Chemical and immunological characterisation of glycophospholipid and phospho-oligosaccharide from mycobacteria

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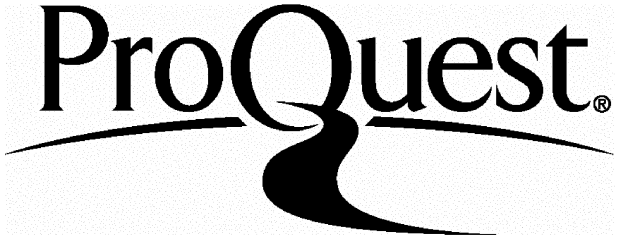

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Abstract

Following ligand binding by a wide variety of cytokines and growth factors, a glycosylphosphatidyl inositol phospholipase D (GPI-PLD) cleaves free membrane glycosylphosphatidyl inositol (GPI) to liberate water-soluble inositol phosphoglycan (IPG) second messengers. IPG are released outside cells and mediate many of the immediate metabolic effects of insulin. Many other cytokines and growth factors use IPG to signal, including IL-2, NGF, IGF-1 and ACTH. The composite structure of IPG molecules is known and they consist of hexose, hexosamine, inositol, phosphate and divalent cations.

The development of clinical tuberculosis in a susceptible host appears to involve a highly complex interaction between the infecting organism and the host immune system, with much or indeed all the tissue damage characteristic of the disease being immune mediated. Many *Mycobacterium tuberculosis* colonised individuals do not develop active disease, and there are demonstrable immunological differences between the asymptomatic immune carrier state and the state of active disease in both humans and animal models.

Mycobacterium tuberculosis contains PLD activity and contains phosphatidyl inositol linked glycans that could theoretically give rise to IPG like structures. Such glycolipids include lipoarabinomannan (LAM), lipomannan (LM) and phosphoinositol mannoside (PIM). Previously it has been shown in our laboratory and that of our collaborators that two strains of the pathogen *M. tuberculosis* and a non-pathogen, *M. vaccae,* contain IPG-like biological activity. This mycobacteria derived material has been provisionally named phospho-oligosaccharide (POS).

The aim of this study was to examine the hypothesis that mycobacteria may contain a homologous system to the mammalian GPI/IPG signalling mechanism. Using an established protocol for the purification of mammalian GPI a glycophospholipid (GPL) was isolated from *M. tuberculosis* and *M. vaccae* that showed similar characteristics to mammalian GPI. This GPL was a substrate for (glycosyl)phosphatidylinositol phospholipase C (GPI-PLC), contained phosphate, sugar residues and did not contain amino groups. GPL was biologically active in a cell free pyruvate dehydrogenase (PDH) phosphatase activation assay and induced inflammatory mediator release from

monocyte/macrophage cells. GPL is distinct from LAM, LM and PIM because it was sensitive to GPI-PLC and incorporated radioactive galactose. POS fractions were isolated from mycobacteria that were biologically active in PDH phosphatase, lipogenesis, cