gently scraped off the well with a cell scraper and transferred into a 1.5 ml Eppendorf tube. Cell lysis was facilitated by passing the suspension lysate through a 26G gauge needle several times. The lysate was centrifuged at 13,000 x g for 5 min to remove insoluble materials. A 50 μl aliquot of the lysate was prepared to measure protein concentration using the Lowry microassay method. The remaining lysates was stored at -20°C until electrophoresis.

## 2.13 SDS-PAGE of proteins

### Materials

*Protogel®:* 30% (w/v) acrylamide, 0.8% (w/v) methylene bisacrylamide, gas stabilized (National Diagnostics, Atlanta, catalogue no. EC-890). Stored at room temperature for up to a year.

*TEMED:* Bio-Rad Laboratories, Hercules, CA. Stored at 4°C.

*10% Ammonium peroxodisulphate (ammonium persulphate):* BDH Laboratory Supplies, Poole, England Electran®, catalogue no. 443073E. Prepared fresh in sterile water on the day of use.

*Buffer 6.8:* 0.5 M Trizma® base and 0.4% (w/v) SDS adjusted to pH 6.8 with 1 M HCl.

*Buffer 8.8:* 1.5 M Trizma® base and 0.4% SDS adjusted to pH 8.8 with 1 M NaOH.

*10% separating gel:* For a 40 ml gel, 13.3 ml of Protogel®, 10 ml buffer 8.8, 16.7 ml water, 563 µl of 10% ammonium peroxodisulphate and 30.7 µl of TEMED were added sequentially and mixed briefly prior to the addition into a gel casting apparatus. The gel casting apparatus was filled up to the mark, 1 cm below the base of the comb. Finally, the gel was layered with 0.1% SDS.

*3% stacking gel:* For a 20 ml gel, 2 ml of Protogel®, 5 ml of buffer 6.8, 12 ml of water, 225 µl ammonium peroxodisulphate and 20 µl of TEMED were added sequentially and mixed briefly. The 0.1% SDS was replaced with the 3% stacking gel and was allowed to completely polymerise for at least 2 h.

*Electrophoresis buffer:* 0.02 M Trizma® base, 0.192 M glycine and 10% SDS.

*2X protein loading dye:* prepared with 1%SDS, 10 mM EDTA, pH 8.0, 10 mM sodium phosphate, pH 7.0, 1% (v/v) 2-mercaptoethanol, 15% (v/v) glycerol, 4 mM PMSF and 0.01% (w/v) bromophenol blue (Bio-Rad Laboratories, Electrophoresis Purity Reagent 161-0404). The dye was filter-sterilised with a 0.45 µm filter and stored as aliquots at -20°C.

*2-mercaptoethanol:* BDH Laboratory Supplies, Poole, England, Molecular Biology grade, catalogue no. 436022A.

*Protein transfer buffer:* 0.025 M Trizma® base, 0.192 M glycine, 20% (v/v) methanol.

*0.45 µm supported nitrocellulose membrane:* (BDH Laboratory Supplies, Poole, England)

**Methods**

The protein samples were diluted with water and mixed with an equal volume of 2X protein loading dye. The protein was denatured by boiling for 3 min, mixed with 2 µl β-mercaptoethanol. The resulting solution was loaded on the gel had been pre-run at a constant current of 10 mA for 15 min. For a single gel, electrophoresis should be performed at a constant current of 24 mA during stacking and 34 mA during separation. The gel was electrophoresed until the bromophenol blue dye was approximately 2 cm from the end of the gel. The stacking gel was removed and the separating gel was blotted onto a supported nitrocellulose membrane. The gel was electro-blotted in a freshly prepared protein transfer buffer using a Trans-

blot™ Cell at a constant current of 100 mA for 12 h at room temperature and the current was increased to 200 mA for an additional hour.

### *Western blotting*

The membrane was immediately placed in blocking buffer and incubated for 1 h at room temperature with gentle agitation. After blocking, the membrane was incubated with the primary antibody diluted in blocking buffer for 1 h at room temperature with agitation. After incubation, the primary antibody was saved for subsequent use and the membrane was rinsed twice with wash buffer and washed once for 15 min and twice for 5 min each. This was followed by incubating the membrane with the secondary antibody again diluted in blocking buffer for another 1 h at room temperature. After incubation, the antibody was discarded and the membrane was washed once for 15 min and twice for 5 min each. The membrane was kept in the wash buffer while the detection reagents were being prepared. An equal volume of reagent 1 and 2 were mixed in an Universal container sufficient to cover the entire surface of the membrane. The excess buffer was drained off and the membrane placed on a piece of Saran wrap with the protein side up. The premixed detection reagent was layered onto the membrane and incubated for exactly 1 min at room temperature. The excess detection reagent was drained off and the gel placed in a Development™ folder. The membrane was finally exposed to a Fuji X-ray film for 5 min to enable visible bands to appear.

## 2.14 Sandwich ELISA for soluble TNF-$\alpha$

### Materials

Quantikine™ mouse TNF-$\alpha$ kit (catalogue no. MTA00) was purchased from R&D Systems, Minneapolis, MN, which included the following reagents:

Mouse TNF-$\alpha$ Microplates (coated with polyclonal antibody specific for mouse TNF-$\alpha$);

Mouse TNF-$\alpha$ Conjugate Concentrate (concentrated solution containing antibody against mouse TNF-$\alpha$ conjugated with horseradish peroxidase, with preservatives);

Mouse TNF-$\alpha$ Conjugate Diluent (diluent for antibody conjugate, with preservatives);

Mouse TNF-$\alpha$ Standard (recombinant mouse TNF-$\alpha$ in a protein base, with preservatives);

Mouse TNF-$\alpha$ Control (mouse TNF-$\alpha$ in a buffered protein base, with preservatives);

Assay Diluent RDW1 (buffered protein solution, with preservatives);

Calibrator RD5Z (buffered protein solution, with preservatives);

Wash Buffer Concentrate;

Color Reagent A (stabilized hydrogen peroxide);

Color Reagent B (stabilized tetramethylbenzidine) and

Stop Solution (diluted hydrochloric acid).

***Reagent Preparation***

Anti-mouse TNF-α Kit Control: reconstituted with 1.0 ml deionised water and assayed undiluted. Used within 8 h, then discarded.

*Anti-mouse TNF-α Conjugate Concentrate:* Diluted 0.5 ml Conjugate Concentrate in 11.0 ml of Conjugate Diluent.

*Wash Buffer:* If crystal formed, concentrate was warmed to room temperature and mixed gently until crystals were completely dissolved. 25 ml of Wash Buffer Concentrate was added to 600 ml of deionised water.

*Substrate Solution:* Color Reagent A and B were mixed in equal volume within 15 min of use.

*Mouse TNF-α Standard:* reconstituted with 2 ml of Calibrator Diluent RD5Z to a stock of 1500 pg/ml. Then, 1 in 2 serial dilutions were prepared starting from 750 pg/ml to 23.4 pg/ml.

**Methods**

The assay was a quantitative sandwich enzyme immunoassay and was performed according to the protocol provided by the manufacturer. Assay Diluent RD1W (50 µl) was added to each well which had been coated previously with a polyclonal antibody specific for mouse TNF-α. This was followed by the addition of 50 µl of the Standard, Control or sample supernatant to each well. The plate was gently tapped, covered with a plate sealer and incubated for 2 h at room temperature. After incubation, the unbound substances were discarded and the plate was washed repeatedly for four times with 400 µl of Wash Buffer. Polyclonal anti-mouse TNF-α

antibody conjugated with HRP (TNF-α Conjugate, 100 μl) was added to each well and incubated for another 2 h at room temperature. The unbound TNF-α conjugate was discarded and the plate was washed repeatedly four times. Tetramethylbenzidine (Color Reagent A) was added to each well (100 μl) and incubated in the dark for 30 min at room temperature to form a blue product. The solution turned yellow when 100 μl of diluted hydrochloric acid solution (Stop Solution) was added to each well. The absorbance of each well was determined using a Dynatech MR5000 plate reader (Guernsey, Channel Islands) set to 450 nm with a correction wavelength at 570 nm. The concentrations of TNF-α were evaluated from the TNF-α standard curve generated from AssayZap version 2.51 (Biosoft®, Cambridge Place, Cambridge). The lowest detection limit was determined and found to be 23.4 pg/ml.

## 2.15 Sandwich ELISA for soluble IL-1β

### Materials

Quantikine™ mouse IL-1β (catalogue no. MLB00) immunoassay kit was purchased from R&D Systems, Minneapolis, MN, which included the following reagents:

Mouse IL-1β Microplates (coated with polyclonal antibody specific for mouse IL-β);

Mouse IL-1β Conjugate Concentrate (concentrated solution containing antibody against mouse IL-1β conjugated to horseradish peroxidase, with preservatives);

Mouse Conjugate Diluent (diluent for antibody conjugate);

Mouse IL-1β Standard (recombinant mouse IL-1β in a buffered protein base, with preservatives)

Mouse IL-1β Control (mouse IL-1β in a buffered protein base with preservatives);

Assay Diluent RD1-14 (buffered protein solution, with preservatives);

Calibrator Diluent RD5T (buffered protein solution, with preservatives);

Wash Buffer Concentrate (concentrated solution of a buffered protein solution, with preservatives);

Color Reagent A (stabilized hydrogen peroxide);

Color Reagent B (stabilized tetramethylbenzidine)

Stop Solution (diluted hydrochloric acid solution)

### ***Reagent Preparation***

*Mouse IL-1β Kit Control:* reconstituted with 1.0 ml deionised water and assayed undiluted. Aliquoted and stored at -20°C for up to 60 days.

*Anti-mouse IL-1β Conjugate Concentrate:* Diluted 0.5 ml of Conjugate Concentrate in 11.0 ml Conjugate Diluent.

*Wash Buffer:* If crystals formed, the concentrate was warmed to room temperature and mixed gently until crystals were completely dissolved. Wash Buffer Concentrate (25 ml) was added to 600 ml of deionised water.

*Substrate Solution:* Color Reagent A and B were mixed in equal volume within 15 min of use.

*Mouse IL-1β Standard:* reconstituted with 5 ml of Calibrator Diluent RD5T to make a stock solution of 500 pg/ml. Doubling serial dilutions were prepared 250 pg/ml to 7.8 pg/ml.

### **Methods**

The assay was performed as previously described with the following exceptions: mouse IL-1β standards and mouse IL-1β control (50 µl each) were added to each well and polyclonal anti-mouse IL-1β conjugated with HRP was used as a IL-1β conjugate. The absorbance was determined using the Dynatech microtitre plate reader set to 450 nm with a correction wavelength at 570 nm. The concentrations of IL-1β were evaluated from the IL-1β standard curve generated from AssayZap version 2.5.1 (Biosoft®, Cambridge Place, Cambridge, England). The lowest detection limit of IL-1β was determined and found to be 7.8 pg/ml.

## 2.16 Results

### LPS synergises with IFN-γ to stimulate macrophages to produce high levels of nitric oxide

Nitrite was detected in the culture supernatant following stimulation of J774A.1 macrophages with recombinant IFN-γ (Fig. 2.1A). The IFN-γ-induced NO production was dose-dependent up to 500 U/ml and reached a plateau above that concentration. The sub-optimal concentration of IFN-γ required to produce half-maximal concentration of nitrite was found to be 250 U/ml. Unlike IFN-γ, LPS alone did not induce NO production in J774A.1 cells over a range of concentrations up to 1 μg/ml. However, when stimulated simultaneously with IFN-γ, LPS could induce high levels of nitrite in J774A.1 cells (Fig. 2.1B).

### Mycobacterial IPG synergises with IFN-γ to stimulate macrophages to produce high levels of nitric oxide

Mycobacterial P- and A-type IPGs were extracted from an environmental isolate of *M. vaccae* and the avirulent strain of *M. tuberculosis* H37Ra and tested for their ability to stimulate J774A.1 cells to synthesise NO. As with LPS, mycobacterial P- and A-type IPGs from *M. tuberculosis* H37Ra or *M. vaccae* alone did not induce NO production. However, mycobacterial IPG P- and A-types, from *M. vaccae* and *M. tuberculosis* H3Ra respectively were able to synergise with IFN-γ to induce the production of NO (Fig. 2.2). The

synergistic induction of NO by IFN-γ plus mycobacterial IPG was dose-dependent as shown in Fig. 2.3. H37Ra P- and A-type IPGs at 1:1,000 dilution were more effective at synergising with IFN-γ in the production of NO than either type of *M. vaccae* IPG. In contrast to mycobacterial IPG, rat liver P- and A-type IPGs failed to synergise with IFN-γ to induce nitrite production in J774A.1 macrophages.

**Effect of polymyxin B on mycobacterial-induced NO production**

The effect of *M. tuberculosis* H37Ra IPG and LAM with IFN-γ on NO production was not affected by the presence of polymyxin B indicative of the absence of LPS in the IPG preparations as shown in Fig. 2.4. In contrast, the effect of LPS was substantially abrogated by the treatment of polymyxin B.

**Effect of anti-LAM monoclonal antibody on ManLAM and AraLAM-induced nitrite production**

The effect of anti-LAM monoclonal antibody was evaluated on its ability to block ManLAM and AraLAM-induced nitrite production with IFN-γ in J774A.1 cells. Anti-LAM used at 10 μg/ml synergised with IFN-γ to stimulate nitrite production in J774A.1 as shown in Fig. 2.5. The levels of nitrite were greatly increase in cells stimulated with either ManLAM or AraLAM with IFN-γ in the presence of the antibody.

## Differential effects of ManLAM and AraLAM-induced nitric oxide production

As already mentioned, ManLAM is found predominantly in the slow-growing mycobacteria which include the virulent strain of *M. tuberculosis* H37Rv, the avirulent strain of *M. tuberculosis* H37Ra, and *M. bovis* BCG. AraLAM, however, is found generally in fast-growing mycobacteria. While NO was not generated by J774A.1 cells stimulated with either AraLAM or ManLAM alone over a range of concentrations up to 10 μg/ml, IFN-γ-induced production of NO was enhanced by co-activation with AraLAM or ManLAM (Fig 2.6). Whereas macrophages activated with 240 U/ml IFN-γ alone produced 15.4 μM nitrite, macrophages activated with IFN-γ and AraLAM produced as much as 37.5 μM nitrite. The synergism of IFN-γ and AraLAM was dose-dependent over a range of concentrations between 0.08–10 μg/ml. In contrast, ManLAM was less effective at enhancing the NO production at all the concentrations tested.

## Effect of L-NMMA and D-NMMA on mycobacterial IPG-induced NO production

The effect of both H37Ra IPG and LPS on the production of NO was unaffected by the presence of D-NMMA (Fig. 2.7). However, the production of NO was markedly inhibited in the presence of L-NMMA. While the activation of J774A.1 cells with H37Ra P-type IPG and IFN-γ in the presence of D-NMMA produced 27.4 μM nitrite, macrophages activated in the presence of L-NMMA produced nitrite levels as low as 7.3 μM, (73 % inhibition).

**Effect of brief exposure of J774A.1 cells to mycobacterial IPG on IFN-γ-induced nitric oxide production**

Macrophages were exposed to IPG P- or A-type of *M. tuberculosis* H37Ra or *M. vaccae* for 4 h and washed twice with medium to remove mycobacterial IPG. This was followed by the stimulation of macrophages with IFN-γ for NO production. Fig 2.8 shows that the exposure of mycobacterial IPG for 4 h was sufficient to trigger maximal production of NO by IFN-γ without the continuous presence of the mycobacterial IPG. Nitrite levels accumulated over a period of 24 h following stimulation with IFN-γ was dependent on the dose of mycobacterial IPG. H37Ra P- and A-type IPGs were potent at synergising with IFN-γ in the production of NO compared with both types of *M. vaccae.* IPG. IFN-γ alone stimulated little or no detectable levels of nitrite but augmented with H37Ra P- and A-type at 1:100 dilution to produce substantial levels of 46.1 μM and 33.2 μM nitrite respectively. In contrast, *M. vaccae* P- and A-type IPG prepared at the same dilutions stimulated with IFN-γ produced a low level of nitrite.

To examine the minimum time required to induce maximal production of NO by mycobacterial IPG, J774A.1 cells were incubated with *M. tuberculosis* H37Ra P- or A-type IPG, or *M. vaccae* P- or A-type IPG all at a dilution of 1:100 for 1/2, 1 and 3 h. Mycobacterial IPG was added first to the culture wells receiving the longest incubation followed by the shortest incubation, IFN-γ was immediately applied to macrophages for 24 h to stimulate NO synthesis. The results showed that macrophages exposed to mycobacterial IPG for only 3 h were sufficient to induce maximal production of NO by IFN-γ

(Fig. 2.9). Again, H37Ra IPGs were more effective at synergising with IFN-γ in the production of NO as compared with *M. vaccae* IPGs.

### Comparison of nitrite induction by IPGs from virulent and avirulent strains of *M. tuberculosis*

Mycobacterial IPG isolated from the virulent strain of M. tuberculosis H37Rv and the avirulent strain of M. tuberculosis H37Ra were compared in their ability to induce NO production in J774A.1 macrophages. As shown in Fig. 2.10 equivalent doses of P- and A-type IPGs from H37Rv or H37Ra induced similar levels of nitrite in J774A.1 macrophages in the presence of IFN-γ.

### Induction of inducible nitric oxide synthase protein expression by IFN-γ and mycobacterial IPG

A 130 kDa-band which corresponded to the same size of the iNOS control protein was seen in the lysates from cells treated with mycobacterial IPG (P or A-type) and IFN-γ (Fig. 2.11; Lane 1–6), LPS and IFN-γ (Fig. 2.11; Lane 9), IFN-γ alone (Fig. 2.11; Lane 7) and LPS alone (Fig. 2.12; Lane 7). The same band was absence in the lysates from cells stimulated with mycobacterial IPG alone (Fig. 2.12; Lane 1–6) and unstimulated control (Fig. 2.11; Lane 8). This was in agreement with the graph showing the absence of nitrite as determined by using the Greiss reaction (Fig. 2.13). As the intensity of the band correlated with the level of iNOS protein generated in the macrophages, the degree of iNOS protein expression caused by different

stimuli could be evaluated. While the cell lysate from the macrophage stimulated with IFN-γ alone gave a light band of 130 kDa, lysates derived from IFN-γ and H37Rv (P- and A-type) IPG, H37Ra (P- and A-type) IPG or LPS gave a darker band of the same size indicating an increase in the expression of the iNOS protein in response to the stimuli.

**Production of TNF-α and IL-1β by J774A.1 macrophages stimulated with IFN-γ and mycobacterial IPG**

Since TNF-α and IL-1β are known to induce iNOS, their detection in the culture supernatants following stimulation of J774A.1 macrophages with H37Ra IPG and LPS would suggest a possible autocrine rôle in inducing and maintaining iNOS expression. The production of NO induced by IFN-γ and H37Ra (P- and A-type) was not associated with high levels of soluble TNF-α at 24 h (Fig. 2.15A) although the induction of TNF-α by H37Ra IPG and IFN-γ was dose-dependent (Fig. 2.16B). Low levels of TNF-α were also detected at 4 h post-stimulation with H37Ra and in the presence of IFN-γ (Fig. 2.16A). However, LPS alone was a good inducer of TNF-α and the level of TNF-α was greatly increased in the presence of IFN-γ.

In Fig. 2.15B shows the levels of secreted IL-1β by J774A.1 macrophages in response to H37Ra (P- or A-type), *M. vaccae* (P- or A-type) or LPS with or without IFN-γ. None of the stimuli alone induced the production of IL-1β. However, with the exception of *M. vaccae* A-type IPG (not determined), all the stimuli including H37Ra IPG, *M. vaccae* IPG and LPS, could co-activate with IFN-γ to elaborate a substantial level of IL-1β. Whilst IFN-γ and LPS

stimulated the macrophages to produce 12.8 pg/ml IL-1β, macrophages activated with IFN-γ and H37Ra P- or IFN-γ and A-type IPG produced 34.1 pg/ml and 24.3 pg/ml IL-1β respectively and yet the levels of nitrite were lower than that induced by LPS (Fig. 2.15C).

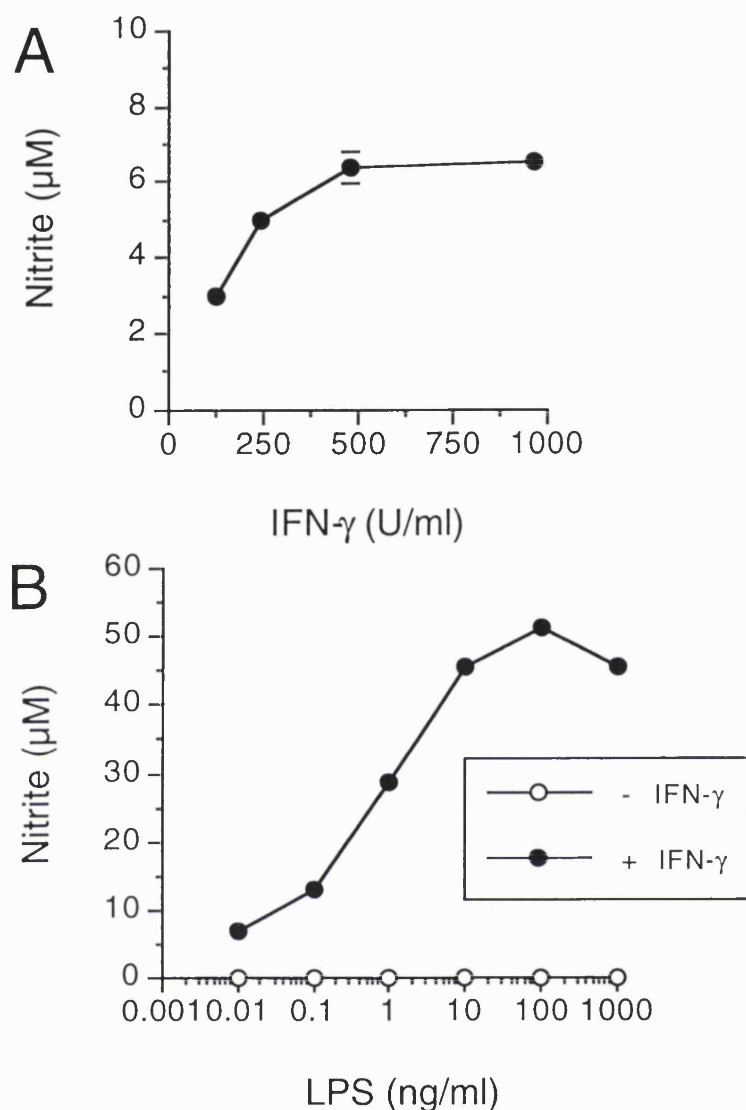



**Fig. 2.1. Dose-response of IFN-γ and LPS-induced nitrite production.** J774A.1 cells (1 x $10^5$) were treated with increasing concentrations of IFN-γ (A) or LPS with or without 240 U/ml IFN-γ (B) for 24 h. Nitrite concentrations were determined by using the Griess reaction. Nitrite was not detected in the supernatants of cells treated with medium or LPS alone. The results were expressed as means ± S.D. of triplicate cultures from one of four similar experiments. Where the error bars are not shown, the values fall within the symbols.

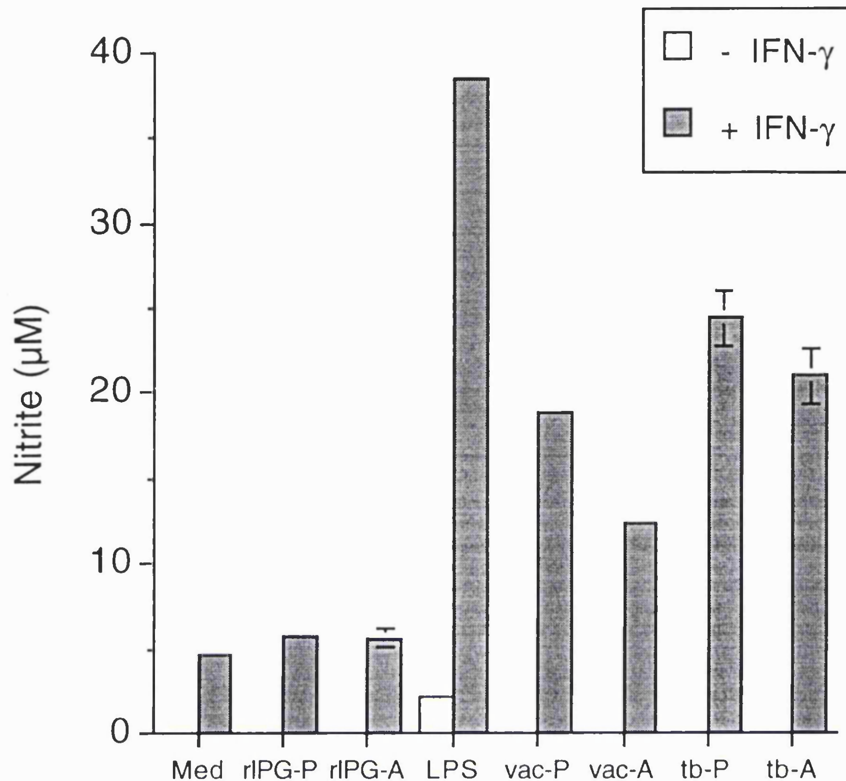



**Fig. 2.2. Synergistic effect of IFN-γ and mycobacterial IPG on the production of nitrite in J774A.1 cells.** J774A.1 cells (1 x $10^5$) were cultured with medium (Med) alone, rat liver IPGs (rIPG-P or A at 100 μg/ml each), 10 ng/ml of LPS, *M. vaccae* IPGs (vac-P or A at 1:100 each), *M. tuberculosis* H37Ra IPGs (tb-P or A at 1:100 each), with or without 240 U/ml IFN-γ for 24 h. Nitrite concentrations were determined by using the Griess reaction. With the exception of LPS, nitrite was not detected in the supernatants of cells incubated with medium alone or in the absence of IFN-γ. The results were expressed as means ± S.D. of triplicate cultures from one of four similar experiments. Where the error bars are not shown, the values fall within the bars.

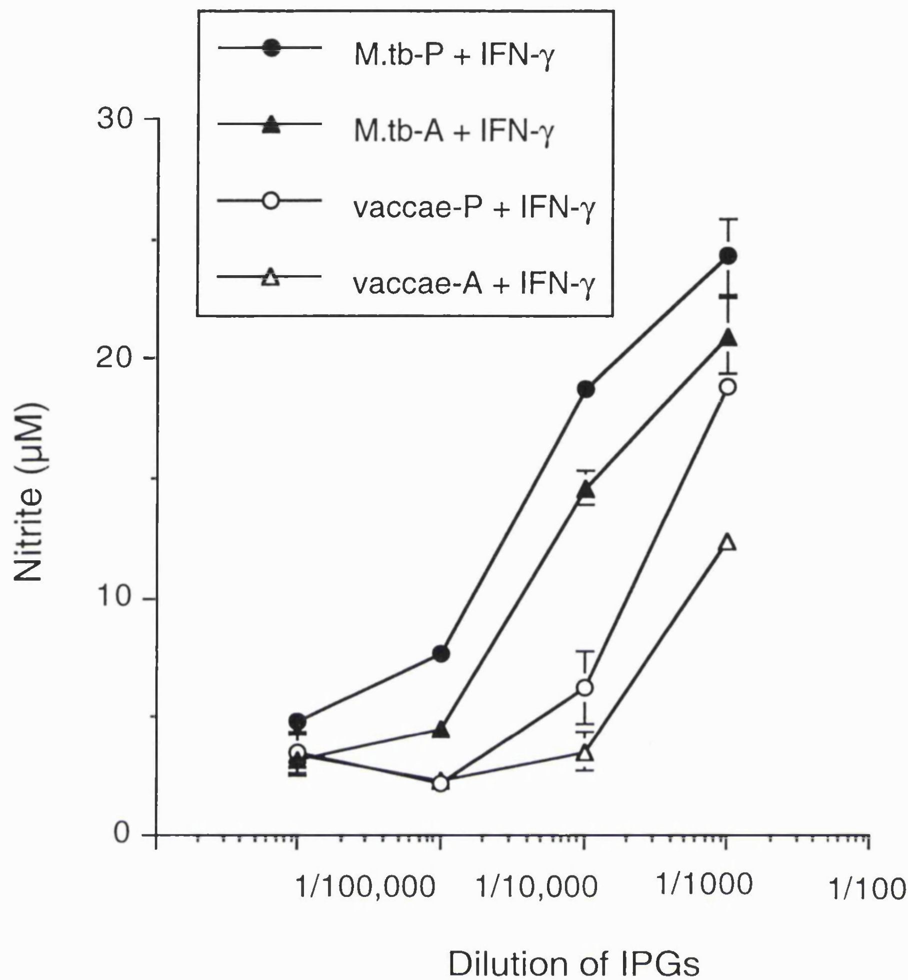



**Fig. 2.3. Dose-response of IFN-γ and mycobacterial IPG-induced nitrite production.** J774A.1 cells (1 x $10^5$) were cultured with medium alone, 240 U/ml IFN-γ alone or increasing concentrations of the avirulent strain *M. tuberculosis* H37Ra P- (M.tb-P) or A-type (M.tb-A) IPGs or *M.vaccae* P- (vac-P) or A-type (vac-A) IPGs in the presence of IFN-γ for 24 h. Nitrite concentrations were determined by using the Griess reaction. The values were expressed as means ± S.D. of triplicate cultures from one of two similar experiments. Nitrite was not detected in supernatants of J774A.1 cells treated with medium alone. Where the error bars are not shown, the values fall within the symbols.

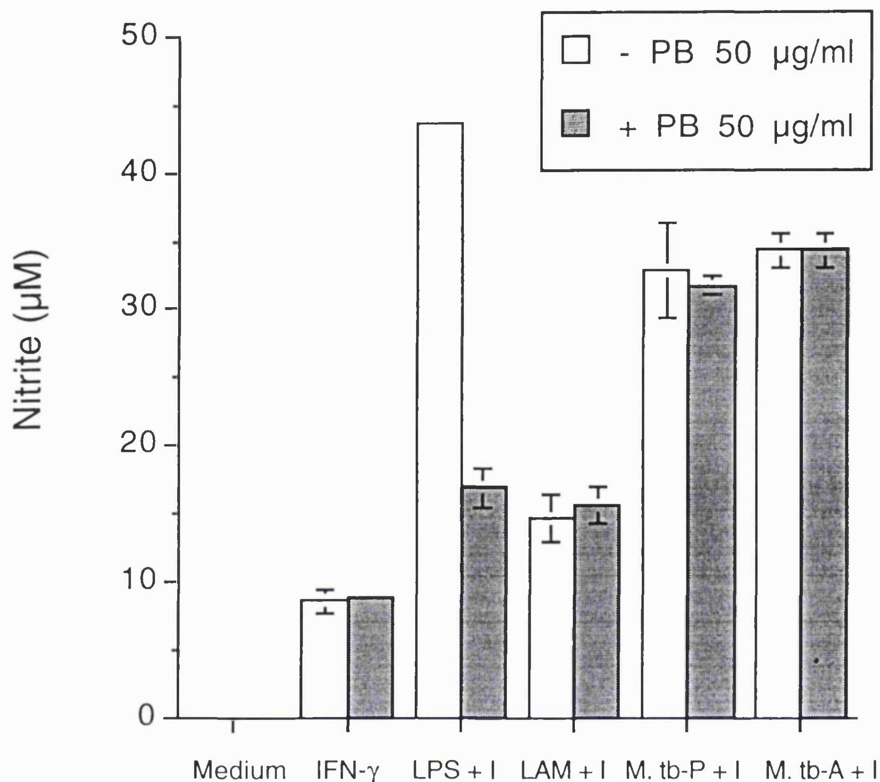



**Fig. 2.4. Effect of polymyxin B on IFN-γ and mycobacterial IPG-induced nitrite production.** J774A.1 cells (1 x $10^5$) were treated with medium alone, medium containing 50 μg/ml polymyxin B, 240 U/ml IFN-γ alone, IFN-γ and 20 ng/ml LPS, IFN-γ and 10 μg/ml *M. tuberculosis* LAM or IFN-γ and *M. tuberculosis* H37Ra (M.tb-P) P- or (M.tb-A) A-type IPG (1:100 each) with or without polymyxin B (PB) for 48 h. Nitrite concentrations were determined by using the Griess reaction. Nitrite was not detected in the supernatants of J774A.1 cells treated with medium or polymyxin B alone. The values were expressed as means ± S.D. of triplicate cultures from a single experiment. Where the error bars are not shown, the values fall within the bars..

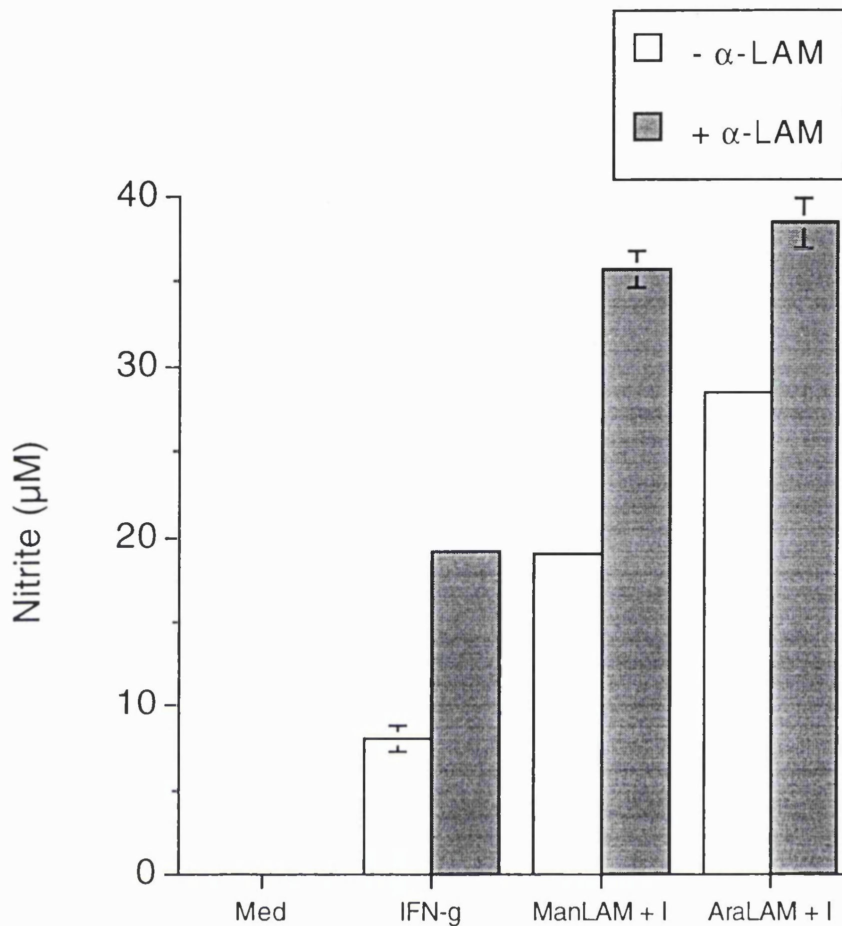



**Fig. 2.5. Effect of anti-LAM monoclonal antibody on ManLAM and AraLAM-induced nitrite production.** J774A.1 ($1 \times 10^5$) were treated with medium (Med) alone, IFN-γ (240 U/ml), ManLAM (10 μg/ml)+IFN-γ (I) or AraLAM (10 μg/ml)+IFN-γ (I) with or without 10 μg/ml of anti-LAM monoclonal antibody for 24 h. Nitrite concentrations were determined by using the Griess reaction. Nitrite was not detected in the supernatants of J774A.1 cells treated with medium or anti-LAM antibody alone. The values were expressed as means ± S.D. of triplcate cultures from a single experiment. Where the error bars are not shown, the values fall within the bars.

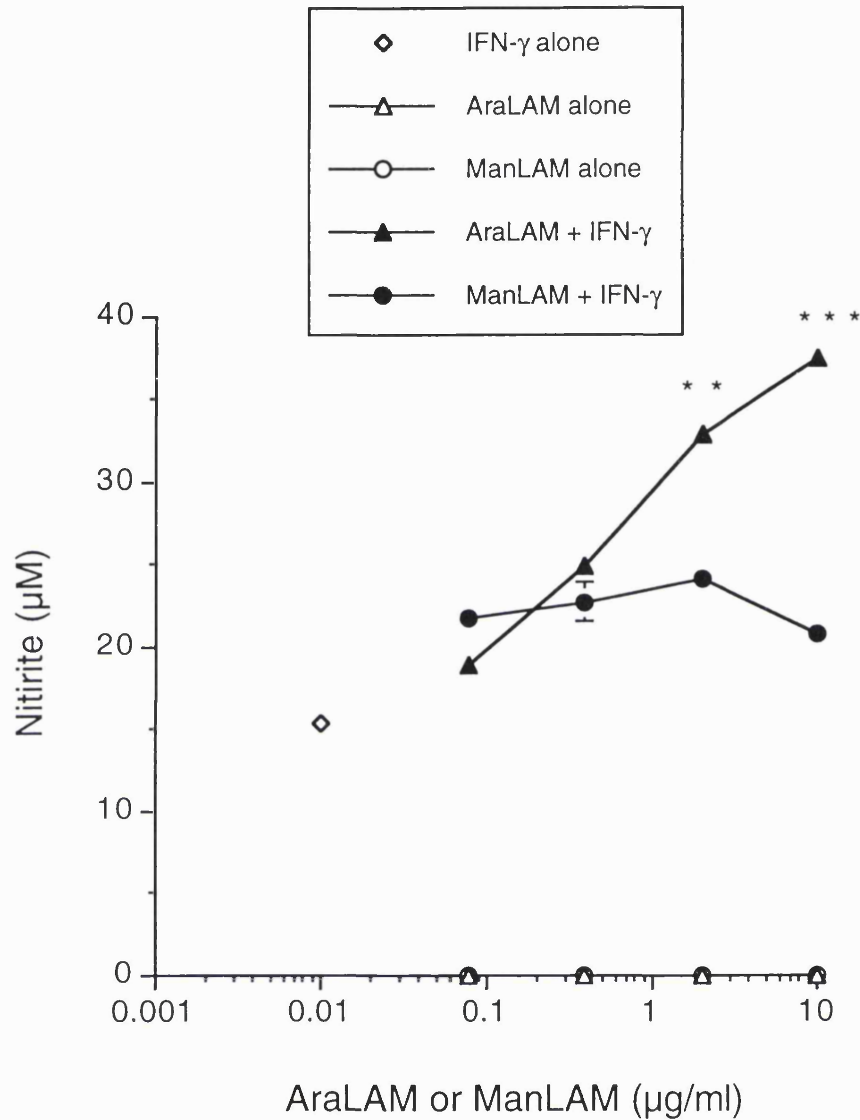



**Fig. 2.6. Differential effect of ManLAM and AraLAM-induced nitrite production in the presence of IFN-γ.** J774A.1 cells ($1 \times 10^5$) were cultured with medium alone, 240 U/ml IFN-γ, or with increasing concentrations of AraLAM or ManLAM and IFN-γ for 24 h. Nitrite concentrations were determined by using the Griess reaction. The values were expressed as means ± S.D. of triplicate cultures from a single experiment. Where the error bars are not shown, the values fall within the symbols. *** P=0.0005, ** P=0.0021 (Student's *t* test)

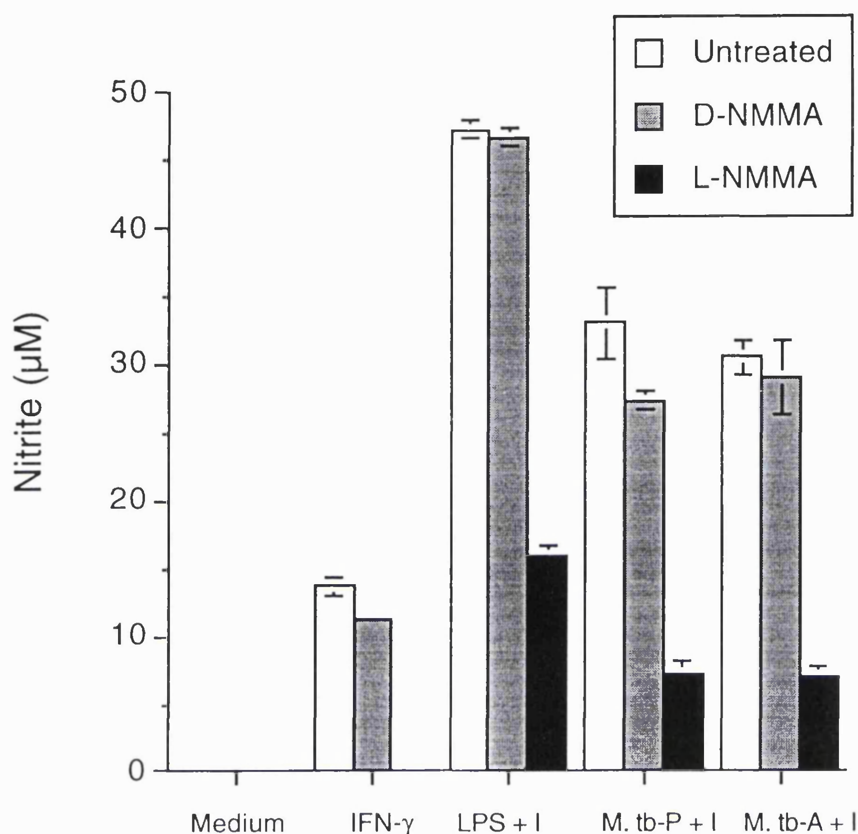



**Fig. 2.7. Effect of $N^G$-monomethyl-L-arginine acetate and $N^G$-monomethyl-D-arginine acetate on mycobacterial IPG-induced nitrite production.** J774A.1 cells (1 x $10^5$) were treated with medium alone, 0.5 M D-NMMA alone, 0.5 M L-NMMA alone, 240 U/ml IFN-γ with or without L-NMMA or D-NMMA (0.5 M each), or IFN-γ and 2 ng/ml of LPS, or IFN-γ with 100 μg/ml of *M. tuberculosis* H37Ra P- (M.tb-P) or 100 μg/ml A-type (M.tb-A) IPG with or without L-NMMA or D-NMMA for 24 h. Nitrite concentrations were determined by using the Griess reaction. Nitrite was not detected in the supernatants of cells stimulated with medium, D-NMMA, L-NMMA alone or IFN-γ+L-NMMA. The values were expressed as means ± S.D. of triplicate cultures from a single experiment. Where the error bars are not shown, the values fall within the bars.

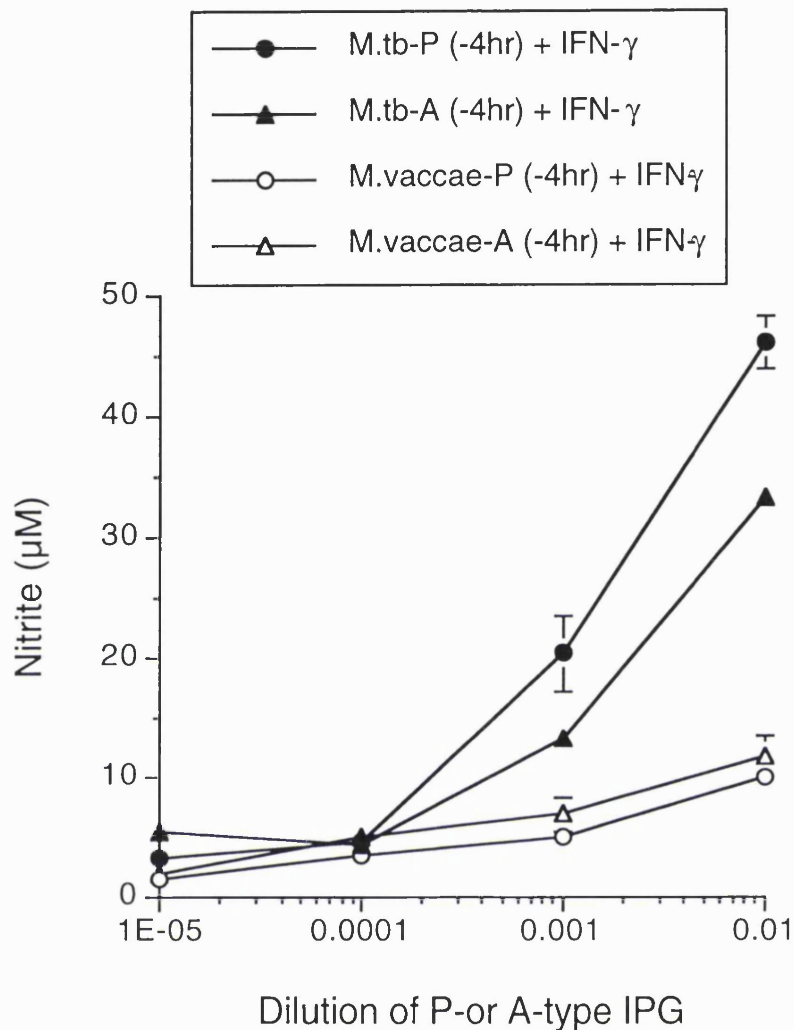



**Fig. 2.8. Short-time exposure of J774A.1 macrophages to mycobacterial IPG on IFN-γ-induced nitrite production.** J774A.1 cells (1 x $10^5$) were cultured with *M. tuberculosis* H37Ra P- or A-type IPG, or *M. vaccae* P- or A-type IPG for 4 h. This was followed by washing the cells twice with DMEM and subsequently stimulating with 240 U/ml IFN-γ for 24 h. Nitrite concentrations were determined by using the Griess reaction. The values were expressed as means ± S.D. of triplcate cultures from a single experiment. Where the error bars are not shown, the values fall within the symbols.

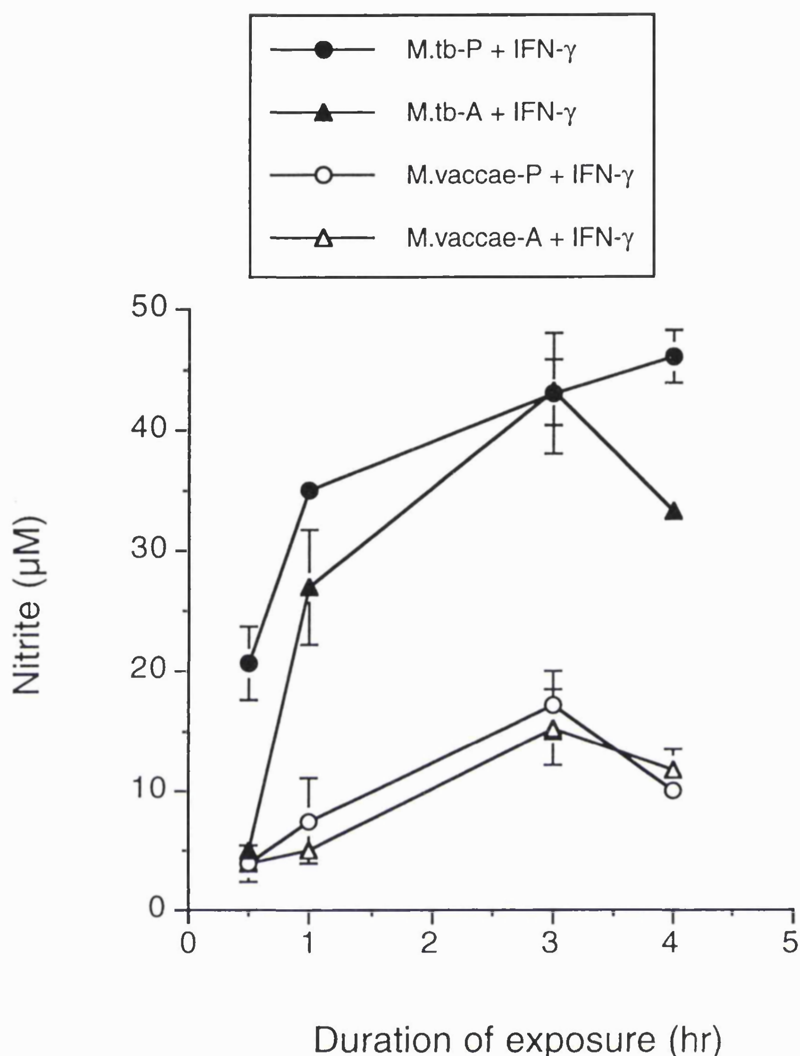



**Fig. 2.9. Time-dependent synergism of nitrite production by mycobacterial IPG and IFN-γ.** J774A.1 cells (1 x $10^5$) were stimulated with 1:100 dilution of *M. tuberculosis* H37Ra P- or A-type IPG or 1:100 dilution of *M. vaccae* P- or A-type IPG for half-an-hour, 1 h or 3 h. This was followed by washing the cells twice with warmed DMEM. The level of nitrite was measured by using the Griess reaction 24 hr after the addition of 240 U/ml IFN-γ. The results were expressed as means ± S.D. nitrite production in triplicate cultures from a single experiment. Where the error bars are not shown, the values fall within the symbols.

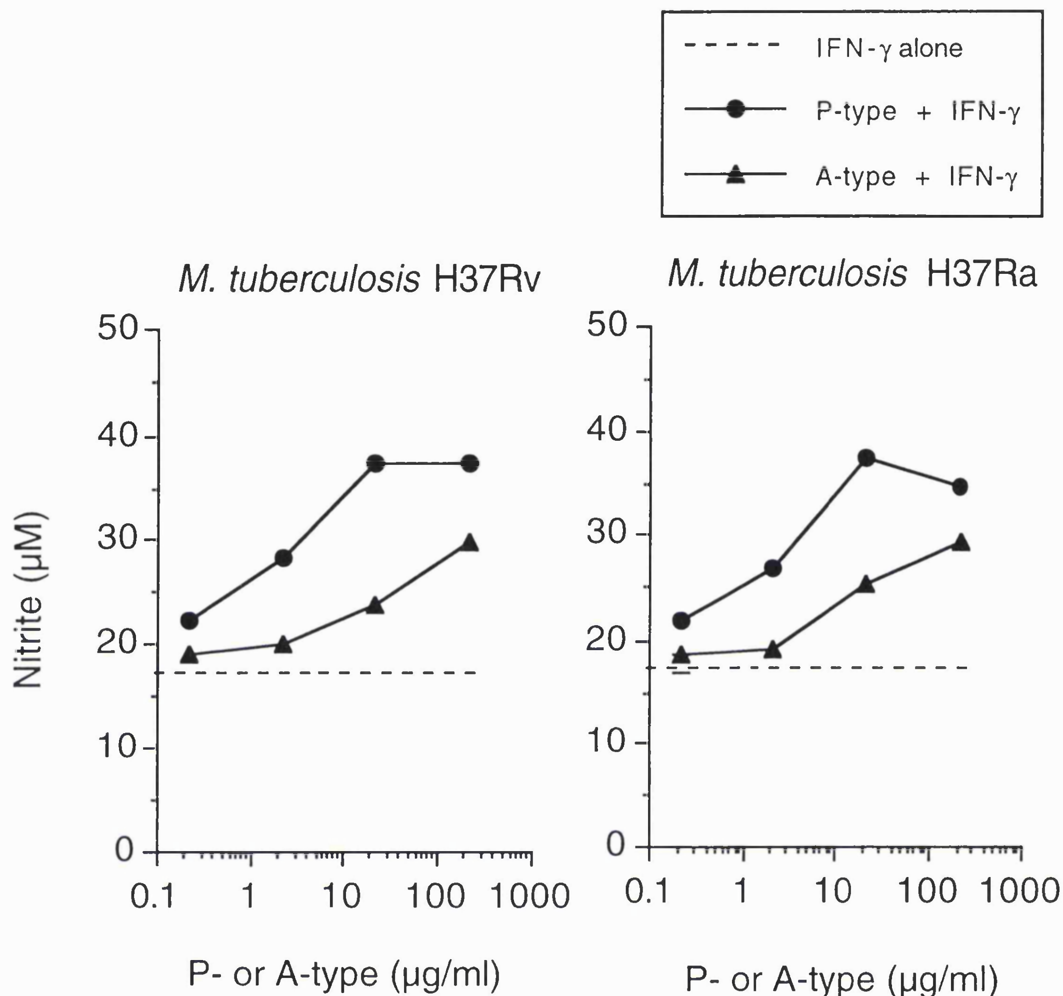



**Fig. 2.10. The level of nitrite generated after exposure to IFN-γ and IPG from H37Rv, compared to the levels generated by IFN-γ and IPG from H37Ra.** J774A.1 (1 x $10^5$) cells were incubated in medium alone, 200 U/ml IFN-γ alone, increasing concentrations of *M. tuberculosis* H37Rv P- or A-type and 200 U/ml IFN-γ, or increasing concentrations of *M. tuberculosis* H37Ra P- or A-type and IFN-γ for 24 h. Nitrite concentrations were determined by using the Griess reaction. The results were expressed as means ± S.D. of triplicate cultures from one of two similar experiments. Where the error bars are not shown, the values fall within the symbols.

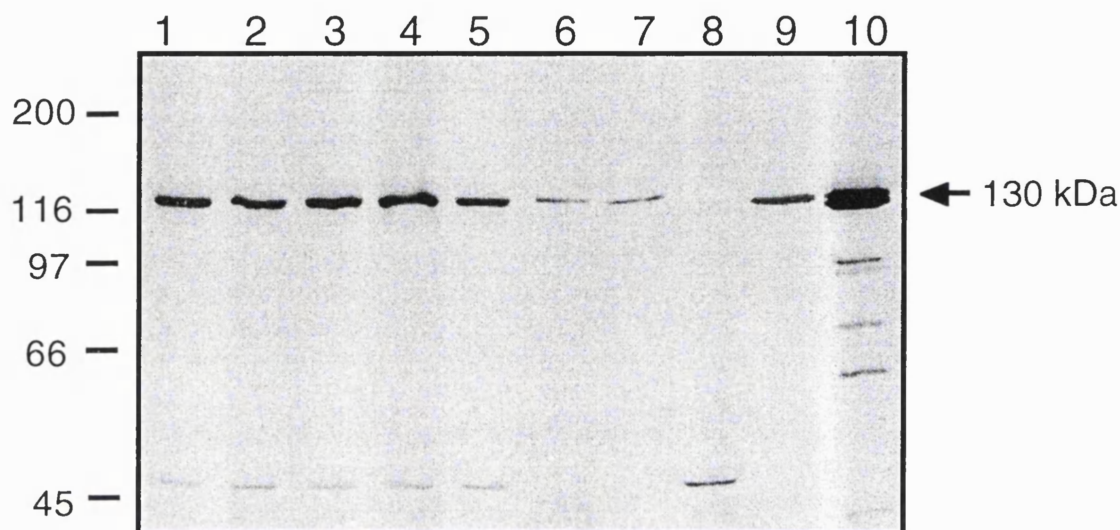



**Fig. 2.11. Western blot analysis of iNOS expression in J774A.1 cells.** Cell lysates containing the equal amount of total protein (100 μg per well) were separated on a 7% SDS-PAGE gel. After transfer to nitrocellulose, 0.4 μg/ml of anti-mouse iNOS monoclonal antibody was applied, followed by staining with 0.4 μg/ml of peroxidase-labelled secondary antibody and developed by enhanced chemiluminescence. J774A.1 cell lysates were prepared after the following treatments for 24 h: Lane 1, H37Rv P-type (110 μg/ml)+IFN-γ (200 U/ml); Lane 2, H37Rv A-type (110 μg/ml)+IFN-γ (200 U/ml); Lane 3, H37Ra P-type (110 μg/ml)+IFN-γ (200 U/ml); Lane 4, H37Ra A-type (110 μg/ml)+IFN-γ (200 U/ml); Lane 5, *M. vaccae* P-type (1:100)+IFN-γ (200 U/ml); Lane 6, *M. vaccae* A-type (1:100)+IFN-γ (200 U/ml); Lane 7, IFN-γ (200 U/ml) alone; Lane 8: medium alone; Lane 9, LPS (10 ng/ml)+IFN-γ (200 U/ml); Lane 10, positive control (total protein 10 μg/ml). Film exposure time: 5 min. Numbers on the left indicate molecular weight protein markers in kiloDalton. The diagram shown is a composite gel.

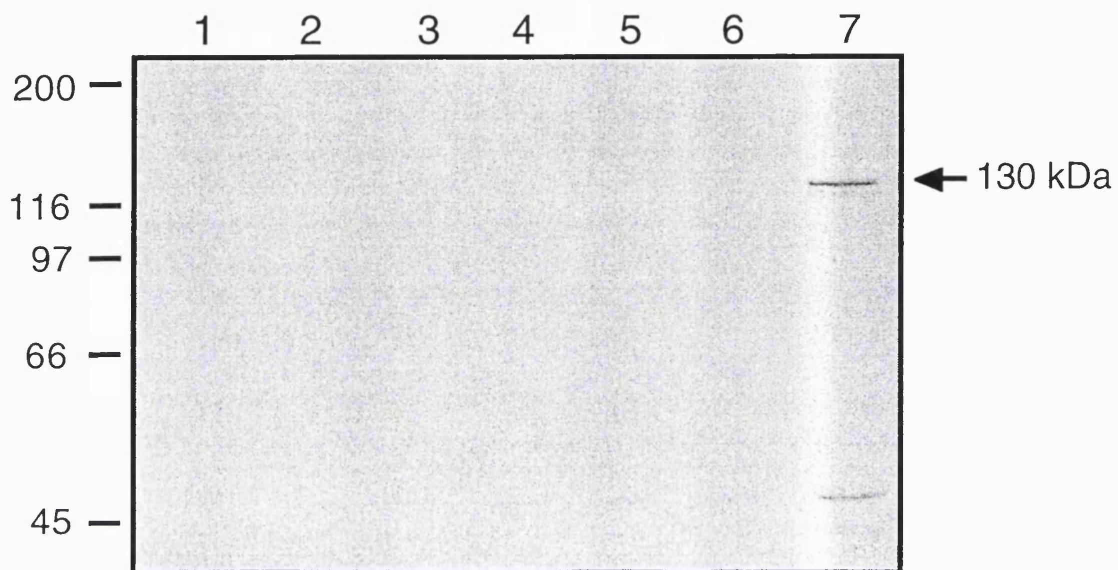



**Fig. 2.12. Western blot analysis of iNOS expression in J774A.1 cells.** Cell lysates (100 µg protein /well) were separated on a 7% SDS-PAGE gel. After transfer to nitrocellulose, 0.4 µg/ml of anti-mouse iNOS monoclonal antibody was applied, followed by staining with 0.4 µg/ml of peroxidase-labeled secondary antibody and developed by enhanced chemiluminescence. J774A.1 cell lysates were prepared after the following treatments for 24 h: Lane 1: 110 µg/ml H37Rv P-type; Lane 2: 110 µg/ml H37Rv A-type; Lane 3: 110µg/ml H37Ra P-type; Lane 4: 110 µg/ml H37Ra A-type; Lane 5: (1:100) *M. vaccae* P-type; Lane 6: (1:100) *M. vaccae* A-type; Lane 7: 10 ng/ml LPS. Film exposure time: 5 min. Numbers on the left indicate molecular weight protein markers in kiloDalton.

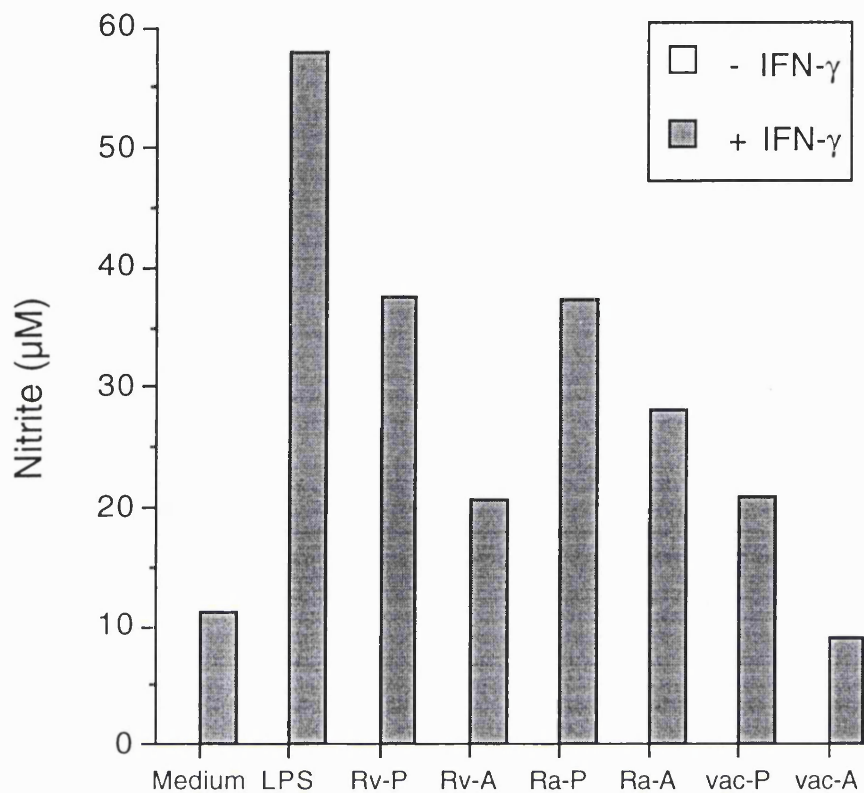



**Fig. 2.13. Stimulation of nitrite production in J774A.1 macrophages by IFN-γ and LPS, *M. tuberculosis* H37Rv IPG, *M. tuberculosis* H37Ra IPG or *M. vaccae* IPG.** J774A.1 cells (3 x $10^6$) were treated with 10 ng/ml LPS, 110 µg/ml H37Rv P- (Rv-P) or A-type (Rv-A) IPG, 100 µg/ml H37Ra P- (Ra-P) or A-type (Ra-A) IPG, (1:100) *M.vaccae* P- (vac-P) or A-type (vac-A) IPG with or without 200 U/ml IFN-γ for 24 h. Nitrite concentrations were determined by using the Griess reaction. Nitrite was not detected in the supernatants of cells incubated with medium alone or in the absence of IFN-γ. The values were expressed as micromolars of nitrite from a single culture.

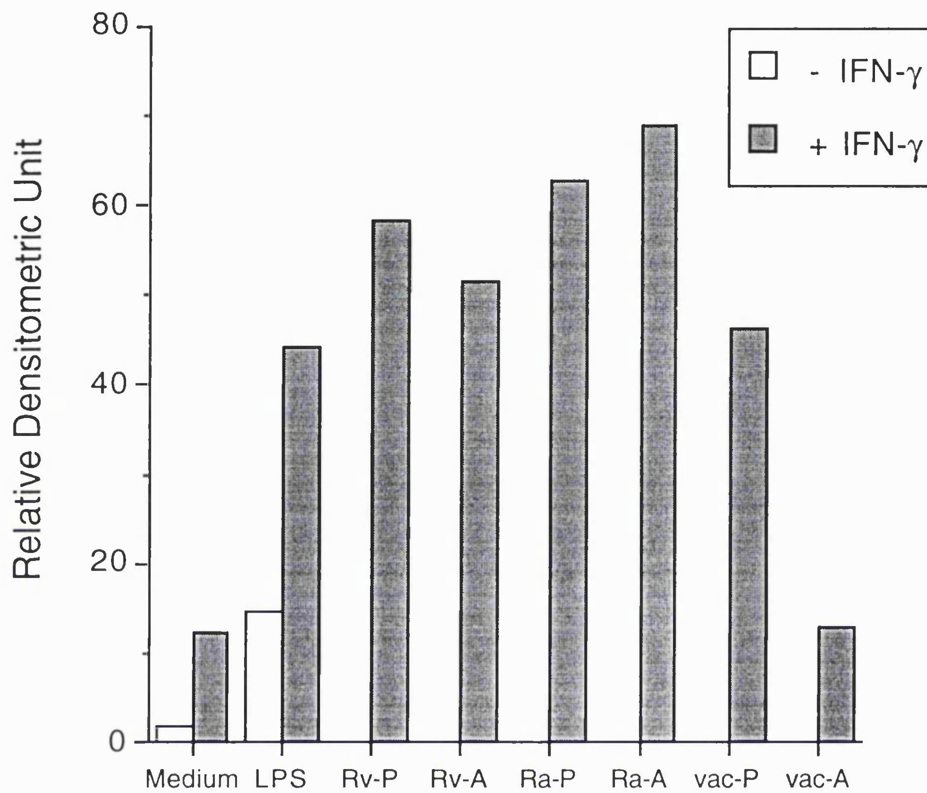



**Fig. 2.14. Induction of the inducible nitric oxide synthase expression in J774A.1 macrophages by IFN-γ and LPS, *M. tuberculosis* H37Rv IPG, *M. tuberculosis* H37Ra or *M. vaccae* IPG.** The intensity of the 130 kDa-band from each lane was determined by using the Image Gauge densitometric software. The level of iNOS protein was expressed as relative densitometric unit.

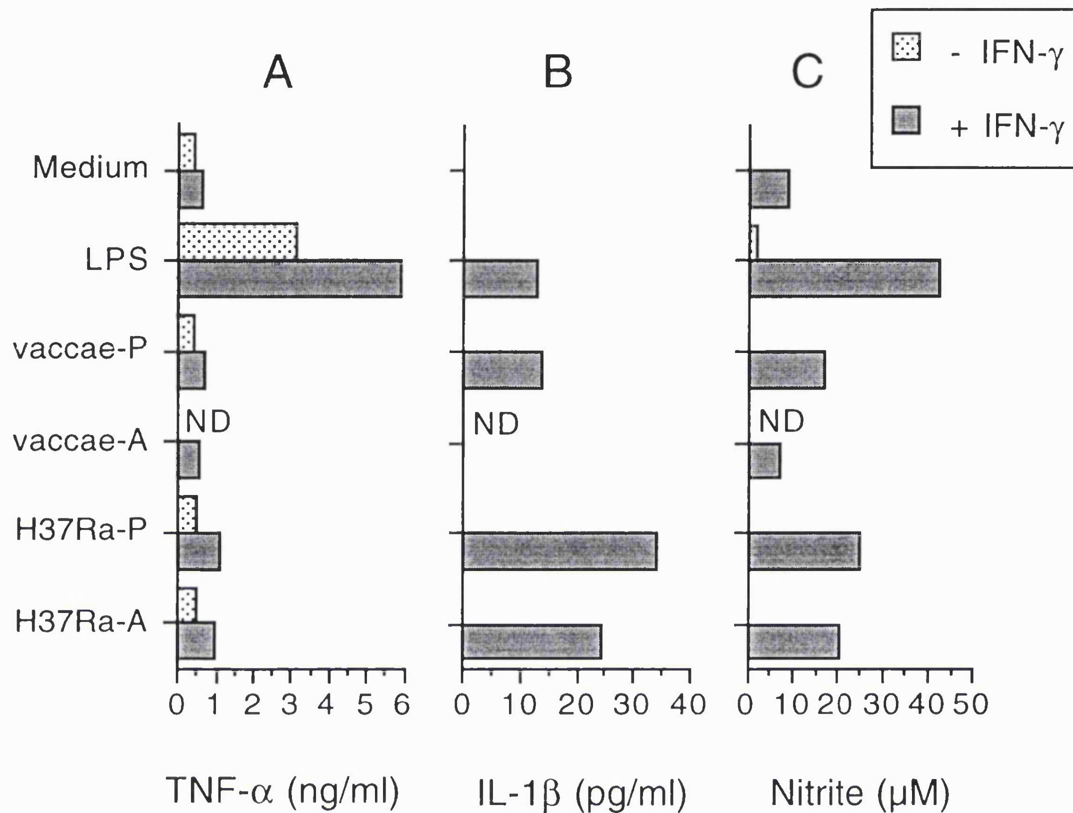



**Fig. 2.15. Production of TNF–α, IL-1β and nitrite in J774A.1 macrophages stimulated with IFN-γ and mycobacterial IPG.** J774A.1 cells (1 x $10^5$) were treated with medium alone, 10 ng/ml LPS, *M. vaccae* 1:100 dilution P- or A-type IPGs, or *M. tuberculosis* H37Ra 1:100 dilution P- or A-type IPGs with or without 200 U/ml of IFN-γ for 24 h. Supernatants were harvested and assayed for TNF-α (Fig. A) and IL-1β (Fig. B) by capture ELISA. Nitrite concentrations were determined using the Griess reaction (Fig. C). The results were expressed as the means of duplicate cultures from a single experiment. ND, not determined.

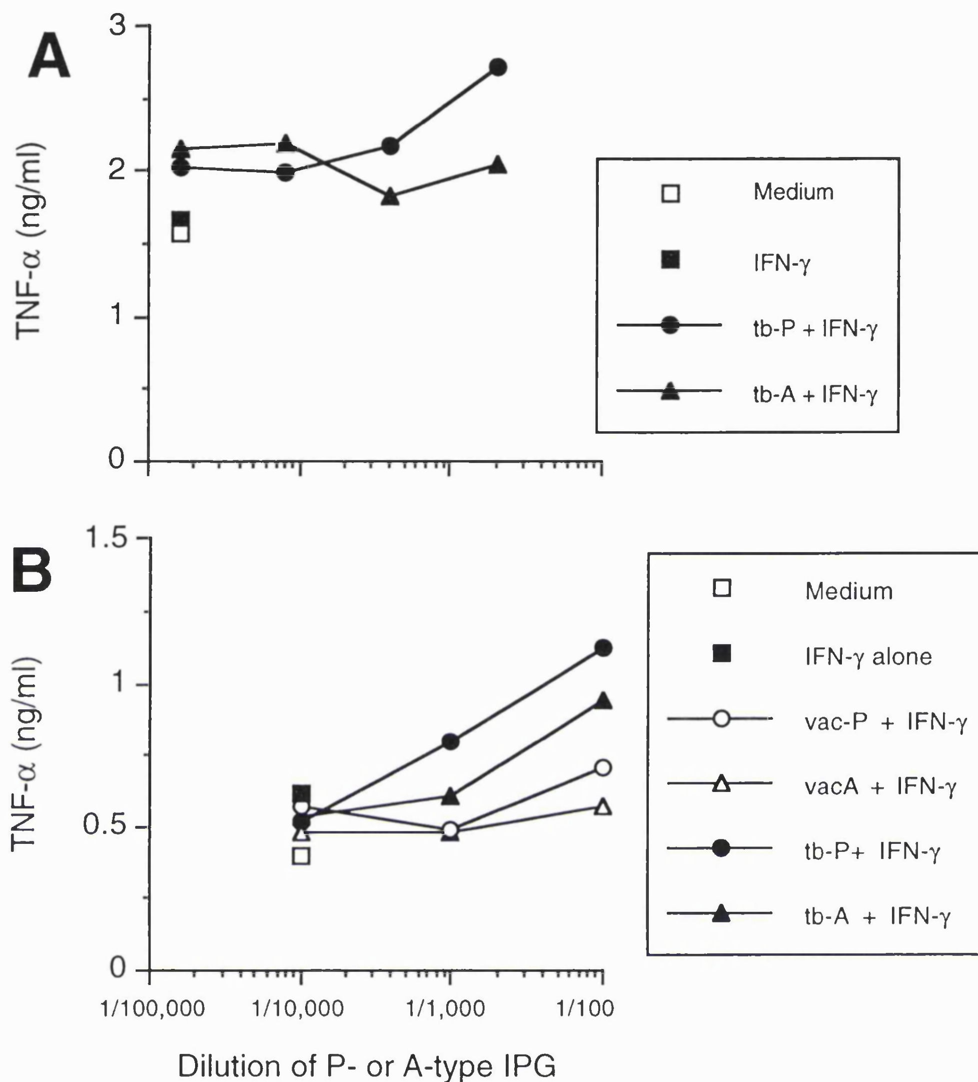



**Fig. 2.16. Dose-response of IFN-γ and mycobacterial IPG-induced TNF-α production.** J774A.1 cells (1 x $10^5$) were incubated with medium, IFN-γ (200 U/ml) alone, increasing concentrations of H37Ra (tb) P- or A-type) or *M. vaccae* (vac) P- or A-type IPGs simultaneously with 200 U/ml of IFN-γ for 4 h (A) or 24 h (B). Secreted TNF-α was measured by capture ELISA. The results were expressed as means of duplicate cultures from a single experiment. *M. vaccae* IPGs were not performed for the 4 h stimulation.

## 2.17 Discussion

The results have demonstrated that mycobacterial IPG can act in synergy with IFN-$\gamma$ to stimulate macrophages to produce NO. Mycobacterial IPG-induced NO production could not be due to the presence of contaminating LPS or LAM that may have been co-isolated with IPG. This is because, in contrast to LPS, mycobacterial IPG-induced NO production and mycobacterial-mediated coagulation of the *Limulus* lysate are unaffected by polymyxin B. Secondly, ManLAM from *M. tuberculosis*, irrespective of its virulence, and *M. bovis* BCG, in which the arabinofuranosyl terminal residues are extensively capped with $\alpha$-mannopyranosyl residues, are poor inducers of NO in murine macrophages (Adams, *et al.*, 1993, Anthony, *et al.*, 1994, Roach, *et al.*, 1995) and TNF-$\alpha$ in murine macrophages and human monocytes (Chatterjee, *et al.*, 1992b, Adams, *et al.*, 1993, Zhang and Rom, 1993, Roach, *et al.*, 1995). On the contrary, the arabinofuranosyl-terminated mycobacterial AraLAM from either a fast growing *Mycobacterium* sp. or *Mycobacterium smegmatis* is a potent inducer of NO and TNF-$\alpha$. Polymyxin B is a series of polypeptide antibiotics produced by *Bacillus polymyxa* and is widely used for inhibiting the activities of LPS. It does not, however, inhibit the effects of LAM on murine macrophages (Adams, *et al.*, 1993) or human macrophages (Moreno, *et al.*, 1989). Therefore, monoclonal antibodies against either ManLAM or AraLAM were used to determine if mycobacterial IPG-induced NO production was a result of LAM. Because the antibodies were not inhibitory to AraLAM or ManLAM-induced NO production in J774A.1 macrophage cells, their use in the nitrite assay was excluded. Since *M. tuberculosis* H37Rv and H37Ra contain ManLAM, and yet their IPGs were effective at inducing NO in macrophages, it is unlikely that the effects were due to ManLAM.

While mycobacterial IPG plus IFN-γ-stimulated NO production coincided with the induction of high levels of iNOS protein, NO production induced by IFN-γ and mycobacterial IPG was substantially inhibited by the L-arginine analogue, L-NMMA. These data strongly support that mycobacterial IPG plus IFN-γ stimulates the activation of iNOS protein to produce NO. It should be noted that as throughout this thesis comparison of relative activity of these material derived from *M. tuberculosis* and *M. vaccae* must be interpreted with caution, because quantification was not possible, and quantities compared were derived from equivalent weight of mycobacterium. Further investigations were also required to ascertain that the phenomenon was the result of a qualitative rather than a quantitative difference. It is likely that the yield obtained from the isolation of *M. vaccae* IPG may differ from the yield obtained from the isolation of *M. tuberculosis* IPG. Thus, similar dilutions of those mycobacterial IPGs might not correspond to equal amounts of IPG used in the stimulation of J774A.1 cells.

It is not clear how mycobacterial IPGs co-activate with IFN-γ to induce the expression of iNOS protein. Previous studies by Vodovotz *et al.* (1993) have shown that IFN-γ alone can increase the iNOS mRNA. Furthermore, IFN-γ can synergise with LPS to enhance iNOS gene transcription in a RAW264.7 macrophage cell line (Xie, *et al.*, 1992). Similarly, mycobacterial IPG may act in synergy with IFN-γ to accelerate the transcription of the iNOS gene resulting in the accumulation of iNOS protein.

It is well recognised that activation of macrophages by IFN-γ and microbial products is mediated primarily by TNF-α. Its release early in an inflammatory response has profound effects on macrophage functions including the induction of iNOS expression in murine macrophages (Drapier, *et al.*, 1988). The levels of TNF-α induced by J774A.1 cells in response to mycobacterial

IPG and IFN-γ were significantly lower than those induced by LPS and IFN-γ. For reasons that are still unclear, these low levels of TNF-α appeared not to diminish the high expression of iNOS protein induced by mycobacterial IPG and IFN-γ. Moreover, the levels of TNF-α assayed at 4 h and 24 h were similar, suggesting that these low levels were not attributed to a delay in TNF-α synthesis. Previous studies have shown that membrane-bound TNF-α retains its biological function and can exert TNF-mediated cytotoxicity in a TNF-α-sensitive cell line (Decker, *et al.*, 1987, Espevik and Nissen-Meyer, 1987). Therefore, the rôle of membrane-bound or cell-associated TNF-α in the induction of iNOS expression and NO synthesis cannot be completely excluded. It is possible that membrane-bound TNF-α on macrophages may synergise with IFN-γ to induce NO production in a cell contact dependent manner. As the release of soluble TNF-α by J774A.1 cells stimulated with LPS was not impaired, activation of NO production is not likely to be caused by membrane-bound TNF-α. It is well-known that metabolites of arachidonic acids such as prostaglandin (PG) $E_2$ can inhibit proinflammatory cytokines release by macrophages *in vitro*. Fieren *et al.* (1992) have shown that $PGE_2$ inhibits TNF-α release in human macrophages. The synthesis of $PGE_2$ is associated with an increase in intracellular cAMP which inhibits TNF-α mRNA expression and protein secretion (Kunkel, *et al.*, 1986, Kunkel, *et al.*, 1988, Tannenbaum and Hamilton, 1989). Furthermore, Strassmann *et al.* (1994) have shown that $PGE_2$ stimulates IL-10 production in LPS-stimulated murine macrophages which results in the inhibition of TNF-α release. However, the presence of IL-10 does not affect the release of nitric oxide by macrophages in response to LPS. Likewise, mycobacterial IPGs and IFN-γ may stimulate J774A.1 cells to release IL-10 which blocks TNF-α but not NO synthesis.

In contrast to TNF-α, the levels of IL-1β induced by *M. tuberculosis* IPGs and IFN-γ were higher than those induced by LPS and IFN-γ. There are two lines of evidence showing that human monocytes can be stimulated to elaborate IL-1β instead of TNF-α. Lee and Rikihisa (1996) have shown that carbohydrate components from a Gram-negative obligate intracellular bacterium, *Ehrlichia chaffeensis*, stimulate IL-1β release but not TNF-α in human monocytes. Similarly, pneumococcal cell wall components have also been shown to the induce production of IL-1β but not TNF-α in human monocytes (Riesenfeld-Orn, *et al.*, 1989). These have led to the speculation that Gram-positive and Gram-negative bacteria may contain IPG or IPG-like molecules which have the ability to stimulate macrophage activation.

# Chapter Three

## Putative Mycobacterial IPG Receptors

## 3.1 Innate immune recognition of microbial antigens

One of the attributes of the immune system is the ability to recognise micro-organisms and their associated components. All multicellular organisms have developed various defence mechanisms to protect themselves by destroying invading pathogens and neutralising their virulent factors. These ancient defence mechanisms are known as the innate immune system which uses germlined encoded receptors to recognise microbial pathogens.

In 1989 Charles Janeway, Jr. proposed that the evolution of innate immunity originated from the recognition of invariant constituents of the microbial pathogens, which are collectively known as **p**athogen-**a**ssociated **m**olecular **p**atterns (PAMPs). It is believed that these PAMPs are essential for the survival of the microbes and therefore are not subjected to variability because mutations affecting these structures are lethal to the microbes. For example, LPS shared by all Gram-negative bacteria is recognised by CD14 found predominantly on macrophages. Recognition of the conserved lipid A of LPS by CD14 implies the presence of Gram-negative bacteria. It is increasingly clear that PAMPs are widely found in many micro-organisms, and they include peptidoglycan and teichoic acids in Gram-positive bacteria, lipoarabinomannan in mycobacteria and glucans in fungi. The host organisms have, however, developed a set of receptors which recognise the PAMPs and are referred to as pattern recognition receptors (Janeway, 1992, Medzhitov and Janeway, 1997).

There is growing evidence that carbohydrates or carbohydrate-containing molecules of Gram-positive, Gram-negative bacteria and mycobacteria have the capacity to stimulate cellular signalling and cytokine synthesis. These

molecules stimulate by binding to the CD14 present on the surface of host cells. A number of reports have shown that LAM from mycobacteria induces cell activation via CD14. Zhang *et al.* (1993) have found that LAM-induced release of TNF-$\alpha$ and IL-1$\beta$ in human blood is inhibited by CD14 monoclonal antibodies. This is confirmed by a report showing that LAM increases the up-regulation of IgM in a murine pre-B cell line (70Z/3) previously transfected with human CD14 suggesting the requirement for membrane CD14 in response to LAM (Pugin, *et al.*, 1994). There is also evidence that CD14 is required for the uptake of *M. tuberculosis* by human microglia (Peterson, *et al.*, 1995).

## 3.2 CD14

CD14 is a membrane glycoprotein originally identified as a myeloid differentiation antigen present in mature monocytes (Hogg and Horton, 1986, Ziegler-Heitbrock and Ulevitch, 1993). Hence, it is widely used as a marker for monocytes. Peripheral blood monocytes have an estimated 50,000 molecules of CD14 expressed in each cell (Van Voorhis, *et al.*, 1983), and polymorphonuclear neutrophils have a tenth of this number. The human gene for CD14 is located on chromosome 5 in a region encoding several genes for growth factors and growth factor receptors, such as IL-3, GM-CSF and PDGF (Goyert, *et al.*, 1990). The glycoprotein is anchored on the outer surface of the plasma membrane via a glycosylphosphatidylinositol (GPI) (Haziot, *et al.*, 1988). The fatty acyl chain forms the lipid bilayer, and the terminal phosphoethanolamine unit of the GPI is linked to the carboxyl-terminus of the protein. Like many other GPI-anchored proteins, they lack a

transmembrane domain and cytoplasmic tail. Thus, GPI-anchored proteins cannot be involved in conventional intracellular signalling.

CD14 is found in two distinct forms: a 54 kDa glycoprotein present as a GPI-anchored membrane protein on myeloid cells and a soluble serum protein lacking the GPI anchor. CD14 binds to LPS from Gram-negative bacteria (Tobias, *et al.*, 1993), cell components from Gram-positive bacteria and LAM from mycobacteria (Pugin, *et al.*, 1994). The evidence comes from studies showing that antibodies to CD14 block LPS-induced activation of leukocytes in whole blood. LPS-induced TNF-$\alpha$ release by monocytes was blocked by anti-CD14 antibodies (Wright, *et al.*, 1990). This is in line with the report showing that transfection of CD14-negative cells with CD14 greatly enhanced the sensitivity to LPS (Lee, *et al.*, 1992, Golenbock, *et al.*, 1993). Studies have shown that CD14 is also involved in the induction of various pro-inflammatory cytokines, including IL-1, IL-6 and IL-8 in human monocytes (Couturier, *et al.*, 1991, Heumann, *et al.*, 1992, Dentener, *et al.*, 1993).

Kirkland *et al.* (1993) have examined the binding of LPS to CD14 in transfected CHO-K1 fibroblasts expressing CD14 and a monocytic cell line THP-1. LPS binding was shown to be rapid and independent of temperature between 10°C and 37°C. The apparent dissociation constant ($K_D$) for LPS is approximately $3 \times 10^{-8}$ M. The $K_D$ for binding of IL-1 to human type 1 IL-1 receptor is in the range of $5 \times 10^{-9}$ to $5 \times 10^{-10}$ M. Thus, the apparent receptor affinity of LPS for cell-bound CD14 is within the lower range of the cytokine receptors. However, it is high enough for agonist-receptor interactions. The stoichiometry of binding of LPS to membrane CD14 is not known but the stoichiometry of binding of LPS to soluble CD14 is 1:1.

## 3.3 Regulation of CD14 expression

The expression of CD14 is upregulated during monocyte maturation. For instance, the expression of CD14 in monocytes is enhanced by 1,25-dihydroxy-vitamin $D_3$. This has led to the suggestion that the expression of CD14 is determined by gene transcription. The 5'-upstream region of the CD14 gene contains binding sites for the transcription factor Sp1 (a member of the zinc-finger group of transcription factors) which is a major regulatory site for this gene (Martin, *et al.*, 1994, Zhang, *et al.*, 1994). There is evidence of differential expression of CD14 among resident macrophage populations. For example, peritoneal macrophages express high levels of CD14, alveolar macrophages express moderate levels, and the intestinal macrophages are deficient in CD14 expression (Andreesen, *et al.*, 1990, Ziegler-Heitbrock and Ulevitch, 1993, Grimm, *et al.*, 1995).

The effect of LPS on the expression of CD14 remains controversial. A number of reports have shown that LPS stimulation of whole blood (Marchant, *et al.*, 1992), peripheral blood monocytes (Brugger, *et al.*, 1991, Birkenmaier, *et al.*, 1992), alveolar macrophages (Kielian, *et al.*, 1995) and monocytic cell lines (Ikewaki, *et al.*, 1993, Katz, *et al.*, 1996) causes an increase in the expression on CD14 on the cell surface. There are, however, reports which show that LPS reduces CD14 expression in monocytes (Bazil and Strominger, 1991) and macrophages (Wright, 1991). This may reflect the differences in the experimental variables such as the source of LPS, LPS concentration, cell type, state of monocycte differentiation and its contamination with biologically active proteins. In a recent study, some of the parameters have been examined using human monocytes and monocyte-derived macrophages (Landmann, *et al.*, 1996). These cells were incubated

with varying concentrations of LPS for different periods of time, and the membrane-bound CD14 (mCD14), soluble CD14 (sCD14) and CD14 mRNA measured. During the first 3 h of LPS exposure there were no changes in all three types of CD14. Between 6 and 15 h, there was a small decrease in mCD14 and in CD14 mRNA, but the sCD14 transiently increased. This is similar to the decrease in CD14 expression reported by Bazil and Strominger (1991). With prolonged exposure to LPS for 48 h, there was an increase in CD14 mRNA and mCD14. It is known that intraperitoneal injection of LPS can result in the up-regulation of mCD14 in various tissues, such as heart, lung, liver and uterus (Fearns, *et al.*, 1995). It has been shown that both sCD14 and mCD14 bind to bacteria (Jack, *et al.*, 1995). Thus, it has been suggested that the up-regulation of mCD14 is necessary for the clearance of Gram-negative bacteria.

A number of reports have suggested that cytokines such as IL-1β, IL-2, IL-3, IL-5, IL-6, TNF-α, GM-CSF and TGF-β have no effects on the expression of CD14 (Landmann, *et al.*, 1990, Landmann, *et al.*, 1991). However, other workers have reported that IL-6 and TNF-α can up-regulate the expression of CD14. Two cytokines have been shown to down-regulate the expression of mCD14: IL-4 (Launer, *et al.*, 1990, Ruppert, *et al.*, 1991) and IL-13 (Cosentino, *et al.*, 1995). These cytokines share structural and functional characteristics and are primarily produced by Th2 cells. IL-13 decreases the pro-inflammatory activity of macrophages (de Waal Malefyt, *et al.*, 1993), and has been shown to inhibit the expression of CD14 on human monocytes in a dose-dependent manner. The down-regulation of mCD14 by IL-13 is not due to the shedding of the surface CD14 but rather, IL-13 inhibits the transcription of the CD14 gene (Cosentino, *et al.*, 1995).

## 3.4 Soluble CD14

Soluble CD14 (sCD14) was first identified in a promyelocytic HL-60 cell line culture medium which contained a factor which prevented the binding of anti-CD14 antibody to myeloid cells (Maliszewski, *et al.*, 1985). sCD14 has also been found in serum and urine, and in the former is present at relatively high concentrations, in the range of 3–6 μg/ml (Bazil, *et al.*, 1986, Grunwald, *et al.*, 1994). sCD14 is shed from monocytes following stimulation with LPS and IFN-γ *in vitro*. However, it is not clear if this is the major physiological pathway by which sCD14 is generated. There are several isoforms of sCD14 which differ in their molecular mass. They include 43, 46, 50 and 53 kDa isoforms (Stelter, *et al.*, 1996). The release of sCD14 may be a result of the action of proteases or to the action of a phospholipase (Bufler, *et al.*, 1995). The precise rôle of sCD14 in the blood is not currently known but a few ideas have been proposed. There has been the suggestion that sCD14 may activate cells (e.g. endothelial and epithelial cells) which do not normally express GPI-anchored CD14 on their cell surfaces (Frey, *et al.*, 1992, Arditi, *et al.*, 1993, Haziot, *et al.*, 1993, Pugin, *et al.*, 1993, Noel, *et al.*, 1995). This is confirmed by reports that sCD14 can confer sensitivity to CD14-negative vascular smooth muscle cells (Loppnow, *et al.*, 1995) as well as monocytes derived from patients with paroxysmal nocturnal haemaglobinuria (Golenbock, *et al.*, 1995), which are deficient in GPI-anchored proteins including CD14. sCD14 is also found to increase in sera of patients with Gram-negative shock and the increased levels are associated with high mortality. Thus, neutralising antibodies against CD14 are protective against LPS-induced septic shock in primates (Leturcq, *et al.*, 1996).

Although sCD14 mediates LPS-induced activation of CD14-negative cells, sCD14 can antagonise LPS-induced activation of CD14-positive cells by competing with membrane CD14 for LPS binding. It has been found that sCD14 inhibits the LPS-induced oxidative burst in cultured human monocytes (Schutt, *et al.*, 1992), and TNF-α production in whole blood (Haziot, *et al.*, 1994). This is further supported by the observation that recombinant sCD14 administered to mice can protect against LPS-induced lethality and TNF-α release (Haziot, *et al.*, 1995) .

## 3.5 The importance of Toll in innate immunity

There is growing evidence that the Toll (also called Toll-like receptor) family of signalling receptors play a crucial rôle in *Drosophila* and mammalian host defence (Knopp and Medzhitov, 1999, Modlin, *et al.*, 1999). For instance, the *toll* genes which control the dorsoventral pattern formation during embryonic development in *Drosophila* (Belvin and Anderson, 1996) are involved in the induction of several genes encoding antimicrobial peptides (Hoffmann and Reichhart, 1997). The Toll proteins or the Toll-like receptors are known to activate intracellular signalling via the transcriptional factor NF (**n**uclear **f**actor)-κB leading to the induction of a variety of genes (Belvin and Anderson, 1996). Several mammalian homologues of *Drosophila* Toll protein have been identified in humans and are referred to as **T**oll-**l**ike **r**eceptors (TLR). Five such proteins have been identified, which include TLR 1, 2, 3, 4 and 5 (Medzhitov, *et al.*, 1997, Rock, *et al.*, 1998). They are homologous proteins that are characterised by an extracellular leucine-rich repeat which forms the ectodomain and a cytoplasmic domain which is responsible for signal transduction. As the cytoplasmic domain of the Toll-

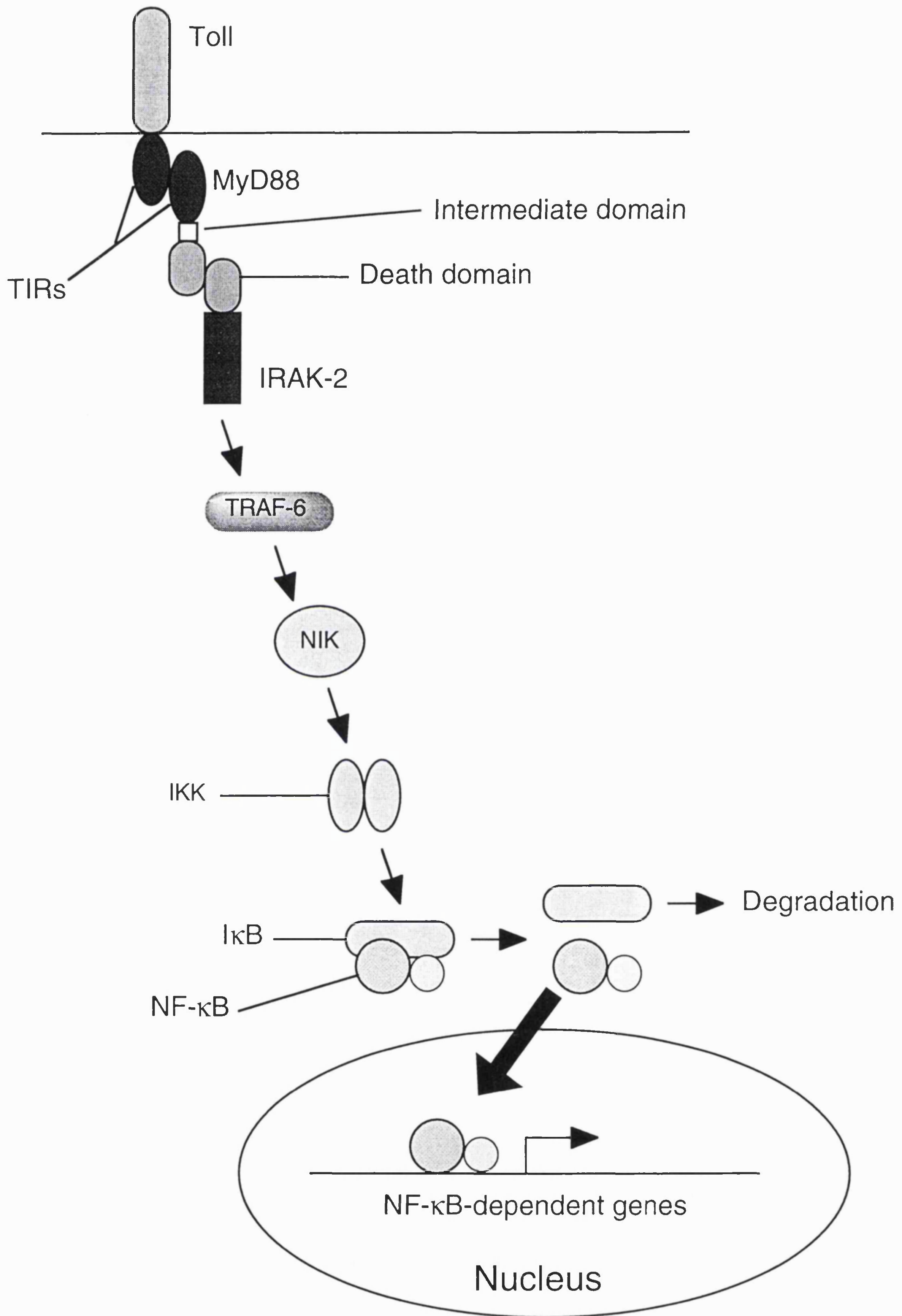
like receptor family is homologous to the cytoplasmic domain of the IL-1 receptor, they are collectively known as the Toll/IL-1R homology (TIR) domain.

Since CD14 is devoid of an intracellular domain, it cannot elicit intracellular events. Therefore, a transmembrane co-receptor has been postulated in LPS responsive cells. Recently, a protein responsible for transmitting signals from LPS has been found from the study of two mouse strains (C3H/HeJ and C57BL/10ScCr) strain known to be defective in response to LPS. Poltorak *et al.* (1998) have found that these two mouse strains had mutations of the toll-like receptor-4 (*Tlr-4* ) gene. The C3H/HeJ strain had a single point mutation at amino acid 712 (histidine to proline) and C57BL/10ScCr lacked the entire Tlr-4 transcript, which resulted in hyporesponsiveness to LPS. This is confirmed by a report showing that expression of human TLR-4 confers responsiveness to LPS-mediated NF-κB activation (Chow, *et al.*, 1999).

Medzhitov *et al.* (1997) have demonstrated that a constitutive active mutant of the Toll-like receptor 4 protein induces the expression of cytokines IL-1, IL-6 and IL-8, and co-stimulatory molecules. A number of reports have shown that expression of Toll-like receptor 2 (TLR2) in a non-myeloid cell line (**h**uman **e**mbryonic **k**idney, HEK 293) confers responsiveness to LPS (Kirschning, *et al.*, 1998), peptidoglycan and lipoteichoic acids (Schwandner, *et al.*, 1999). Co-expression of CD14 and TLR2 in 293 cells result in a dramatic increase in LPS-mediated signalling which suggests TLR2 is a signal transducer for LPS. TLR2 is expressed in peripheral blood leukocytes, spleen, and lung. In a recent report, TLR2 has been detected in a number of monocytic cell lines (THP-1, U937, and Mono Mac-6 cells) (Kirschning, *et al.*, 1998).

Activation of the Toll or TLR is initiated through binding of PAMP ligands resulting in the recruitment of a cytoplasmic adaptor protein, myeloid differentiation factor 88 (MyD88) (Fig. 3.1). MyD88 is a transcriptional factor which contains three functional domains: amino-terminal death domain, an intermediate domain and a carboxyl-terminal TIR domain. Overexpression of full-length MyD88 protein or a mutant protein containing only the death domain and intermediate domain leads to activation of NF-κB (Medzhitov, *et al.*, 1998). MyD88 is stimulated in response to IL-1 and IL-18. This is confirmed by a report that MyD88-deficient mice are defective in signalling mediated through IL-1R and IL-18R (Adachi, *et al.*, 1998).

The carboxyl-terminal TIR domain of MyD88 interacts with the cytoplasmic domain of Toll whereas the amino-terminal death domain of MyD88 binds and recruits a down-stream target containing the death domain, **IL-1R-a**ssociated **k**inase-2 (IRAK-2). IRAK-2 is a member of the serine-threonine kinases family which participate in signalling pathways during an innate immune response. IRAK is found in plants, invertebrates and vertebrates. IRAK is recruited to the Toll/MyD88 receptor complex, which becomes autophosphorylated. The autophosphorylated form dissociates and binds to a **TNFR-a**ssociated **f**actor (TRAF) family adaptor, TRAF-6. TRAF-6 in turn binds to a member of the protein kinase family, **N**F-κB-**i**nducing **k**inase (NIK). NIK phosphorylates another protein kinase, **I**κB **k**inase (IKK), which in turn phosphorylates IκB. This is followed by the ubiquination and degradation of IκB which releases NF-κB to translocate into the nucleus where it can induce expression of immunomodulatory genes.




Taken and adapted from Medzhitov and Janeway, in Curr. Opin. Immunol. 10:12-15, 1998.

**Fig. 3.1 Signalling of Toll-like receptor.** Activation is initiated by the binding of pathogen-associated molecular structures (PAMPs) with the Toll or Toll-like receptor. Toll contains the Toll/IL-1 homology domain (TIR) and binds to MyD88. MyD88 is a transcriptional factor which recruits IL-1R associated kinase-2 (IRAK-2) through the death domain. IRAK-2 is activated by autophosphorylation. Active IRAK-2 dissociates from the receptor complex and associates with an adaptor protein TNF-α receptor-associated-factor-6 (TRAF-6). TRAF-6 interacts with and activates NF-κB-inducing kinase (NIK). NIK phosphorylates IκB kinase (IKK) and IKK in turn phosphorylates IκB. Phosphorylated IκB is degraded releasing NF-κB to translocate into the nucleus to activate transcription of NF-κB-dependent genes.

## 3.6 Objectives of this chapter

The objective of this chapter was to determine if mycobacterial IPGs stimulate nitrite production through CD14 on J774A.1 cells. The approaches were outlined as follows:

1) evaluate the levels of nitrite following stimulation with mycobacterial IPGs plus IFN-$\gamma$ in the presence of an anti-CD14 monoclonal antibody.

2) determine the levels of nitrite produced by PI-PLC treated J774A.1 cells in response to mycobacterial IPGs plus IFN-$\gamma$. Since CD14 is anchored to the membrane by linkage to glycosyl-phosphatidylinositol (GPI), treatment with PI-PLC cleaves between the phosphatidylinositol and the diacylglycerol, releases the protein from the cell surface.

3) determine the levels of nitrite produced by *Rhodobacter sphaeroides* LPS (RsLPS)-treated J774A.1 cells stimulated with mycobacterial IPGs plus IFN-$\gamma$. RsLPS has been shown to block LPS-induced TNF-$\alpha$ in monocytes (Golenbock, *et al.*, 1991) and macrophages (Takayama, *et al.*, 1989). It has also been shown to inhibit LPS-induced IL-1$\beta$ release in macrophages (Qureshi, *et al.*, 1991) and TNF-$\alpha$ and IL-6 release in J774A.1 cells (Kirikae, *et al.*, 1994). RsLPS competes with LPS for binding to CD14 on macrophages which results in the inhibition of LPS activity (Kirikae, *et al.*, 1994).

4) compare the levels of nitrite produced by J7.DEF.3 and J774A.1 cells in response to mycobacterial IPGs plus IFN-$\gamma$. It has been shown that J7.DEF.3

cells lack the expression of surface CD14 (Goyert, *et al.*, 1992) and is therefore defective in LPS binding.

## 3.7 Quantification of nitrite levels by the Griess reaction

### Materials

All reagents were obtained from various sources as described previously.

J7.DEF.3 cells were obtained from Dr Ken Kengatharan (The William Harvey Research Institute, St. Bartholomews and the Royal London Hospital School of Medicine and Dentistry). J7.DEF.3 cells were originated from Dr Teruo Kirikae (Jichi Medical School, Department of Microbiology), and they were derived by treating J774.1 cells with a mutagen (1-methyl-3-nitro-1-nitrosoguanidine) and isolating those cells which were defective in binding to $^{125}$I-labelled LPS in the presence of serum (Kirikae, *et al.*, 1991).

### Methods

J774A.1 and J7.DEF.3 cells were maintained in Dulbecco's modified Eagle medium (DMEM) without sodium pyruvate, supplemented with 10% foetal calf serum, 2 mM L-glutamine, 5 mM non-essential amino acids, 100 U/ml of penicillin, and 100 µg/ml of streptomycin in 80 $cm^2$ Nunc™ tissue culture flasks, under 5% $CO_2$ at 37°C. Cells were pretreated with DMEM, anti-CD14, phosphatidylinositol phospholipase C, or *Rhodobacter sphaeroides* LPS and subsequently stimulated with IFN-γ and LPS, AraLAM, or H37Ra P-type IPG. The supernatants were collected after 24 h and assayed for nitrite levels using the Griess reaction as described previously in 3.9.

## 3.8 Pretreatment of J774A.1 cells with anti-mouse CD14, phosphatidylinositol phospholipase C or *Rhodobacter sphaeroides* LPS

### Materials

*Anti-mouse CD14:* Purified rat anti-mouse $IgG_1$, κ antibody (Clone: rmC5-3) was purchased from Pharmingen, San Diego, CA, catalogue no. 09470D. Prepared as a 50 µg/ml in DMEM just before use.
*Phosphatidylinositol phospholipase C (PI-PLC)*: from recombinant *Bacillus thuringiensis* purchased from Oxford GlycoSystems, Inc., Abingdon, Oxford catalogue no. GPI-02. Prepared as a 4 U/ml solution in DMEM just before use.
*Lipopolysaccharide:* from *Rhodobacter sphaeroides* (RsLPS) ATCC #17023 purchased from List Biological Laboratories, Inc., Campbell, CA. catalogue no. 414. Prepared as a 1 mg/ml stock solution in sterile water and stored at 4°C.

### Methods

#### *Anti-CD14 treatment*

Membrane CD14 on the surface of J774A.1 cells was neutralised by the addition of anti-CD14 monoclonal antibody and incubated at 37°C for 1 h.

Control or anti-CD14 macrophages were incubated with LPS and IFN-γ to induce nitrite production.

### *PI-PLC treatment*

The treatment of J774A.1 cells with PI-PLC was based on the method described by Haziot (1988). J774A.1 ($1 \times 10^5$) cells were incubated with 4 U/ml PI-PLC for 1 h at 37°C. After the incubation, the cells were washed twice with 100 µl of warmed DMEM and incubated with LPS and IFN-γ.

### **R. sphaeroides** *LPS treatment*

The treatment of J774A.1 cells with RsLPS was based on the method described by Kirikae (1994). J774A.1 cells were preincubated with increasing concentrations of RsLPS at 37°C for 2 h prior to the addition of LPS, AraLAM or H37Ra P-type IPG and IFN-γ.

## 3.9 Immunofluorescence and flow cytometry of surface CD14

### Materials

*Phosphate buffered saline (PBS):* See Appendix

*Staining buffer:* prepared as 1% bovine serum albumin fraction V (Sigma A-9647) and 0.1% sodium azide in PBS and stored at 4°C for up to three weeks.

*Fixing buffer:* prepared as 1% paraformaldehyde in PBS and stored at 4°C for up to three weeks.

*Anti-CD14:* Flurorescein isothiocynate (FITC)-conjuated rat anti-mouse CD14 monoclonal $IgG_1$, κ antibody (Clone: rmC5-3) was purchased from Pharmingen, San Diego, CA, catalogue no. 09474D.

*Isotype control:* FITC-conjugated rat anti-mouse $IgG_1$, κ antibody (Clone: R3-34) with unknown specificity was purchased from Pharmingen, catalogue no. 11014C.

*Cell scraper, sterile:* purchased from Greiner Labortechnik Ltd, Gloucestershire, U.K., catalogue no. 541070.

### Methods

J774A.1 and J7.DEF.3 cells were grown in 80 $cm^2$ Nunc™ tissue culture flask as previously described. When the cells reached 80-90% confluence, they were ready for immunostaining. The culture medium was discarded and

the cells were resuspended in 8 ml of cold PBS. Cells were gently scraped with a sterile cell scraper to detach the adherent cells from the bottom of the culture plate. The number of viable cells was evaluated by trypan blue dye staining and counting on a haemocytometer. J774A.1 and J7.DEF.3 cells (1 x $10^6$ each) were aliquoted into tubes and centrifuged at 150 x g for 5 min at room temperature. The cells were washed once with 1 ml of cold staining buffer and centrifuged at 150 x g for 5 min at room temperature. The supernatant was discarded. The cells were incubated with 5, 10 or 20 µg/ml FITC anti-mouse CD14 or FITC isotype- matched control in the dark for 20 min at 4°C. The cells were washed twice with 1 ml cold staining buffer, each time centrifuged at 150 x g for 5 min at room temperature, supernatant was discarded and cells were resuspended in 400 µl of 1% paraformaldehyde. Fluorescence was evaluated by quantitative flow cytometry on a FACSCalibur (Becton Dickinson, San Jose, CA) using CellQuest software.

## 3.10 Results

### Effect of anti-CD14 on IFN-γ and LPS-induced nitrite production

These experiments investigated the ability of mycobacterial IPGs to induce nitrite production in J774A.1 cells through CD14 in the presence IFN-γ. Since LPS at low concentrations is known to activate macrophages through CD14, J774A.1 cells were preincubated with anti-CD14 monoclonal antibodies and subsequently stimulated with LPS and IFN-γ. Anti-CD14 antibodies tested on a separate occasion were ineffective at blocking IFN-γ- and LPS-induced nitrite production (Fig 3.2). This is in line with reports

showing that various commercially available anti-mouse CD14 antibodies were unable to block LPS-induced activation of macrophages (Netea, *et al.*, 1998). Thus, the antibody could not be used as a neutralising antibody.

## Effect of *R. sphaeroides* LPS (RsLPS) on IFN-γ and LPS, IFN-γ and AraLAM or IFN-γ and *M. tuberculosis* H37Ra P-type IPG-induced nitrite production

IFN-γ and LPS-induced nitrite production were partially inhibited by the treatment with RsLPS (Fig. 3.3). There was no statistical difference between J774A.1 cells stimulated in the presence or absence of RsLPS ($P>0.05$ using Mann-Whitney test). RsLPS was inhibitory to IFN-γ and AraLAM or H37Ra P-type IPG-induced nitrite production but only at an unacceptably high concentration (Fig. 3.4). Of interest is that the inhibition of AraLAM and H37Ra IPG were more pronounced than inhibition of LPS. These results suggest that RsLPS may compete for the same receptor shared by AraLAM and H37Ra IPG in the activation of J774A.1 cells.

## Surface expression CD14 on J774A.1 and J7.DEF.3 cells

The surface expression of CD14 in J774A.1. and J7.DEF.3 cells was determined by flow cytometry. J774A.1 cells stained with increasing concentrations of rat monoclonal anti-CD14 displayed high levels of surface CD14 as shown in Fig. 3.5. The expression of CD14 was not due to the non-

specific binding to the cells surface molecules such as the Fc-receptors because J774A.1 cells stained with varying concentrations of isotype-matched control did not generate similar levels of fluorescence obtained with anti-CD14. J7.DEF.3 cells however, expressed few or no surface CD14 by comparison to J774A.1 cells.

**Nitrite production of J774A.1 and J7.DEF.3 cells stimulated with IFN-γ and LPS or mycobacterial IPGs**

To determine if CD14 is required for the induction of nitrite production stimulated with IFN-γ and mycobacterial IPGs, J774A.1 and J7.DEF.3 cells were stimulated with equal concentrations of mycobacterial IPGs plus IFN-γ or LPS plus IFN-γ. In J774A.1 cells, LPS synergised with IFN-γ to produce high levels of nitrite. In contrast, there was a reduced capacity to synthesise nitric oxide in J7.DEF.3 cells in response to LPS plus IFN-γ as shown in Fig. 3.6. The baseline release of nitric oxide in the presence of IFN-γ alone was similar in J774A.1 and J7.DEF.3 cells in the experiment shown in Fig. 3.6, but in the experiment shown in Fig. 3.7 the baseline IFN-γ-induced nitric oxide was greater in J774A.1 cells. Whether in J774A.1 or J7.DEF.3 cells, *M. tuberculosis* IPGs (P and A-type) dose-dependently synergised with IFN-γ to elaborate high levels of nitrite (Fig. 3.7). H37Rv and H37Ra IPGs with IFN-γ were more effective inducers of nitric oxide in comparison with *M. vaccae* IPGs. However, IFN-γ and mycobacterial IPG-induced nitrite production in J7.DEF.3 cells were substantially lower than those induced in J774A.1 cells. In view of the differing baseline in J774A.1 and J.DEF.3 cells, this indicates that CD14 is not involved in the effect of IPGs.

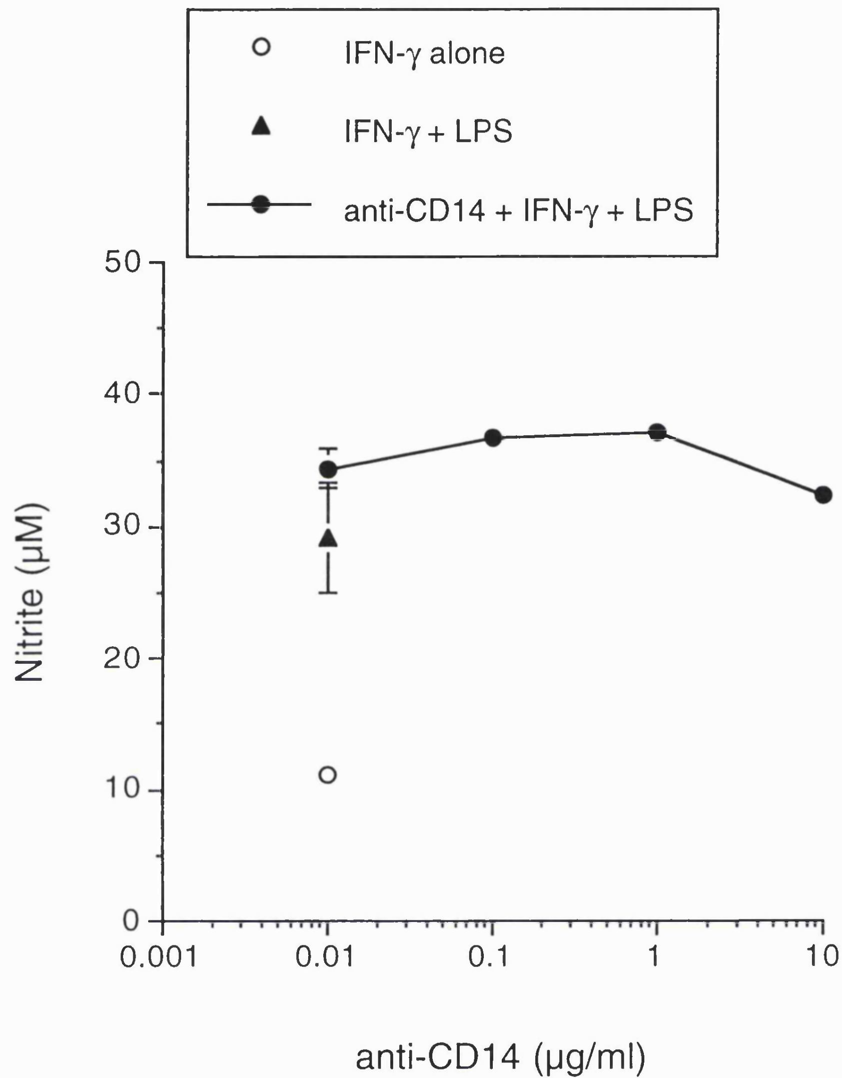



**Fig. 3.2. Effect of anti-CD14 on IFN-γ and LPS-induced nitrite production.** J774A.1 cells (1 x $10^5$) were pretreated with or without increasing concentrations of anti-CD14 monoclonal antibody at 37°C for 1 h prior to stimulation with 200 U/ml IFN-γ or IFN-γ and 10 ng/ml LPS for 24 h. The nitrite concentrations were determined by using the Griess reaction. The values were expressed as means ± S.D. of triplicate cultures from a single experiment. Where the error bars are not shown, the values fall within the symbols.

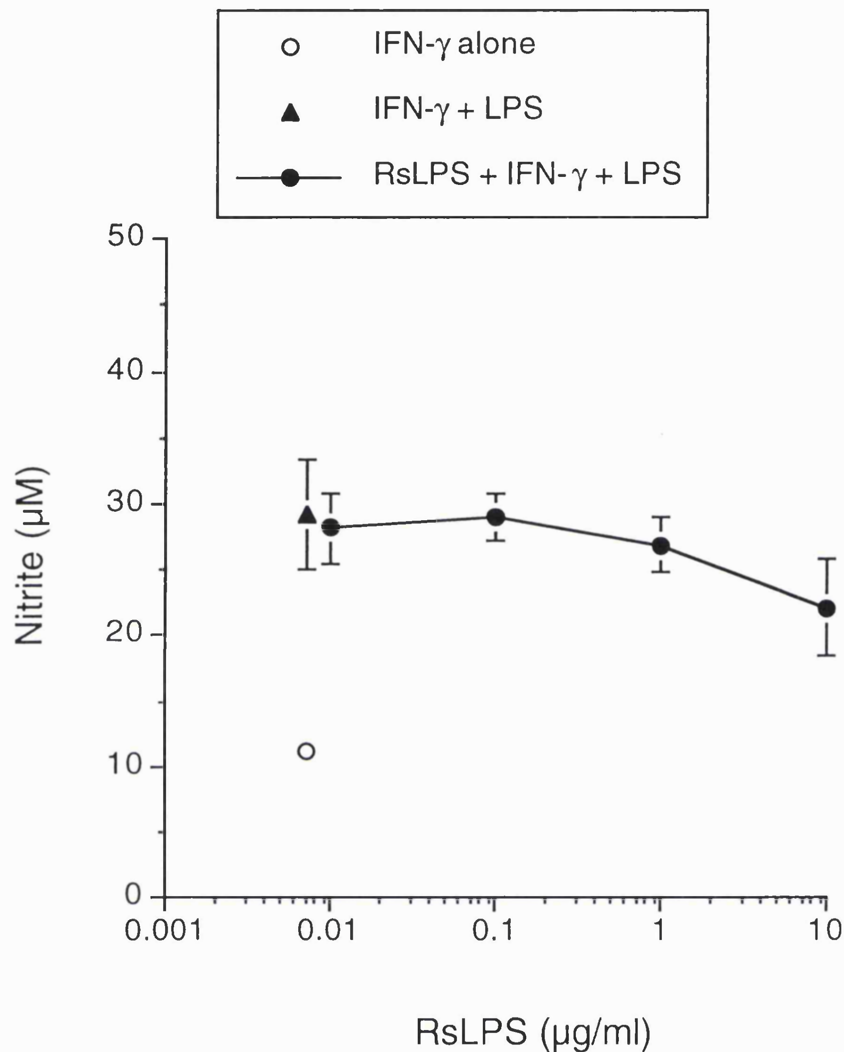



**Fig. 3.3. Effect of *R. sphaeroides* LPS (RsLPS) on IFN-γ and LPS-induced nitrite production.** J774A.1 cells (1 x $10^5$) were pretreated with or without increasing concentrations of RsLPS at 37°C for 2 h prior to stimulation with 200 U/ml IFN-γ or IFN-γ and 10 ng/ml LPS for 24 h. The nitrite concentrations were determined by using the Griess reaction. The values were expressed as means ± S.D. of triplicate cultures from a single experiment. Where the error bars are not shown, the values fall within the symbols.

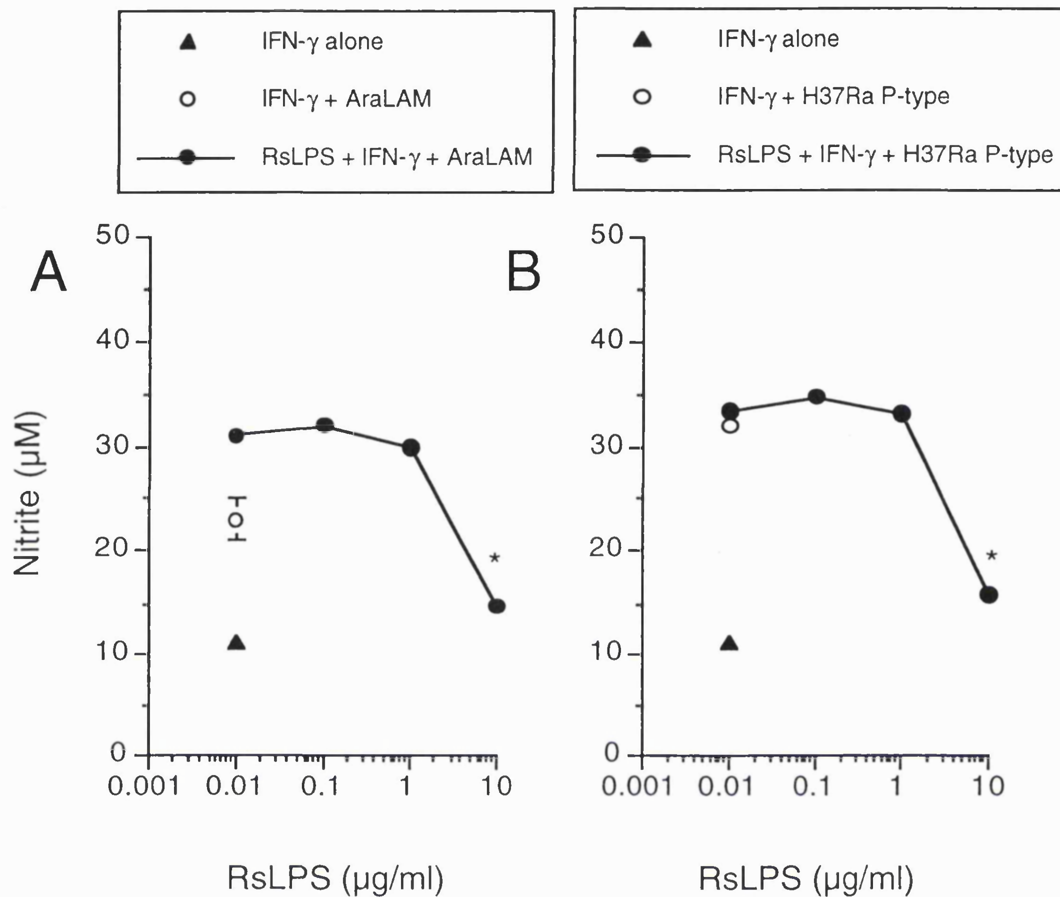
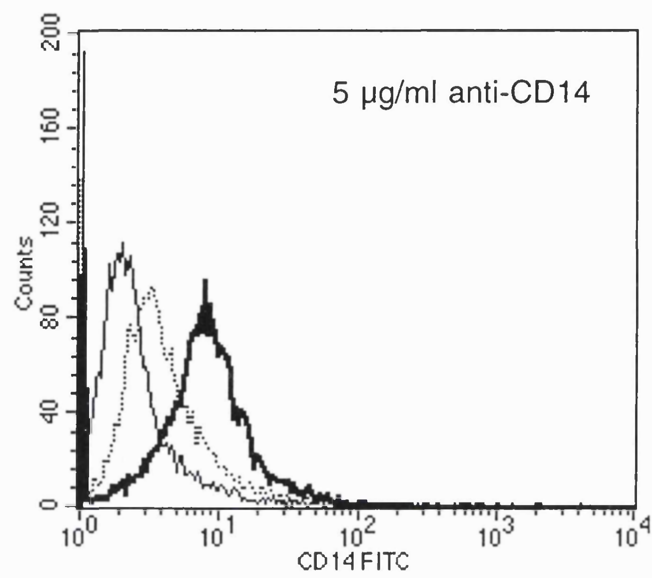
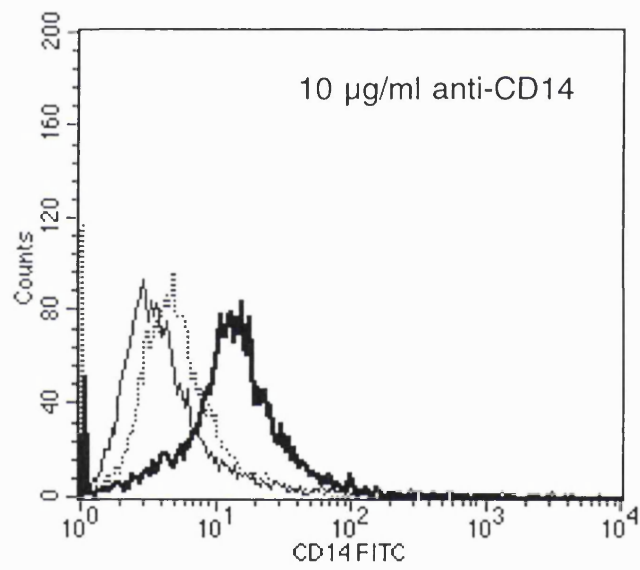



**Fig. 3.4. Effect of *R. sphaeroides* LPS (RsLPS) on IFN-γ and AraLAM or *M. tuberculosis* H37Ra P-type IPG-induced nitrite production.** J774A.1 cells (1 x $10^5$) were pretreated with or without increasing concentrations of RsLPS at 37°C for 2 h prior to stimulation with 200 U/ml IFN-γ or IFN-γ and 10 µg/ml AraLAM as shown in (A) or IFN-γ and H37Ra P-type (1:1250) as shown in (B) for 24 h. The nitrite concentrations were determined by using the Griess reaction. The values were expressed as means ± S.D. of triplicate cultures from a single experiment. Where the error bars are not shown, the values fall within the symbols. * $P<0.05$ (Mann Whitney test). RsLPS on its own did not trigger nitrite production in J774A.1 cells.

Fig. 3.5



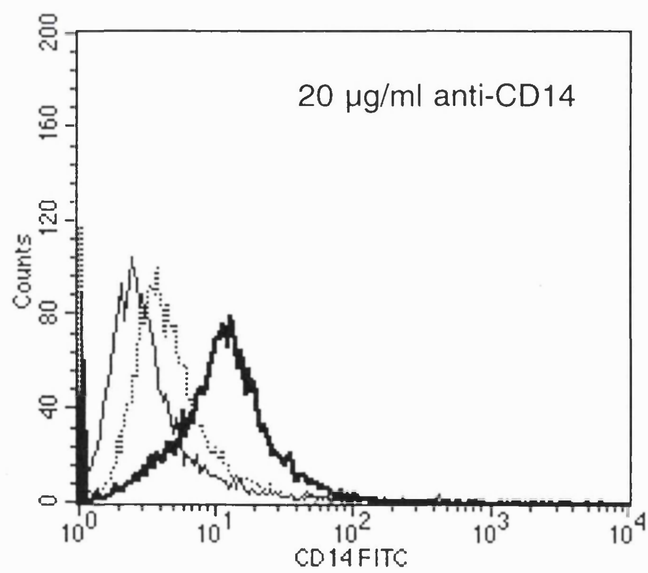



**Fig. 3.5. Surface expression of CD14 on J774A.1 and J7.DEF.3 cells.** J774A.1. (thick line) and J7.DEF.3 (thin line) cells (1 X $10^6$ each) were stained with increasing concentrations of FITC anti-mouse CD14 for 20 min at 4°C. The same number of J774A. 1 cells was stained with increasing concentrations of FITC isotype matched control (dotted line) under the same conditions. After incubation, the cells were analysed for surface CD14 expression by flow cytometry. The results shown are from a single experiment.

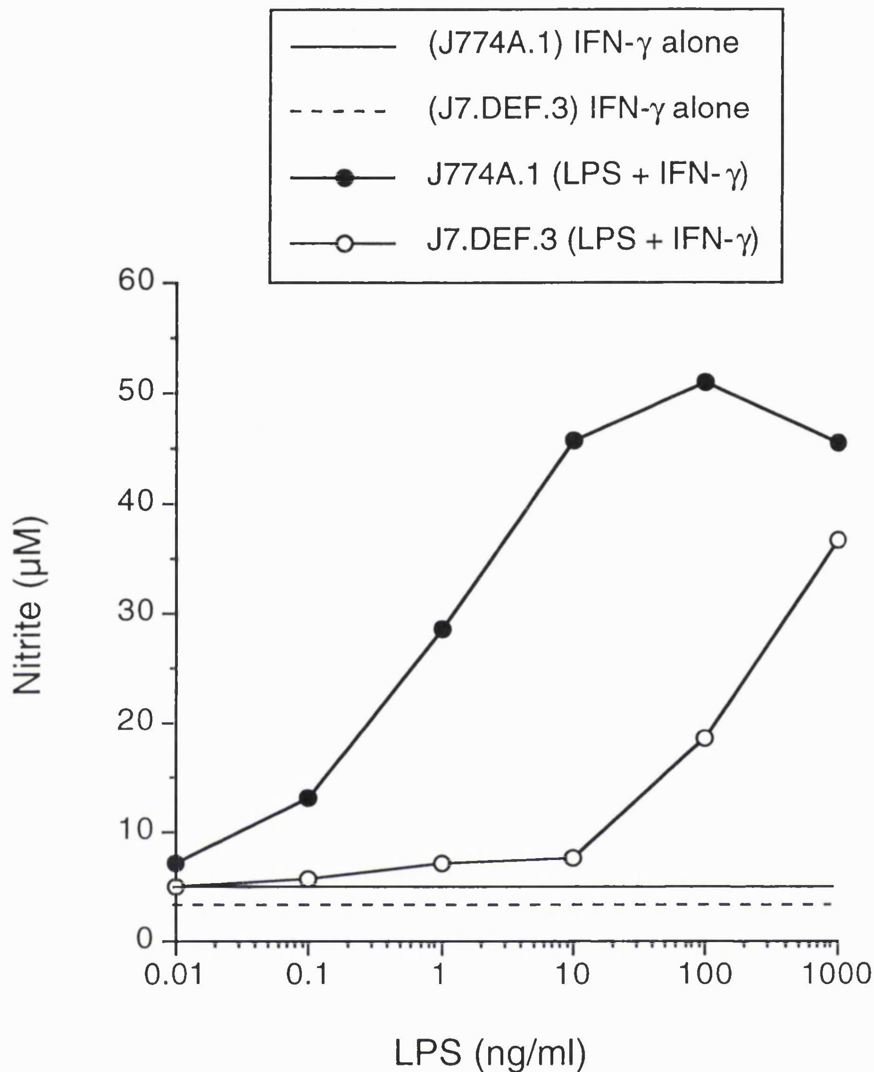



**Fig. 3.6. Nitrite production of J774A.1 and J7.DEF.3 cells stimulated with IFN-γ and LPS.** J774A.1 and J7.DEF.3 cells (1 x $10^5$ each) were incubated with 200 U/ml of IFN-γ or IFN-γ with increasing concentrations of LPS for 24 h. Nitrite concentrations were determined by using the Griess reaction. The results were expressed as means ± S.D. of triplicate culture from a single experiment. Where the error bars are not shown, the values fall within the symbols.

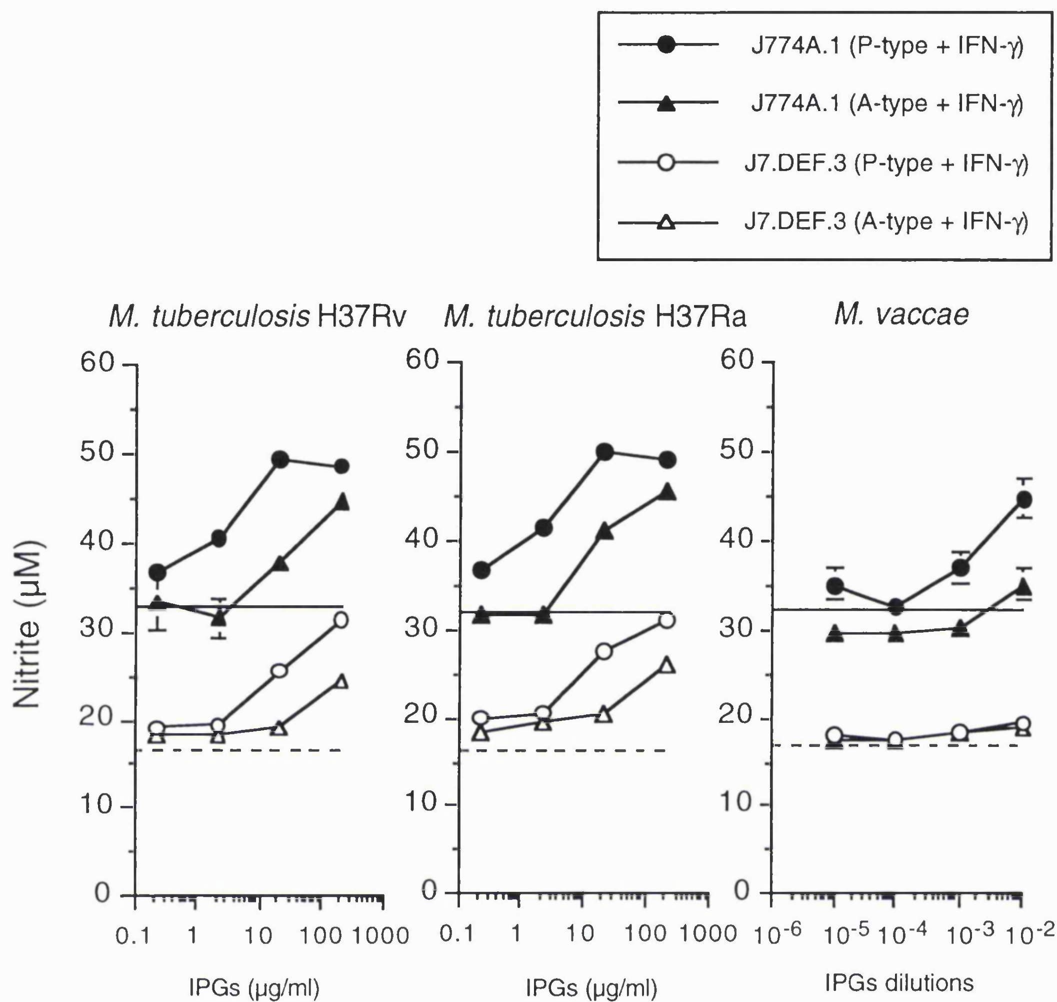



**Fig. 3.7. Nitrite production of J774A.1 and J7.DEF.3 cells stimulated with IFN-γ and mycobacterial IPGs.** J774A.1 and J7.DEF.3 cells (1 x $10^6$ each) were incubated with medium alone, IFN-γ (160 U/ml) or IFN-γ with increasing concentrations of H37Rv (P- or A-type) IPGs, H37Ra (P- or A-type) IPGs or *M. vaccae* (P- or A-type) IPGs for 24 h. Nitrite concentrations were determined by using the Griess reaciton. The values were expressed as means ± S.D. of triplicate cultures from a single experiment. Solid lines and dotted lines indicate the levels of nitrite induced by IFN-γ in J774A.1 and J7.DEF.3 cell respectively. Where the error bars are not shown, the values fall within the symbols.

## 3.11 Discussion

CD14 is one of several pattern recognition receptors which has evolved to recognise pathogen-associated molecular patterns (PAMPs) found in many different micro-organisms (Pugin, *et al.*, 1994). Previous studies have shown that PAMPs such as LAM from mycobacteria activate CD14 on macrophages to release a wide range cytokines (Zhang, *et al.*, 1993) which include TNF-$\alpha$, IL-1$\beta$ and IL-6, and nitric oxide (Anthony, *et al.*, 1994, Pugin, *et al.*, 1994). This is confirmed by the report showing LAM increases the expression of surface IgM on a pre-B cell line (70Z/3) transfected with human CD14 (Lee, *et al.*, 1992). In this study, the rôle of CD14 in the production of nitric oxide by J774A.1 cells stimulated with mycobacterial IPGs was investigated. Anti-CD14 monoclonal antibody obtained from Pharmingen was unable to neutralise LPS-induced nitrite production in J774A.1 cells. This is in contrast with one report by Hattori (1997) showing that anti-CD14 monoclonal antibody from the same source is inhibitory to LPS-induced nitrite production in J774A.1 cells. However, studies from three independent laboratories have shown that none of the commercially available antibodies have neutralising properties (Kengatharan, 1998, Netea, *et al.*, 1998, Tobias, 1998).

A number of reports have shown that treatment of monocytes (Haziot, *et al.*, 1988) and macrophages (Pugin, *et al.*, 1993, Netea, *et al.*, 1998) with PI-PLC removes the membrane-bound CD14 (mCD14). Preliminary results have shown that the nitrite production is unaltered following stimulation with LPS in J774A.1 cells pretreated with PI-PLC (data not shown). It is likely that the mCD14 was not removed by PI-PLC because immunostaining was not performed to assessed levels of CD14 before and after PI-PLC treatment. It is noteworthy that complete removal of mCD14 cannot be accomplished with

high concentrations of PI-PLC and/or prolonged treatment with PI-PLC. This is because previous studies have shown that either method leads to a decrease in cell viability (Haziot, *et al.*, 1988).

Previous studies have shown that LPS derived from *R. sphaeroides* (RsLPS) inhibits TNF-α and IL-6 production in J774A.1 cells stimulated by LPS (Kirikae, *et al.*, 1994). RsLPS competes with LPS for binding to CD14 which results in the inhibition of LPS activity (Kirikae, *et al.*, 1994). There was no significant decrease in the levels of nitrite in RsLPS-treated J774A.1 cells stimulated with LPS. In contrast, RsLPS reduced nitrite production from cells stimulated by AraLAM and *M. tuberculosis* P-type IPG. However, for reasons that are still unclear, about thirty times more RsLPS were required to achieve significant inhibition than previously described by other investigators (Kirikae, *et al.*, 1994, Kirikae, *et al.*, 1995). Therefore, the biological significance of this observation is unclear.

Kirikae *et al.* (1991) previously isolated J7.DEF.3 cells which are defective in LPS-binding. In contrast to J774A.1 cells, J7.DEF.3 cells have a reduced TNF-α and nitric oxide production in response to LPS (Kirikae, *et al.*, 1991). It has been shown that these defects are attributed to the lack of CD14 expression on J7.DEF.3 cells (Goyert, *et al.*, 1992). The present studies have shown that J7.DEF.3 cells lack surface CD14 expression and produce a lower levels of nitrite in response to low concentrations of LPS as compared to J774A.1 cells. Similarly, J7.DEF.3 cells were found to produce lower levels of nitrite in response to mycobacterial IPGs. With the exception of *M. vaccae* IPGs, the nitrite levels could be markedly increased by using high concentrations of *M. tuberculosis* IPGs. It is possible that the J7.DEF.3 cells

which have little or no CD14 may have a lower background level of activation, because they are insensitive to low levels of *M. vaccae* IPGs as well as background levels of contaminating LPS. These data, together with the effects of RsLPS, suggest that mycobacterial IPG plus IFN-$\gamma$ may stimulate NO production independent of CD14, but neither experiment is conclusive. This is because: 1) the RsLPS required an excessive concentration; 2) mycobacterial IPG were able to drive NO production in CD14-negative J7.DEF.3 cells. As the dose-response curve has shifted to the right by about 1 log, the interpretation is obfuscated by the lower baseline in the J7.DEF.3 cells. 3) J7.DEF.3 cells may have other defects such as the ability to synthesise NO in addition to the absence of surface CD14.

Recent studies have shown that transfection of a human Toll-like receptor (TLR)-2 confers responsiveness to peptidoglycan and lipoteichoic acids from Gram-positive bacteria (Kirschning, *et al.*, 1998, Yoshimura, *et al.*, 1999). Furthermore, Brightbill *et al.* (1999) have shown that a 19 kDa lipoprotein from *M. tuberculosis* stimulate IL-12 production in macrophages via TLR-2. Since the activation of macrophages by *M. tuberculosis* IPGs and LPS could occur in the absence CD14, TLRs other than CD14 may be involved in the activation of macrophages to stimulate nitric oxide production. More recently, Fenton *et al.* (1999) have found a protease resistant and heat stable mycobacterial ligand activates a TLR-2. Unlike the mycobacterial ligand, mycobacterial IPGs require CD14 to generate an optimal response. Thus, it is conceivable that mycobacterial IPGs may use TLR-2, which acts in synergy with CD14 to trigger macrophage activation.

# Chapter Four

## The Rôle of GPI-specific Phospholipase D in Macrophage Activation

## 4.1 Phospholipases

A number of GPI-anchored proteins have already been described in Chapter 1. GPI-anchored proteins are functionally diverse proteins which include cell surface receptors, complement regulatory proteins and virulence factors (as reviewed in Low and Saltiel, 1988, Low, 1989, Cross, 1990, Thomas, *et al.*, 1990, McConville and Ferguson, 1993, Gerold, *et al.*, 1996). One proposed function for the GPI-anchor is that it facilitates the release from the membrane by serving as a target substrate for anchor-specific-phospholipases (Low and Saltiel, 1988, Low, 1989). The classification of phospholipases is based on the types of bond they cleaves and the location of that bond within the phospholipid molecule (Roberts, 1996). Phospholipase A hydrolyses the carboxylic ester bond of the phospholipid to produce fatty acid and lysophospholipid. Two types of phospholipase A have been characterised: phospholipase $A_1$ and phospholipase $A_2$ where the subscript 1 and 2 indicate carbon-1 and carbon-2 of the glycerol backbone respectively. Phospholipase C hydrolyses the phosphodiester bond to produce 1,2-diacylglyerol or alkylacylglycerol and a phosphorylated polar head group. Phospholipase D hydrolyses the phosphodiester bond to produce a phosphorylated 1,2-diacylglycerol (also known as phosphatidic acid) or phosphorylated alkylacylglycerol (also known as lysophosphatidic acid) and a polar head with a exposed hydroxyl group. Most phospholipases are non-specific and they do not have ability the discriminate different types of phospholipids. There are, however, some phospholipases which have specificity for structural features of some phospholipids. These specific phospholipases include: phosphatidylinositol (PI)-phospholipase(PL) C, glycosyl(G)PI-specific-PLC and GPI-specific-PLD (Brodbeck and Bütikofer, 1994).

## 4.2 Phosphatidylinositol-specific phospholipases C

PI-PLCs have been purified from a number of bacteria and they include *Bacillus thuringiensis*, *Bacillus cereus*, and *Staphylococcus aureus*. (Low, 1990). PI-PLC derived from *B. thuringiensis* and *S. aureus* have been widely used in the detection and characterisation of GPI-anchored proteins. The former PI-PLC is preferred for routine screening because its specific activity is approximately 15-50 fold higher than the *S. aureus* PI-PLC. Another reason is that there are large variations in the synthesis of PI-PLC between strains of *S. aureus*. PI-PLC cleaves GPI-anchored proteins in addition to phosphatidylinositols. It is noteworthy that $Ca^{2+}$-dependent phospholipases, which are involved in signal transduction in mammalian cells, can also cleave PIs. Unlike the $Ca^{2+}$-dependent phospholipases, bacterial PI-PLCs are not sensitive to inhibition by divalent cation chelators such as EGTA and 1,10 phenanthroline.

## 4.3 GPI-specific phospholipase C

GPI-PLC was first purified from *Trypanosoma brucei* (Bülow and Overath, 1986, Hereld, *et al.*, 1986). The first evidence came from the observations that virtually all the **m**embrane-**f**orm **v**ariant **s**urface **g**lycoproteins (mfVSG) on the surface of the trypanosome were released as **s**oluble variant surface glycoproteins (sVSG) following lysis (Cardoso de Almeida and Turner, 1983). However, the conversion to sVSG was averted by boiling the trypanosomes in sodium dodecyl sulphate prior to the isolation of the mfVSG. The mfVSG contains a GPI-anchor which anchors the protein to the

plasma membrane via two myristic acid chains. Previous studies have shown that the mfVSG is converted to soluble VSG (sVSG) by a GPI-specific phospholipase C found in trypanosomes (Bülow and Overath, 1986, Hereld, *et al.*, 1986). The GPI-PLC cleaves at a phosphodiester bond to liberate 1,2-dimyristyl diacylglycerol. The conversion of mfVSG to sVSG exposes a carboxyl terminal inositol 1,2-cyclic phosphate, also known as cross-reacting determinant (CRD) found common in all VSGs as well as in a number of GPI-anchored proteins such as *Leishmania major* gp63 surface protease, thymocyte Thy-1 antigen and human erythrocyte acetylcholiesterase (Zamze, *et al.*, 1988). By comparison, mfVSG is cleaved more rapidly than PI suggesting the specificity for the former as the substrate.

Although the mfVSG is the substrate for GPI-PLC *in vitro*, the biological function of GPI-PLC in trypanosomes is not clear. There has been suggestion that GPI-PLC is involved in the release of sVSG during differentiation from the bloodstream forms to procyclic forms (Webb, *et al.*, 1994). mfVSG is expressed in the infective metacyclic forms found in the tsetse fly salivary gland and the bloodstream forms but not in the non-infective procyclic forms in the insect midgut. It has been found that there is little GPI-PLC activity (Bülow and Overath, 1985) and GPI-PLC mRNA (Carrington, *et al.*, 1989) in the procyclic forms suggesting that the enzyme and the substrate are synthesised at the same time. The purified GPI-PLC represents 0.04% of the total trypanosome protein (Hereld, *et al.*, 1986). There are an estimated 400 mfVSG molecules per molecule of GPI-PLC. Under *in vitro* conditions, each molecule of GPI-PLC cleaves about 100 mfVSG in one minute suggesting this enzyme may be responsible for the rapid release of sVSG during lysis. GPI-PLC does not require the presence of $Ca^{2+}$ for activity, and it is stimulated by chelating agents and dithiothreitol. Some but not all mammalian GPI-anchored proteins are cleaved by *T. brucei*

GPI-PLC. For instance, the human erythrocyte acetylcholinesterase which has an acyl chain linked to the inositol ring is resistant to GPI-PLC.

Since the discovery of GPI-PLC in trypanosomes, efforts have been concentrated to find similar enzyme in mammalian cells. Two reports have shown that GPI-PLC activity is detected in rat liver (Fox, *et al.*, 1987) and mouse brain (Fouchier, *et al.*, 1990). However, this activity has yet to be established in other mammalian cells.

## 4.4 GPI-specific phospholipase D

In contrast to GPI-PLC, GPI-PLD activity in mammals is well-established (Metz, *et al.*, 1991a, Brodbeck and Bütikofer, 1994). GPI-PLD was originally discovered in bovine and human sera (Davitz, *et al.*, 1987, Cardoso, *et al.*, 1988, Low and Prasad, 1988). To date, two GPI-PLD genes have been cloned from human liver and pancreatic complementary DNA libraries (Tsang, *et al.*, 1992). The protein was identified by its ability to cleave mfVSG to release sVSG and dimyristyl phosphatidic acid (Malik and Low, 1986). Neither recombinant, purified nor crude GPI-PLD releases GPI-anchored proteins from intact cell (Low and Huang, 1991). However, this restriction can be overcome by the addition of detergents. Similar GPI-PLD activity has been found in rat and rabbit sera as well as human placenta which also release other GPI-anchored proteins such as alkaline phosphatase and 5'nucleotidase. Studies have shown that GPI-PLD is specific for the GPI-anchor but it does not cleave phosphatidylinositol, phosphatidylethanolamine and phosphatidylcholine. Unlike PI-PLC and GPI-PLC, cleavage of mfVSG with GPI-PLD does not expose the cross-

reacting determinant (CRD). Treatment of the mfVSG removes the terminal phosphate on the inositol ring destroying the CRD epitope (Cardoso de Almeida and Turner, 1983, Shak, *et al.*, 1988). SDS-PAGE analysis of the purified GPI-PLD from human serum has a molecular weight of 110,000 (Davitz, *et al.*, 1989). Davitz and Huang estimated that the plasma concentration of GPI-PLD was 5–10 μg/ml (Huang, *et al.*, 1990). GPI-PLD activity is not inhibited by serine esterase inhibitor phenylmethylsulfonyl fluoride (PMSF) but is inhibited by thio-blocking agent p-chloromercuriphenyl-sulfonic acid (Davitz, *et al.*, 1989).

**Structural features of GPI-specific phospholipase D**

Complementary DNA sequence analysis revealed four repeats that show a high degree of similarity with the metal-ion binding domains in the $\alpha$ subunits of proteins belonging to the integrin family of cell surface adhesion molecules (Huang, *et al.*, 1990, Scallon, *et al.*, 1991). These repeats within GPI-PLD and integrin sequence contains sequences which show some similarity with E-F hand found in a number of calcium-binding proteins such as parvalbumin, troponin C and calmodulin. However, unlike E-F hand motif and integrin metal-ion binding sites, the $Ca^{2+}$-binding sites in GPI-PLD are irreversibly disrupted by denaturation. A recombinant $\alpha$ integrin fragment has been shown to bind $Ca^{2+}$ as well as RGD (Arg-Gly-Asp)-containing ligands such as collagen, fibrinogen and fibronectin (Gulino, *et al.*, 1992).

Previous studies have shown that GPI-PLD is inhibited by divalent cation chelators (Malik and Low, 1986, Davitz, *et al.*, 1987, Low and Prasad, 1988, Davitz, *et al.*, 1989, Hoener, *et al.*, 1990, Huang, *et al.*, 1990) such as EGTA

and 1,10 phenanthroline. The latter is more effective inhibitor of GPI-PLD than the former (Low and Prasad, 1988). This has led to the suggestion that divalent cations are essential for GPI-PLD activity. It is found that $Zn^{2+}$ is more effective than $Ca^{2+}$ at blocking the effect of both EGTA and 1,10 phenanthroline suggesting that the inhibition is not attributed to removal of $Ca^{2+}$ from GPI-PLD (Li, *et al.*, 1994). This is also supported by the observation that the pretreatment of GPI-PLD with 1,10 phenanthroline does not block the binding of $^{45}Ca^{2+}$. Metal analysis of GPI-PLD revealed that there are as many as 10 atoms of zinc per molecule compared to 5 atoms of calcium per molecule of GPI-PLD (Li, *et al.*, 1994). A zinc active site is required for phosphate ester binding and hydrolysis by several enzymes such as P1 nuclease, alkaline phosphatase and phosphatidylcholine phospholipase C (Coleman, 1992). Unlike GPI-PLD, these phosphohydrolases contain only 2–3 atoms of zinc per molecule. A high number zinc atoms has been found in some proteins containing multiple copies of zinc finger structural motif rich in cysteine-histidine residues. However, no such motif has been identified in the GPI-PLD sequence. Thus, the mechanism of zinc binding and its involvement in the catalytic activity remains to be elucidated.

Treatment of bovine serum purified GPI-PLD with trypsin resulted in three polypeptides with molecular weight of 33, 39 and 47 kDa (Heller, *et al.*, 1994). The active site is found to be the 39 kDa polypeptide which corresponds to the amino-terminal region of GPI-PLD. Whilst the amino-terminus may be involved in the catalytic activity of GPI-PLD, carboxyl-terminal deletions of GPI-PLD resulted in a loss of activity suggesting that the carboxyl-terminus is essential for the activity of GPI-PLD (Stadelmann, *et al.*, 1997). Trypsinised GPI-PLD has an approximately 3–4-fold increase in enzymatic activity (Li, *et al.*, 1994). A similar increase of activity is achieved

when the purified GPI-PLD is treated with other proteases such as chymotrypsin, proteinase K and L-1-tosylamido-2-phenylethyl-chloromethyl-ketone (TPCK)-trypsin. However, trypsinisation of GPI-PLD does not overcome the inability to cleave GPI-anchored proteins in intact membranes. Studies from Hoener *et al.* (1994) have shown that a small amount of 47 kDa may exit as an active form. Furthermore, the purified GPI-PLD can be processed into a 47 kDa active form by *in vitro* treatment with lysosomal extract and with cathepsin D (Hoener, *et al.*, 1994).

**Tissues expression of GPI-PLD**

Although GPI-PLD is found in abundance in mammalian sera, it remains to be established which tissue or cell types are the principle source of the circulating GPI-PLD. Hoener *et al.* (1993) have shown that GPI-PLD in serum associates with high density lipoprotein (HDL). Studies using density centrifugation revealed that GPI-PLD interacts with a major apolipoprotein in HDL, apolipoprotein A-I (Hoener and Brodbeck, 1992). In the absence of apo A-I or detergent, GPI-PLD exists as an inactive aggregates (Hoener, *et al.*, 1993). However, in the presence of apo A-I or detergent, GPI-PLD becomes dispersed and is able to hydrolyse GPI-anchored substrates. This is supported by the results showing that apo A-I increases GPI-PLD activity up to 5-fold (Hoener and Brodbeck, 1992).

GPI-PLD is widely distributed in a number of tissues including brain, cerebrospinal fluid and milk (Hoener and Brodbeck, 1992). Many cells have been shown to contain GPI-PLD including hepatocytes, keratinocytes (Xie, *et al.*, 1993), pancreatic islet cells (Metz, *et al.*, 1991b) and a number of myeloid

cell lines (Xie and Low, 1994). However, only pancreatic islet cells and myeloid cell lines have been demonstrated to secrete GPI-PLD. It is plausible that the circulating GPI-PLD in human serum originates from the pancreatic islet cells because amino acid sequence of the serum GPI-PLD corresponds to the predicted human pancreatic complementary DNA. Recent studies from Deeg and Verchere (1997) have shown that a mouse pancreatic insulinoma cell line (βTC3) secretes GPI-PLD into the media in response to phorbol myristic acid. Although insulin secretagogues such as carbachol, glucagon-like peptide I and isobutyl-methylxanthine do not stimulate βTC3 cells to secrete GPI-PLD, they can synergise with glucose to stimulate GPI-PLD secretion.

Studies have shown that a number of myeloid cell lines including the mouse J774 macrophage cell, the human U937 promyelocytic cell and the human monocytic THP-1 cell contain GPI-PLD activity (Xie and Low, 1994). This is supported by the positive immunofluorescent staining with a monoclonal antibody which stained in the perinuclear region and around the cell surface in those three cell lines. The GPI-PLD has been shown to localise within secretory vesicles because disruption of the cells with streptolysin O releases the cytosolic enzyme, lactate dehydrogenase but not GPI-PLD into the medium. Recent studies from Hari *et. al.* (1998) have shown that GPI-PLD is found in the lysosomal fraction of rat liver.

**Function of the GPI-specific phospholipase D**

It is well known the GPI-PLD is unable to release GPI-anchored proteins from native cell membrane unless the plasma membrane is solubilised with

detergent (Low and Huang, 1991). Nonetheless, studies from Low *et al.* (1991) have shown that GPI-PLD in the presence of detergent is able to release most of the GPI-anchored proteins on the surfaces of the cell membrane in less than one minute. This has led to the suggestion that GPI-PLD may be under tight regulation to preclude the complete loss of GPI-anchored proteins on the cell membrane.

Studies by Scallon *et al.* (1991) have shown that bovine GPI-PLD cDNA co-transfected with the GPI-anchored protein, **pl**acental **a**lkaline **p**hosphatase (PLAP), into COS cells greatly increase the release of PLAP from the cell as compared to COS cells transfected with placental alkaline phosphatase alone. The release of PLAP was not the result of the action of the secreted GPI-PLD in the medium because no PLAP was released from the cell when PLAP-transfected cells were incubated with medium containing active GPI-PLD. Thus, the cleavage of the PLAP is likely to occur intracellularly rather than on the cell surface. This is supported by the fact that GPI-PLD has a broad pH range which permits the enzyme to operate in an intracellular compartment. Furthermore, a number of GPI-anchored proteins including the macrophage marker CD14, the complement regulatory factor CD55 (also known as **D**ecay **A**ccelerating **F**actor or DAF) and the thymocyte antigen Thy-1 have been found as soluble forms. Analysis of the carboxyl terminal of the soluble Thy-1 is in agreement with the hydrolysis of the intact Thy-1 molecule.

Another proposed function of GPI-PLD is that it may be involved in the release of inositol phosphoglycan (IPG) second messengers from free GPIs but not GPI-anchored proteins (as reviewed in Rademacher*, et al.*, 1994, Varela-Nieto*, et al.*, 1996, Jones and Varela-Nieto, 1998). Unlike GPI-anchored proteins, free GPIs contain structural components are not found in

GPI-anchored proteins (Rademacher, *et al.*, 1994). At least two structurally distinct mammalian IPGs have already been described of which each is capable of activating key enzymes involved in cell metabolism (Kunjara, *et al.*, 1995). IPG-P activates pyruvate dehydrogenase phosphatase to induce glycogen synthesis whereas IPG-A activates acetyl-CoA carboxylase to drive lipogenesis (Kunjara, *et al.*, 1995). It has been shown that phosphatidic acid and lipid A are inhibitory to the GPI-PLD activity (Low and Huang, 1993). More recently, it has been shown that serum GPI-PLD activity is markedly decreased in patients with systemic inflammatory response syndrome, sepsis and septic shock (Rhode, *et al.*, 1999).

## 4.5 Objectives of this chapter

The objective was to elucidate the effects of mycobacterial IPG on macrophage function in the absence of mammalian IPG. The approaches were as follow:

1) obtain mfVSG from *T. brucei* as a substrate for the study of GPI-PLD activity. With the help of Drs Wendy Gibson and Brian De Souza, trypanosomes were propagated in mice infected with *T. brucei*. The bloodstream trypanosomes were passed through a DE52 anion exchange column to separate the trypanosomes from the blood (Lanham, 1968). Since [$^3$H] myristic acids are readily incorporated into the mfVSG to generate [$^3$H] myristate mfVSG which can be isolated to purity (Ferguson and Cross, 1984), the trypanosomes were metabolically labelled with [$^3$H] myristic acid and processed accordingly to isolate [$^3$H] myristate mfVSG. It was assessed on its sensitivity to GPI-PLC from *T. brucei* and GPI-PLD from bovine serum.

2) purify GPI-PLD from human serum using ammnonium sulphate precipitation and HPLC.

3) determine the effect of AraLAM and ManLAM on GPI-PLD activity as assessed by the release of myristate-labelled phosphatidic acid.

4) compare nitrite production in J774A.1 cells grown in untreated and alkaline treated serum following stimulation with IFN-γ and LPS, since GPI-PLD activity is abrogated following alkaline treatment (Küng, *et al.*, 1997).

## 4.6 Isolation and metabolic labelling of bloodstream trypanosomes

### Materials

*Trypanosome brucei* of variant antigen type 117 and 221 kindly provided by Drs Wendy Gibson (Bristol University) and David Horn (London School of Hygiene and Tropical Medicine) respectively.

*Phosphate-buffered saline-glucose (PSG):* 53 mM anhydrous disodium hydrogen orthophosphate, 3 mM anhydrous monosodium dihydrogen orthophosphate, sodium chloride and L-glucose, pH 8.0.

*DEAE cellulose, DE52:* Pre-swollen microgranular cellulose (Whatman International Ltd, Kent, England, catalogue no., 4057 050).

*[9, 10(n)-$^3$H] myristic acid:* specific activity 51 Ci per mmol, 1 mCi per ml (Amersham International plc, Little Chalfont, England, catalogue no. TRK907).

*Bovine serum albumin (BSA):* Prepared from Fraction V albumin (A-4503), fatty-acid free BSA purchased from Sigma Chemical Co., St. Louis, MO, catalogue no. A-6003

*Labelling medium:* RPMI 1640 medium (Life Technologies Ltd, Paisley, U.K.) supplemented with 1 mg/ml of fatty-acid free BSA and 25 mM HEPES, pH 7.4.

*Lysis buffer:* 10 mM sodium phosphate solution: 3.9 mM anhydrous monosodium dihydrogen orthophosphate and 6.1 mM anhydrous disodium hydrogen orthophosphate, pH 7.4 .

*Protease inhibitors:*

*Leupeptin hydrochloride:* a thiol protease inhibitor purchased from Sigma Chemical Co., St. Louis, MO, catalogue no. L-0349, prepared as a 5 mg/ml stock solution in cold sterile water and stored as aliquots at -20°C.

*Nα-p-Tosyl-L-Lysine chloromethyl ketone (TLCK), hydrochloride:* purchased from Sigma Chemical Co., catalogue no. T-7254. prepared as a 100 mM solution in sterile water on the day of use.

*Sodium p-chloromercuriphenylsulfonic acid, monosodium salt:* purchased from Sigma Chemical Co., St. Louis, MO, catalogue no. C-4503. prepared as a 100 mM stock solution in 0.1 M NaOH.

## Methods

### *Equilibration of DEAE-column*

DEAE celluose DE52 (2 kg) was suspended in 5 litres of PSG. The resulting slurry was adjusted to pH 8.0 with dilute orthophosphoric acid (1:20, v/v). The slurry was mixed for 30 min with a magnetic stirrer and allowed to stand without stirring for 15 min. The supernatant was discarded leaving the cellulose. The DE52 was subjected to another 6 washes each time with 5 litres of PSG. The slurry was stored at 4°C.

## Infection with *Trypanosoma brucei*

F1 (BALB/C x CBA) mice weighing 20-25 g were infected intraperitoneally with stabilate of *T. brucei* strain 427 or 221 with 5 x $10^5$ or 1 x$10^6$ trypanosomes. The infection was assessed by estimating the number of trypanosomes from tail bleeds. When parasitaemia reached 1–5 x$10^8$/ml (usually after 3 days following infection). the mice were sacrificed.

*Trypanosome brucei* of variant antigen type 221 (kindly provided by Dr David Horn, London School of Hygiene and Tropical Medicine) were grown in F1 mice until parasitaemia reached approximately 2 x $10^9$ per ml. The trypanosomes were diluted 1 in 4 with PSG containing 10 U per ml heparin and kept on ice before layering on to a Büchner glass funnel with a slit sieve plate.

## *Isolation of bloodstream trypanosomes*

The preparation of the column and separation of trypanosomes were performed at room temperature (Lanham, 1968, Lanham and Godfrey, 1970). A Whatman No. 41 filter paper was moistened with PSG and placed on the plate of the Büchner funnel. Approximately 40 ml of slurry containing the DE52 cellulose was poured into the funnel. The excess liquid was run out gently through the column controlled with a metal clip fitted on the rubber tubing. The liquid was allowed to run out leaving the cellulose to form a firm horizontal surface. The outlet was closed and another moistened Whatman No. 41 filer paper was placed on the cellulose bed. The cold diluted blood

was layered carefully on to the surface. A slow flow rate was maintained generating a sharp descending front of the erythrocytes. When all the blood had entered the column, trypanosomes were eluted with 6 columns volume of PSG each time with 20 ml. The trypanosomes were collected in a conical flask kept cooled on ice. The yield of the trypanosomes was enumerated using a haemacytometer. The fractionated trypanosomes were washed once with 20 ml of ice-cold PSG and centrifuged at 220 x g for 10 min at 4°C. The pellet containing the trypanosomes was ready for metabolic labelling.

***[$^3$H] myristate labelling of bloodstream trypanosomes***

The myristate labelling was based on the method described by Ferguson and Cross (1984). [9, 10(n)-$^3$H] myristic acid was dried, redissolved in 5 µl of 95% ethanol, and mixed with equimolar fatty-acid free BSA (20 mg/ml in $H_2O$). The trypanosomes were suspended in labelling medium ($10^8$ per ml). The cells were incubated at 37°C for 15 min, and then supplemented with [9, 10(n)-$^3$H] myristic acid at 100 µCi per ml and incubation at 37°C for 1 h.

***Isolation of [$^3$H]myristate-labelled membrane-formVSG***

The isolation of [$^3$H]myristate-labelled mfVSG was based on the method described by Hereld *et al.* (Hereld, *et al.*, 1986, Hereld, *et al.*, 1988). The labelled trypanosomes were cooled to 0°C and centrifuged at 1,019 x g for 10 min at 4°C. The medium was saved for subsequent labelling. The cell pellet was washed with ice-cold PSG, centrifuged at 1,019 x g for 10 min to

discard supernatant. The trypanosomes were lysed osmotically in 10 ml of 10 mM sodium phosphate solution containing 1 μg/ml leupeptin, 0.1 mM TLCK and 5 mM sodium p-chloromercuriphenylsulfonate. Sodium p-chloromercuri-phenylsulfonate inhibits VSG lipase and sulfhydryl proteases. After 10 min on ice, the lysate was centrifuged at 5,900 x g for 5 min at 4°C and the pellet was washed with 10 ml of the same solution. The pellet which contained the membrane-form (mf)VSG was transferred into a 15 ml glass Corex® tube, extracted with 10 ml of $CHCl_3/CH_3OH$ (2:1) at 20°C and centrifuged at 13, 200 x g for 5 min at 20°C. The pellet was dissolved in 5 ml of 1% SDS by heating at 100°C for 10 min and vortexing. The remaining [$^3$H]-labelled lipids was removed by extracting twice with 6 ml of *n*-butyl alcohol. For each extraction, the solution was vigorously homogenized and centrifuged at 10,400 x g for 5 min at 20°C to resolve the lower aqueous and upper organic phase. The latter was discarded. In the third extraction, 9 ml of water-saturated *n* -butyl alcohol was used to maintain the aqueous phase. In the fourth extraction, the aqueous was eliminated by using 9 ml of *n*-butyl alcohol (not water-saturated). The resulting gummy precipitate was recovered by centrifugation at 9,600 x g for 5 min at 4°C and washed with anhydrous ether, air-dried, and dissolved in 1 ml of 1%SDS by heating at 100°C for 10 min. Insoluble materials were removed by centrifugation at 13,000 x g for 5 min at 20°C. The product was assayed by SDS-PAGE and Coomassie staining. Autoradiography showed that virtually all the radioactivity was incorporated in the mfVSG. The protein concentration of the [$^3$H]-myristate-labelled mfVSG was 1.5 mg per ml as assayed by Lowry and the specific activity was about 4,000 cpm per μg.

## 4.7 Autoradiography of SDS-PAGE gel

### Materials

*Fixing solution:* 10%(v/v) glacial acetic acid, 30%(v/v) methanol and 70%(v/v) distilled water.

*Coomassie Brilliant blue stain:* 0.2% Brilliant blue, 10% glacial acetic acid, 45% methanol and 45% water.

*Destaining solution:* 10%(v/v) glacial acetic acid, 45%(v/v) methanol and 45%(v/v) distilled water.

*Autoradiography Enhancer**, *EN$^3$HANACE™:* purchased from E.I. du Pont de Nemours and Co., Inc., Boston, MA., NEN® Research Products, NEF-981.

*X-ray film:* New Rx Fuji X-ray film purchased from Fuji Photo Film Co. Ltd, Tokyo, catalogue no. 03 E270

### Method

After electrophoresis, the gel was fixed with 200 ml of fixing solution containing at room temperature for 1 h with gently agitation. The fixing solution was discarded and the gel was stained with 200 ml Coomassie Brilliant blue. The dye was through a 3MM Whatman filter paper for 2 h at room temperature with gentle agitation. The gel was destained with 200 ml of destaining solution overnight at room temperature. Destaining was continued with another three washes of destaining solution, each time with 200 ml of destaining solution leaving a clear background. After destaining, the gel was impregnated with 200 ml EN$^3$HANCE™ at room temperature for 1 h with gentle agitation. This was followed by precipitation of the fluorescent material inside the gel with 300 ml of distilled water for 30 min with gentle agitation. After precipitation, the gel was dried on a 3MM Whatman filter paper at 70°C for 1 h under vacuum on a slab gel drying apparatus. Finally, the dried gel was exposed to New Rx Fuji X-ray film overnight at -70°.

## 4.8 GPI-PLD activity assay

### Materials

*Glycosylphosphatidylinositol phospholipase D (GPI-PLD):* bovine serum GPI-PLD obtained from Boehringer Mannheim, Mannheim, Federal Republic of Germany, catalogue no. 1500 651. One unit of activity is defined by the

amount of enzyme required to hydrolyse 1 pmole of bovine erythrocyte acetylcholinesterase per 1 min at 37°C and pH 7.4.

*Glycosylphosphatidylinositol phospholipase C (GPI-PLC):* from *Trypanosoma brucei* purchased from Oxford GlycoSystems, Inc., Abingdon, Oxford, catalogue no. GPI-03. One unit of activity is defined by the amount of enzyme required to convert 10 μg mfVSG to sVSG in 30 minutes in Tris buffered saline pH 7.4 containing 0.1% Triton X-114 at 30°C.

*Maleic acid or cis-Butenedoic acid:* prepared as a 1 M solution in deionised water (Sigma Chemical Ltd, St. Louis, MO, catalogue no. M0375).

*Trizma® base*: prepared as a 200 mM stock solution (Sigma Chemical Ltd, St Louis, MO, catalogue no. T-8524) in deionised water and adjusted to pH 7.2 with 1 M maleic acid.

*Nonidet P-40:* prepared as a 1% (v/v) stock solution in deionised water (ICN Biochemicals, catalogue no. 155942).

*Ammonia solution:* $NH_3$ = 17.03, catalogue no. 10011, AnalaR®, specific gravity 0.91, about 25% (w/w) was purchased from BDH Laboratories Supplies, Poole, England.

*1 M Ammonium hydroxide saturated n-*butanol*:* was prepared by mixing approximately 90 ml of 1 M $NH_4OH$ with approximately 200 ml of *n*-butanol and allowed to stand for 3 h. The lower phase was discarded and the upper phase was used in the assay.

*Ecoscint™ A:* purchased from National Diagnostics, Atlanta, GA, U.S.A., catalogue no. TS-273.

### Methods

The GPI-PLD degradation assay was based on the method described by Xie and Low (1994). The sample containing GPI-PLD was first diluted in 40 mM Tris-maleate in 1.5 ml Eppendorf tube and the reaction was started by adding 0.1 ml of diluted sample to 0.1 ml 60 nM mfVSG in 40 mM Tris-maleate (pH 7.2), 0.1%(v/v) Nonidet P-40. A negative control containing all the reagents except GPI-PLD was included in each set of experiment and processed in parallel with the other samples. 7,000 c.p.m. [$^3$H] myristate-labelled mfVSG was used. The resulting mixture was incubated at 37°C for 30 min or as indicated and the reaction was stopped by adding 0.5 ml of 1 M ammonium hydroxide-saturated n-butanol. The tube was thoroughly mixed by vortexing and phase separation was achieved by centrifugation at 10,000 x g for 5 min. Finally, 0.3 ml of the upper organic phase was sampled and mixed with 10 ml Ecoscint™A. The radioactivity was determined by counting in a 1212 MiniBeta liquid scintillation counter (LKB Wallac, Türku, Finland).

## 4.9 Thin layer chromatography (TLC) analysis of VSG-degradation products

### Materials

*Silica-gel TLC plate:* 20 x 20 cm, 60 layers K6, purchased from Whatman International Ltd., Kent, England., catalogue no. 48060 820).
*Vehicle:* Chloroform/methanol (2:1, v/v).

*Solvent:* Chloroform/methanol/0.25% KCl (55:45:10, by volume).

*EN$^3$HANCE™ Spray:* purchased from E.I. du Pont de Nemours and Co., Inc., Boston, MA., NEN® Research Products, NEF-970G.

### Methods

The upper butanol phase (0.4 ml) was retrieved and dried using a Speed-Vac concentrator. The dried samples were redissolved in chloroform/methanol (2:1, v/v) and spotted on a silica-gel TLC plate. The plate was developed in chloroform/methanol/0.25% KCl, air-dried and sprayed with EN$^3$HANCE™ spray and subjected to fluorography for 2 weeks at -80°C.

## 4.10 Partial Purification of human serum GPI-PLD

### Materials

*Ammonium sulphate:* AnalaR® obtained from BDH Laboratories Supplies, Poole, England, catalogue no. 100333B. Prepared as a saturated ammonium sulphate solution. About 40 g ammonium sulphate in 50 ml of deionised water.

*Dialysis tubing:* 12-14 kDa cut-off tubing was purchased from Medical International Ltd., London, U.K.

*Dialysis buffer:* Prepared as a 20 mM bis-Tris-HCl, pH 6.5 containing 0.01 M NaCl and 2.6 mM $CaCl_2$.

*Strong anion exchanger:* Vydac 300VHP575 was purchased from Vydac/The Separations Group, Inc., CA.

**Methods**

Human blood (120 ml) was obtained from the author and allowed to clot for 2 h at 37°C. The clot was removed by centrifugation at 10,000 x g for 10 min at 4°C and the supernatant (serum) was dialysed overnight at 4°C against the dialysis buffer with three changes of the buffer. The serum (approximately 50 ml) was transferred into a glass beaker and placed on a magnetic stirrer. While the serum was stirring, saturated ammonium sulphate (50 ml) was added slowly to the serum until the ammonium sulphate solution reached a final concentration of 50% saturation. The resulting solution was stirred for 12 h at 4°C. The supernatant was separated from the aggregates by centrifugation at 10,000 x g at 4°C for 5 min. The supernatant was dialysed against using the same buffer for 2 consecutive days with three changes of the buffer. Approximately 15 mg of protein were injected into a strong anion exchange column, Vydac 300VHP575 mounted on a preparative HPLC system (Gilson). The column was eluted with a linear gradient from 0.01 M NaCl to 0.5 M NaCl. GPI-PLD activity eluted at a broad peak between 0.35 M and 0.44 M NaCl. No flow-through activity was observed. The active fractions were pooled together and concentrated with a 100,000 M.W. cut-off Vivaspin 4 concentrator (Vivascience Ltd., London) by centrifugation at 5,000 x g at 4°C for 3 min. The retentate was subjected to SDS-PAGE on a 10–15% gradient PhastGel® system (Pharmacia Biotech AB., Uppsala, Sweden) and visualised by silver staining.

## 4.11 Silver staining

### Materials

Silver Stain Plus (catalogue no. 161-0449) was purchased from Bio-Rad Laboratories, Hercules, CA. which included the following reagents:

Fixative Enhancer Concentrate

Silver Complex Solution (contains $NH_4NO_3$ and $AgNO_3$)

Reduction Moderator Solution (contains tungstosilicic acid)

Image Development Reagent (contains formaldehyde)

Development Accelerator Reagent (contains $NaCO_3$)

*Fixative Enhancer solution:* was prepared from 50%(v/v) methanol, 10%(v/v) acetic acid, 10%(v/v) Fixative Enhancer Concentrate and 30%(v/v) deionised water on the day of use.

*Staining solution:* prepared from 1 ml of Silver Complex Solution, 1 ml of reduction Moderator Solution and 1 ml of Image Development Reagent within 5 min of use. Accelerator Solution 10 ml was added to the mixture immediately before use.

### Methods

The polyacrylamide gel was stained with the Fixative Enhancer solution in a glass vessel for 20 min at room temperature with gentle agitation. After 20 min, the gel was rinsed twice with 10 ml of deionised water, each time for 10

min with gentle agitation. The gel was stained with Staining solution at room temperature for about 20 min until desired staining intensity was reached. The staining reaction was stopped with 10 ml of 5%(v/v) acetic acid. Finally, the gel was rinsed with 10 ml of deionised water for 5 min and dried on a 3MM Whatman filter paper at 70°C for 15 min under vacuum on a slab gel dryer.

## 4.12 Inactivation of GPI-PLD in foetal bovine serum

### Materials

*Foetal bovine serum:* was purchased from Life Technologies, Paisley, U.K.
*NaOH solution:* prepared as a 1 M NaOH solution from NaOH pellets and sterilised before use.
*HCl solution:* prepared as a 1 M HCl solution from a 6 M HCl, catalogue no. 24309, constant boiling, Sequenal grade (Pierce, IL.)

### Methods

The inactivation of GPI-PLD in serum was based the method described by Küng *et al.* (1997). Serum (10 ml) was treated with 400 µl of sterile 1 M NaOH to increase the serum to pH 11 and incubated for 1 h at 37°C. The pH was adjusted to pH 7.4 with approximately 800 µl of sterilised 1 M HCl

solution. After this treatment, the GPI-PLD activity was no longer detectable as assessed by degradation of $[^3H]$ myristate mfVSG.

## 4.13 Results

### Metabolic labelling of *Trypanosoma brucei* with $[^3H]$ myristic acid

An estimated 1 $x10^9$ trypanosomes were isolated from blood from three mice. These numbers were required for metabolic labelling of trypanosomes with $[^3H]$ myristic acid. SDS-PAGE analysis of the fractionated material revealed a major protein with an estimated molecular weight of 60,000 which is consistent with the molecular weight of variant surface glycoprotein (Fig. 4.1A). Analysis of this material by fluorography showed that mfVSG was the labelled component as shown in Fig. 4.1B. The specific activity of the $[^3H]$ myristate labelled mfVSG was found to be $4.2 \times 10^6$ cpm/mg of protein.

To determine if $[^3H]$ myristate labelled mfVSG is sensitive to the action of GPI-PLD, increasing concentrations of the labelled mfVSG were incubated with bovine GPI-PLD. As the product $[^3H]$1,2-dimyristyl phosphatidic acid partitions to the upper butanol phase, it could be used to measure the activity of GPI-PLD. The amount of $[^3H]$ mfVSG converted was assessed by the levels of radioactivity detected in the butanol phase (Fig. 4.2). The results were expressed as a linear graph using the Lineweaver-Burk plot. The points at which the graph cut the y-axis and x-axis give Vmax and K*m* respectively. The Vmax and K*m* of the enzyme were found to be 1,000 unit/min and 1 μM respectively.

## Analysis of the $[^3H]$ myristate labelled mfVSG-degradation products

To ascertain whether the labelled mfVSG degradation product following GPI-PLD hydrolysis was $[^3H]$ phosphatidic acid, the reaction products were analysed by thin layer chromatography. As shown in Fig. 4.4, bovine GPI-PLD (lane 1), partially purified human GPI-PLD (lane 4 and 5) and foetal bovine serum (lane 6) generated $[^3H]$ dimyristyl phosphatidic acid when incubated with labelled mfVSG. In contrast, GPI-PLC from *T. brucei* generated $[^3H]$ 1,2-diacylglycerol (Fig. 4.4, lane 2). The $R_f$ of $[^3H]$ dimyristyl phosphatidic acid and $[^3H]$ 1,2-diacylglycerol were found to be 0.69 and 0.91 respectively. These values are in agreement with the $R_f$ values of 1,2-diacylglycerol and phosphatidic acid described in Xie and Low (1994). Lane 7 shows effect of alkaline treatment on foetal bovine serum. Lane 8 shows reaction performed in the presence of buffer alone. Lane 9 and 10 depict extraction control (ammonium hydroxide saturated *n*-butanol) and carrier control (chloroform/methanol) respectively.

**Effect of AraLAM and ManLAM on GPI-PLD activity**

Previous studies have shown that GPI-PLD are sensitive to inhibition by phosphatidic acid and lipid A (Low and Huang, 1993). Since LAM is a major glycolipid found in mycobacteria, we examined the effect of AraLAM and ManLAM on [$^3$H] myristate labelled mfVSG-degradation by GPI-PLD. As shown in Fig. 4.5, ManLAM was inhibitory to GPI-PLD activity on mfVSG degradation and the inhibition by ManLAM was dose-dependent. In contrast, AraLAM showed little or no inhibition on GPI-PLD activity.

**Partial purification of human GPI-PLD**

A 110 kDa band corresponded to the expected size of GPI-PLD was observed in Fig. 4.6. There were other bands which reflected other proteins found in the serum.

**Effect of J774A.1 cells grown in alkaline treated serum on nitrite production**

J774A.1 cells grown in alkaline treated serum were more sensitive to IFN-$\gamma$-induced nitrite production as compared to those grown in untreated serum as shown in Fig. 4.7 and 4.8. The synergy between IFN-$\gamma$ and LPS for nitrite production was observed in cells grown in untreated serum whereas high levels of LPS were required to synergise with IFN-$\gamma$ to induce nitrite

production in cells grown in alkaline treated serum (Fig. 4.7). In contrast, *M. tuberculosis* H37Ra synergised with IFN-$\gamma$ to stimulate nitrite production in J774A.1 cells cultured in alkaline treated serum as shown in Fig. 4.8.

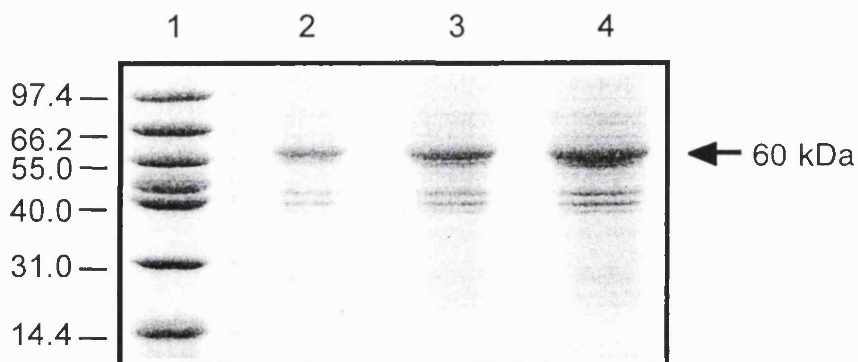



**Fig. 4.1A. SDS-PAGE of *T. brucei* mfVSG.** The isolated mfVSG was separated on a 10% SDS-PAGE gel. This was followed by staining the gel with Coomassie blue dye. Lane 1, mid-range molecular weight protein markers in kiloDalton (Promega Corporation); Lane 2; 3 and 4; mfVSG at 5 μg, 10 μg and 15 μg per well respectively.

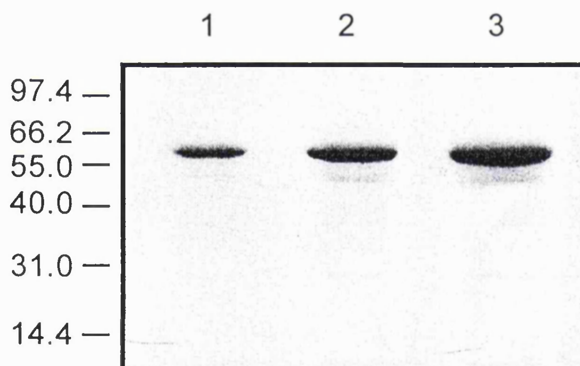



**Fig. 4.1B. Fluorography of the mfVSG from *T. brucei*.** The gel was fixed, stained with Coomassie blue dye, destained and treated with $EN^3HANCE^{TM}$ solution. The resulting gel was dried at 70°C under vacuum and exposed on to a X-ray film for 24 hr at -70°C. Lane 1, 2 and 3, mfVSG at 5, 10 and 20 μg respectively. The numbers on the left indicate the mid-range molecular weight protein markers in kiloDalton (Promega Corporation).

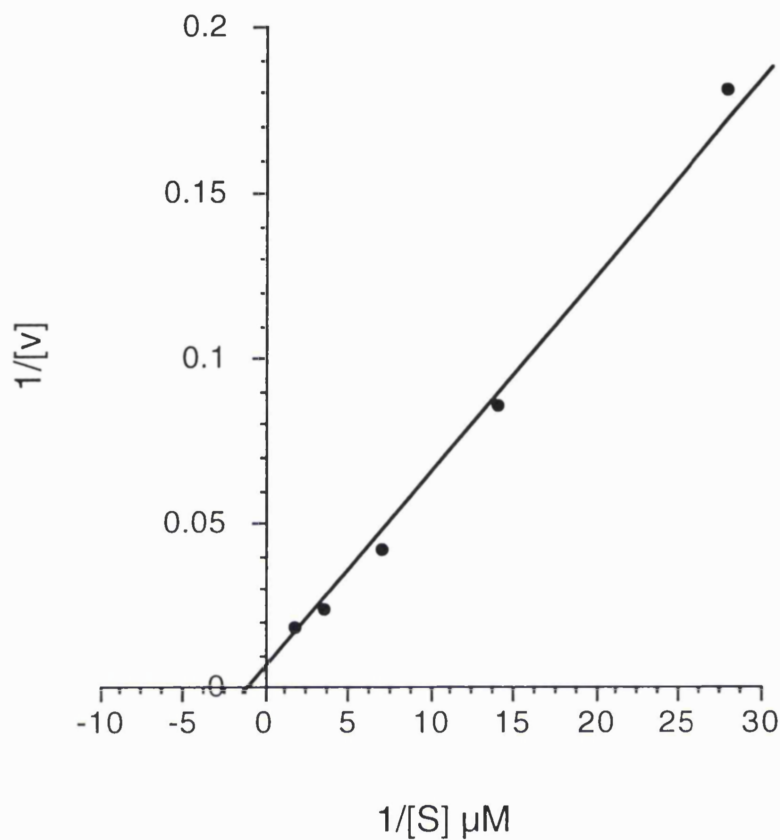



**Fig. 4.2. Lineweaver-Burk plot of bovine GPI-PLD cleaving [$^3$H] myristate labelled VSG.** Bovine GPI-PLD (0.02 U) was incubated with increasing concentrations of [$^3$H] myristate labelled mfVSG in 200 µl of 20 mM Tris-maleate, 0.075% NP-40, pH 7.0, at 37°C for 30 min. The reaction was stopped by the addition of 0.5 ml of 1 M ammonium hydroxide saturated n-butanol. The radioactivity in 300 µl of the organic phase was measured and total radioactivity of converted mfVSG was calculated. The radioactivity of the negative controls without GPI-PLD containing 2, 4, 8, 16 and 31 µgm/l mfVSG were 46, 52, 70, 106 and 173 cpm respectively. The results shown are representative of two similar experiments.

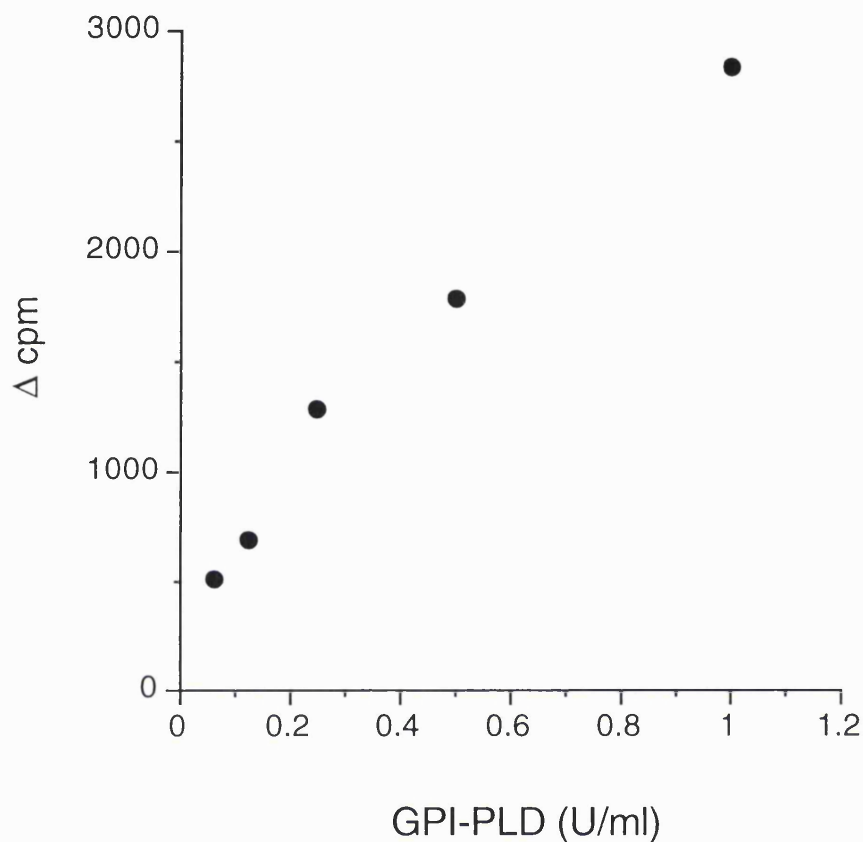



**Fig. 4.3. GPI-PLD assay with varying concentration of GPI-PLD.** [$^3$H] myristate-labelled mfVSG (31 µg/ml) was incubated with increasing concentration of bovine GPI-PLD in 200 µl of 20 mM Tris-maleate, 0.075% NP-40, pH 7.0, at 37°C for 30 min. The reaction was stopped by the addition of 0.5 ml of 1 M ammonium hydroxide saturated n-butanol. The radioactivity in 300 µl of the organic phase was measured and the total amounts of mfVSG converted were calculated. The radioactivity of the negative control without GPI-PLD containing 31 µg/ml was 169 cpm. The results shown are representative of two similar experiments.

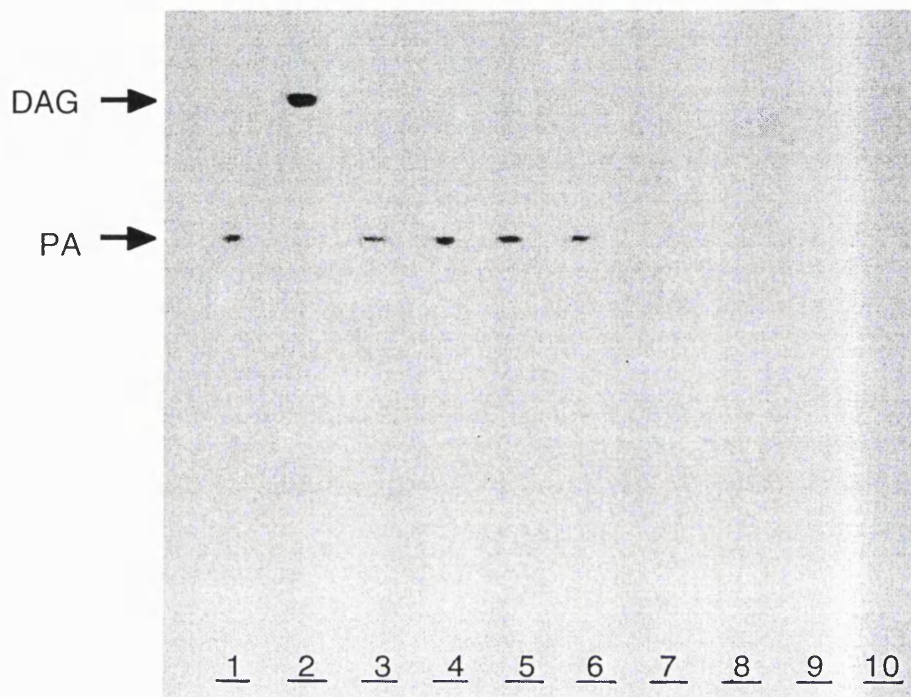



**Fig. 4.4. Analysis of the [$^3$H] myristate labelled mfVSG-degradation products.** [$^3$H] myristate labelled mfVSG was incubated for 3 h at 37 °C as follows: lane 1 and 3, bovine serum GPI-PLD (0.5 U/ml); lane 2, *T. brucei* GPI-PLC (0.5 U/ml); lane 4 and 5, partially purified human GPI-PLD; lane 6, foetal bovine serum (220 µg/ml); lane 7, foetal bovine serum (220 µg/ml) incubated at pH 11 for 1 h at 37°C, lane 8, 20 mM Tris-maleate solution containing 0.05% NP-40; lane 9, 1 M ammonium hydroxide saturated n-butanol; and lane 10, chloroform/methanol (2:1). GPI-PLD and GPI-PLC cleaved [$^3$H] myristate mfVSG to form [$^3$H] 1,2-dimyristoyl phosphatidic acid (PA) and [$^3$H] 1,2-dimyristoly glycerol (DAG) respectively. The results shown are from a single experiment.

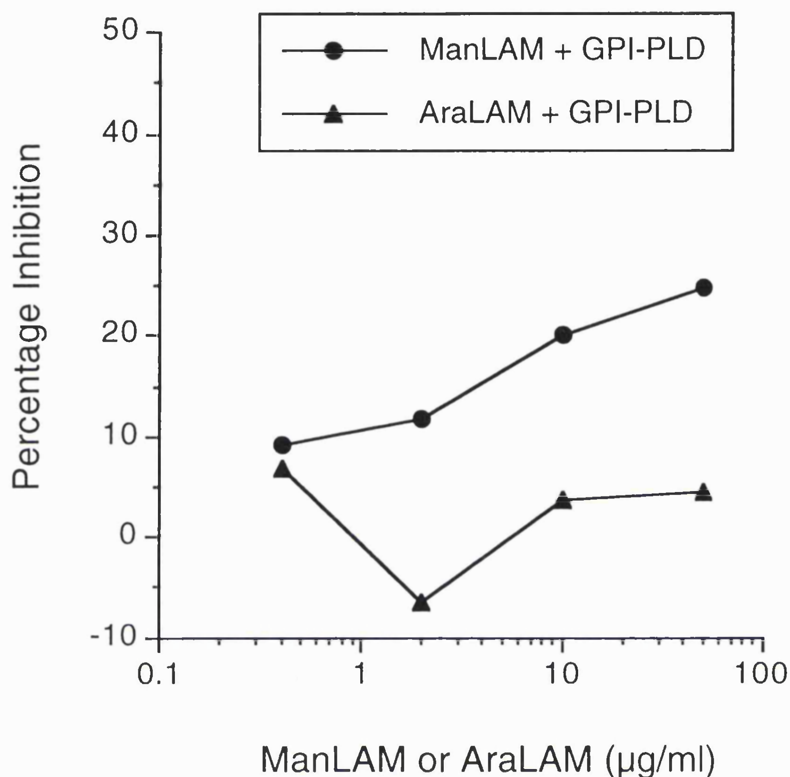



**Fig. 4.5. Effect of AraLAM and ManLAM on GPI-PLD activity.** [$^3$H] myristate labelled mfVSG (31 μg/ml) was preincubated with increasing concentrations of AraLAM or ManLAM on ice for 30 min. The reaction was started by the addition of 0.05 U of bovine GPI-PLD and the mixtures were incubated at 37°C for 30 min. The reaction was stopped by the addition of 0.5 ml of 1 M ammonium hydroxide saturated *n*-butanol. The radioactivity in 300 μl of the organic phase was measured and the total amounts of mfVSG converted were calculated. The radioactivity in Eppendorf tube with GPI-PLD and without GPI-PLD were 2,790 and 272 cpm respectively. The results shown are from a single experiment.

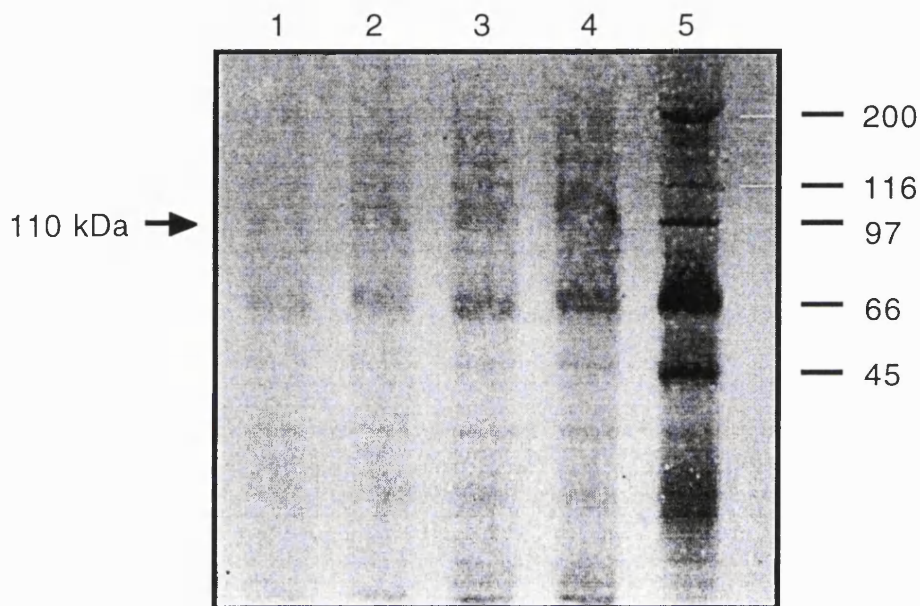



**Fig 4.6. SDS-PAGE of the partially purified human GPI-PLD.** The active pooled fractions were separated on a 10–15% gradient PhastGel®. This was followed by staining the gel with sliver staining. Lane 1, 0.01 µg GPI-PLD; Lane 2, 0.02 µg GPI-PLD; Lane 3, 0.05 µg GPI-PLD; Lane 4, 0.1 µg GPI-PLD and Lane 5, high-range molecular weight protein standard markers in kDa (Bio-Rad Laboratories).

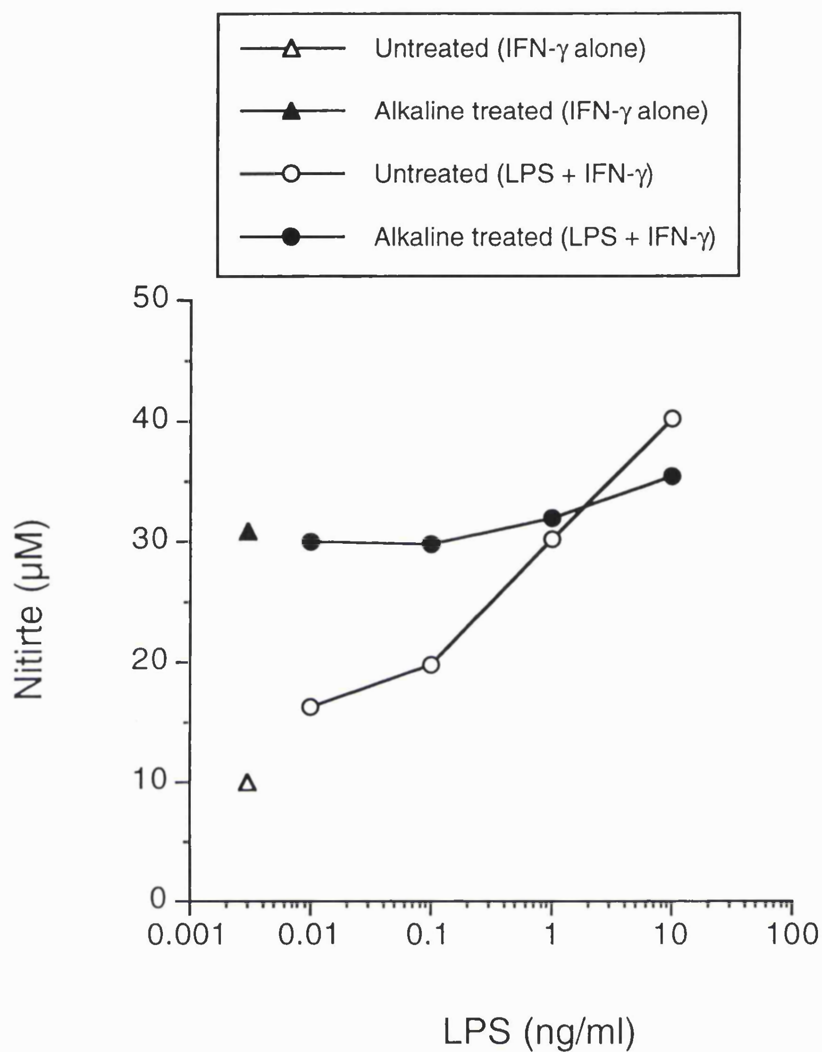



**Fig. 4.7. Effect of alkaline treatment on IFN-γ and LPS-induced nitrite production.** J774A.1 cells (1 x $10^5$) previously grown in 10% FBS or 10% alkaine treated FBS were incubated with 200 U/ml of IFN-γ or IFN-γ with increasing concentrations of LPS for 24 h. Nitrite concentrations were determined by using the Griess reaction. The results were expressed as means ± S.D. of triplicate culture from a single experiment. Where the error bars are not shown, the values fall within the symbols.

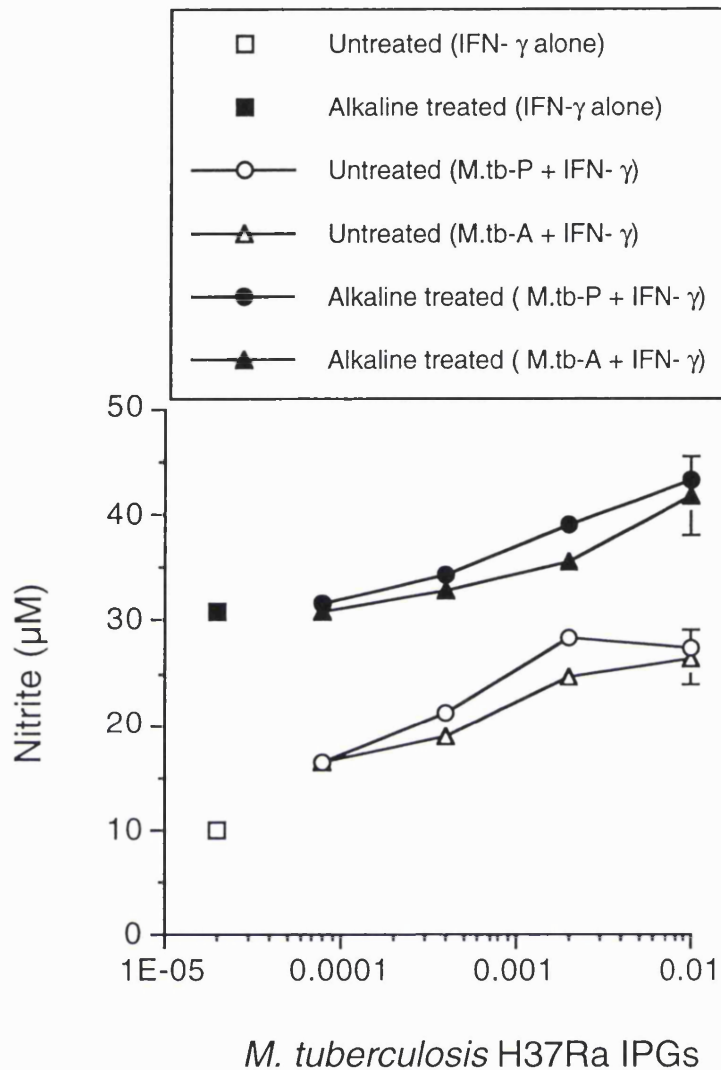



**Fig. 4.8. Effect of alkaline treatment on IFN-γ and *M. tuberculosis* IPG-induced nitrite production.** J774A.1 cells (1 x $10^5$) previously grown in 10% FBS or 10% alkaline treated FBS were incubated with 200 U/ml of IFN-γ or IFN-γ with increasing concentrations of *M. tubeculosis* H37Ra P-type or A-type IPGs for 24 h. Nitrite concentrations were determined by using the Griess reaction. The results were expressed as means ± S.D. of triplicate culture from a single experiment. Where the error bars are not shown, the values fall within the symbols.

## 4.14 Discussion

Variant surface glycoprotein (VSG) is a GPI-anchored protein (Ferguson, *et al.*, 1988) found in abundance in trypanosomes which includes *Trypanosoma brucei*, a pathogenic protozoan which causes sleeping sickness in humans and Nagana in cattles. It has been shown that an estimated 10 million copies of VSG is present on a single trypanosome (Jackson, *et al.*, 1985). Thus, it can serve as a good source of GPI-anchored proteins. In this study, a large number of trypanosomes could be isolated from mice infected with *T. brucei.* Their membrane form VSGs were labelled with [$^3$H] myristic acid prior to isolation. The [$^3$H] myristate labelled mfVSG was purified to near homogeneity and mfVSG was the only labelled component as assessed by SDS-PAGE and fluorography respectively. The labelled mfVSG was sensitive to cleavage by both *T. brucei* GPI-PLC and mammalian GPI-PLD which were consistent with the published results (Bülow and Overath, 1986, Hereld, *et al.*, 1986, Malik and Low, 1986).

It has been shown that GPI-PLC cleaves at the phosphodiester bond of the mfVSG to liberate 1,2-dimyristyl glycerol. In contrast, GPI-PLD hydrolyses the mfVSG to release 1,2-dimyristyl phosphatidic acid (Malik and Low, 1986). 1,2-Dimyristyl phosphatidic acid differs from 1,2-dimyristyl glycerol in that the former has a phosphate attached to the glycerol backbone. Thus, both products migrate at different rates on a t.l.c plate which result in different R*f* values. The data showed that the labelled mfVSG was cleaved by bovine and human GPI-PLD to generate 1,2-dimyristyl phosphatidic acid.

Since GPI-PLD activity is inhibited by phosphatidic acid and lipid A (Low and Huang, 1993), the ability of mycobacterial LAMs to inhibit GPI-PLD *in vitro*

was examined. The results showed that ManLAM but not AraLAM was inhibitory to GPI-PLD-mediated release of [$^3$H] dimyristyl phosphatidic acid. GPI-PLD inhibition by ManLAM was not attributed to the effect of LPS present in the preparation because similar levels of LPS were only slightly inhibitory to GPI-PLD activity. As the concentration of ManLAM required to achieve 50% inhibition of GPI-PLD activity was higher than that of LPS, the significance of this finding under physiological conditions remains to be established. However, there has been a report showing that mycobacterial LAM accumulates within macrophages following infection with *M. tuberculosis* (Xu*, et al.*, 1994). This has led to the speculation that ManLAM may incapacitate the ability of membrane associated GPI-PLD to generate IPG second messengers leading to the perturbation of macrophage activation. The hypothesis could be tested by raising antibodies against the partially purified GPI-PLD which would block the mammalian cells' ability to generate their own IPGs, and thereby substitute with mycobacterial IPGs to influence their effect on macrophage activation as illustrated in Fig. 4.9. Küng *et al.* (1997) have shown that GPI-PLD activity is inactivated by alkaline treatment. Preliminary data showed that J774A.1 cells cultured in the alkaline treated serum displayed an increased response to IFN-$\gamma$ and LPS, and *M. tuberculosis* IPGs.

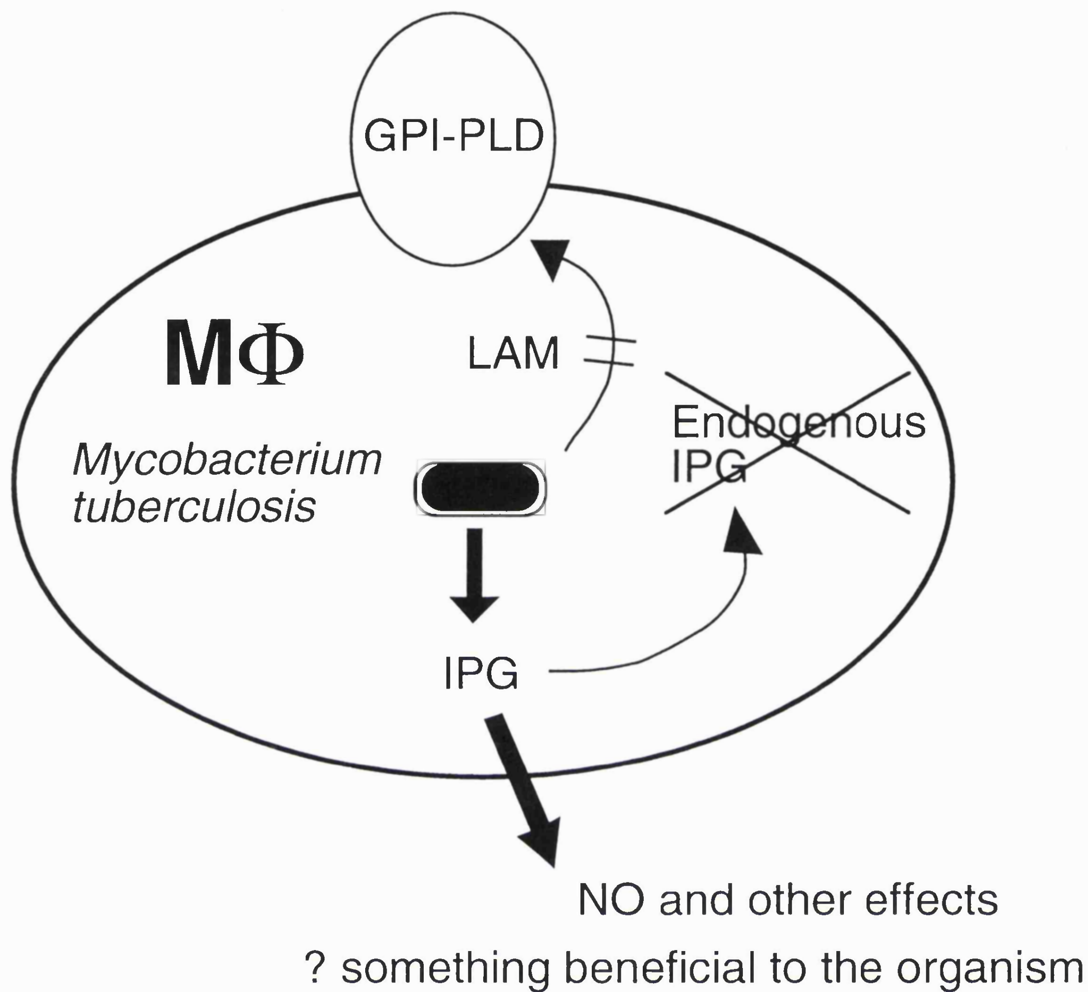



**Fig. 4.9 Hypothetical "take-over" of IPG function in a cell infected with *M. tuberculosis*.** LAM inhibits GPI-PLD, and therefore blocks endogenous IPG release, leaving all IPG-mediated control of enzyme function to the very different mycobacterial IPG.

# Chapter Five

## General Discussion

## 5.1 General discussion

It has been shown that mammalian and mycobacterial IPGs can be isolated using a AG1-X8 strong anion exchange column. Since the isolation of IPG involves boiling in acid and elution with acid, they are likely to be heat and acid resistance. Like the mammalian IPGs, the mycobacterial IPGs mimic the effects of insulin in a number of enzymatic assays and bioassays. Hitherto, two types of mammalian IPGs have been described: P-type and A-type. P-type IPGs from non-pathogenic saprophyte (*M. vaccae*) and an obligate intracellular pathogen (*M. tuberculosis* H37Rv) activate pyruvate dehydrogenase (PDH) phosphatase, a key enzyme involves in glycogenesis. In contrast, A-type IPGs from *M. vaccae* and *M. tuberculosis* H37Ra activate acetylCoA carboxylase to stimulate lipogenesis in adipocytes. The molecular mechanisms by which mycobacterial IPGs activate those two enzymes remain to be elucidated. However, previous studies have shown that the IPGs isolated from rat liver increase the affinity of PDH phosphatase for divalent cations (Lilley, *et al.*, 1992). This is in agreement with a study showing that IPGs are markedly released following treatment with ATP-$Mn^{2+}$ (Suzuki, *et al.*, 1987). Preliminary studies using ion chromatography have shown that the P-type and A-type IPG from rat liver contains $Mn^{2+}$ and $Zn^{2+}$ respectively. This has led to the suggestion that IPGs may function as metal shuttles which transport divalent cations to the target enzyme leading to the activation of the enzyme.

It is well recognised that many metal ions function as activators for numerous enzymes and physiological processes. Their uptake and secretion are imperative for the maintenance of homeostasis in cell growth, division and resistance to mycobacterial infection (as reviewed in Supek, *et al.*, 1997,

Govani and Gros, 1998). Supek *et al.* (1996) have shown that mutation of a Smf1 manganese transporter in yeast results in the cessation of growth in the presence of EGTA and low concentrations of $Mn^{2+}$. However, the growth was restored by the overexpression of Smf1 and addition of $Mn^{2+}$ in the culture medium. Amino acid anlaysis of Smf1p protein shows 30% identity with the human and mouse **n**atural **r**esistance **a**ssociated **m**acrophage **p**rotein. This protein is encoded by the *Nramp* gene in the mouse which confers resistance to mycobacterial infection. Moreover, the *Nramp* gene is identical to the *Ity* and *Lsh* gene conferring resistance to infection by *Salmonella typhimurium* and *Leishmania donovani* respectively. More recently, a Nramp homologue has been found in *M. tuberculosis* which is responsible for transporting $Mn^{2+}$ and $Zn^{2+}$ (Agranoff, *et al.*, 1999). Since the survival of intracellular pathogen against respiratory burst is partly mediated by the microbial superoxide dismutase (Welch, *et al.*, 1979), which contains $Mn^{2+}$ or $Fe^{2+}$ in its centre, the function of the Nramp may be to limit $Mn^{2+}$ within the phagosome which leads to the killing of the pathogen. It is conceivable that mycobacterial IPGs may function as a $Mn^{2+}$ chelator which competes for $Mn^{2+}$ within the macrophage to preclude the depletion of $Mn^{2+}$. Thus, the notion that mycobacterial IPGs carry metal ions awaits metal analysis.

Unlike mammalian IPGs extracted from rat liver and human placenta, mycobacterial IPGs could synergise with IFN-$\gamma$ to stimulate nitrite production in J774A.1 macrophages. Mycobacterial IPG and IFN-$\gamma$-induced nitrite production was associated with the up-regulation of iNOS protein. It has been shown that *M. tuberculosis* (H37Rv and H37Ra) IPGs are more effective nitrite inducers that *M. vaccae* IPGs. This difference in potency may reflect the structural differences between *M. tuberculosis* and *M. vaccae* IPGs.

Previous studies have shown that AraLAM differs from ManLAM in that the former has an extensive arabinan side chain and therefore, is a potent inducer of iNOS and nitric oxide (Anthony, *et al.*, 1994, Roach, *et al.*, 1995). In contrast, ManLAM has arabinan side chains which are masked by short mannan segments and hence are poor inducer of nitric oxide. Preliminary data using **m**atrix **a**ssisted **l**aser **d**esorption **i**onisation (MALDI) **f**ourier **t**ransformed (FT) mass spectrometry have shown that *M. tuberculosis* H37Rv P-type and A-type IPGs compose of three major compounds, which include dihexose inositol monophosphate, dihexose monophoshate and trihexose monophosphate. However, it requires further investigations to determine which compound(s) are the active fractions.

The induction of nitric oxide production in response to IFN-$\gamma$ and TNF-$\alpha$ is a well documented phenomenon. TNF-$\alpha$ on its own is completely inactive, however, it synergise with IFN-$\gamma$ to stimulate macrophages to release high levels of nitric oxide. Gram-positive bacteria, Gram-negative bacteria, LPS, muramyl dipeptide and bacterial toxins are potent inducer of TNF-$\alpha$, which synergises with IFN-$\gamma$ to stimulate macrophages to synthesise and respond to TNF-$\alpha$ in an autocrine manner. Interestingly, mycobacterial IPGs differ from other stimuli in that they lack the ability to generate TNF-$\alpha$ even in the presence of IFN-$\gamma$. However, this lack of TNF-$\alpha$ release did not preclude the induction of iNOS expression and NO synthesis in J774A.1 cells. These phenomena may be unique to mycobacterial IPGs. $PGE_2$ and IL-10 have been implicated in the inhibition of TNF-$\alpha$ synthesis without affecting NO production. Thus, the release of $PGE_2$ and IL-10 induced by mycobacterial IPGs will have a profound effect on macrophage functions. It will be interesting to determine if mycobacterial IPGs can stimulate J774A.1 cells to synthesise $PGE_2$ and IL-10. It will be imperative to evaluate the levels of

TNF-α in J774A.1 cells pretreated with mycobacterial IPGs following stimulation with LPS.

IL-1β is released primarily by activated macrophages in response to microbial stimuli and proinflammatory cytokines. Previous studies have shown that IL-1β activates phospholipase $A_2$ and cyclooxygenase to synthesise prostaglandins. *M. tuberculosis* IPGs synergised with IFN-γ to release high levels of IL-1β, the lack of soluble TNF-α may be attributed to $PGE_2$ stimulated by IL-1β. This is reminiscent of a report showing carbohydrate components from *Ehrlichia chaffeensis* stimulate IL-1β but not TNF-α release in human monocytes (Lee and Rikihisa, 1996). Similarly, IL-1β but not TNF-α is also released by human monocytes following stimulation with pneumococcal cell components (Riesenfeld-Orn, *et al.*, 1989).

Whilst the precise mechanism of macrophage activation by mycobacterial IPGs remains elusive, the study of the effect of mycobacterial IPG on macrophage functions may provide clues to the pathogenesis of tuberculosis.

**Future work**

Although the purification of the human GPI-PLD was not pursued due to the lack of time, a recombinant GPI-PLD protein has been made by a colleague. Thus, neutralising antibodies could be raised against GPI-PLD to block endogenous IPG production. Firstly, such antibodies could be used to investigate the effect of mycobacterial IPGs on macrophage functions. Secondly, these antibodies could be conjugated to a column to prepare GPI-

PLD-depleted medium. This would verify the high levels of nitrite produced by J774A.1 cells grown in the absence of GPI-PLD as opposed to cells cultured in alkaline-inactivated medium in response to IFN-$\gamma$. Preliminary data using reverse-transcription polymerase chain reaction (RT-PCR) have shown that J774A.1 cell contains an amplified product. Further studies using DNA sequencing are required to ascertain the identity of the RT-PCR product. There are two isoforms of GPI-PLD: isoform 1 and 2 which are expressed in a $\beta$TC3 pancreatic cell line and a HepG2 hepatic cell line respectively (O'Brien, *et al.*, 1999). Previous studies have shown that GPI-PLD isoform 1 is expressed in human macrophages. Thus, a similar GPI-PLD isoform may be expressed in J774A.1 cells.

Since LPS and mycobacterial IPGs share their ability to induce nitrite production, it is likely that they utilise similar receptors such as CD14 and the TLRs to trigger NF-$\kappa$B activation which leads to the induction of iNOS. The approach would be to evaluate the nitrite levels following stimulation with mycobacterial IPG and IFN-$\gamma$ in the presence of antibodies against the TLRs. Alternatively, whether mycobacterial IPG activates the TLRs could also be investigated on a cell line transfected with both TLR expression vector and a NF-$\kappa$B dependent reporter gene. The activation of the TLRs by the mycobacterial IPGs would result in the transcription of the NF-$\kappa$B dependent reporter gene.

Previous studies have shown that antibodies against LAM have been found in patients with active tuberculosis (Li, 1990, Da Costa, *et al.*, 1993, Del Prete, *et al.*, 1998, Prignot, 1998, Sousa, *et al.*, 1998). Whether patients with tuberculosis have neutralising antibodies against mycobacterial IPGs remains to be elucidated.

# Appendix

**Coomassie Brilliant Blue Stain**

0.2%(w/v) Brilliant blue 6

10%(v/v) glacial acetic acid

45%(v/v) methanol

45%v/v) deionised water

**Coomassie Blue Destain**

10%(v/v) glacial acetic acid

45%(v/v) methanol

45%(v/v) deionised water

**2X Protein Loading dye**

1%(v/v) SDS

10 mM EDTA

10 mM Sodium phosphate, pH 7.0

1%(v/v) 2-mercaptoethanol

15%(v/v) glycerol

0.01%(w/v) bromophenol blue

4 mM PMSF

**Phosphate-buffered Saline, pH 7.4**

137 mM sodium chloride

1.47 mM anhydrous potassium dihydrogen phosphate

8 mM anhydrous disodium hydrogen orthophosphate

2.7 mM potassium chloride

0.05% (v/v) Tween 20

**Lowry Assay of Protein Concentration**

**Materials**

*Potassium sodium tartarate:* prepared as a 2% (w/v) solution in deionised water and stored at room temperature.
*Cupric sulphate penhydride:* prepared as a 1% (w/v) solution in deionised water and stored at room temperature.
*Sodium hydroxide:* prepared as a 0.1 M solution in deionised water stored at room temperature.
*Sodium carbonate:* prepared fresh as a 2% (w/v) solution in 0.1 M sodium hydroxide.
*Folin and Ciocalteau phenol reagent*

**Methods**

Protein concentration was determined using the Lowry reaction. This was based on the reaction of protein and copper tartarate under alkaline condition. The copper treated protein subsequently reduced the Folin reagent to one or few reduced species which formed a characteristic blue colour.

Sample (20 µl) was added into Nunc-Immuno™ plate 96-well flat bottom plate (Nalge Nunc International, Denmark). This was followed by the addition of 80 µl of reagent A (2%(w/v) potassium sodium tartarate, 1%(w/v)

cupric sulphate penhydride and freshly made 2%(w/v) sodium carbonate in 0.1 M sodium hydroxide in a ratio of 1:1:50 (v/v/v) respectively). The plate was allowed to stand for 10 min before adding 100 µl reagent B (1 ml of Folin Ciocalteau phenol reagent diluted with 13.6 ml of water). It was allowed to stand for 10 min prior to reading the plate at 650 nm using a (ThermoMax, Molecular Devices). The protein concentrations were determined using bovine serum albumin standards.

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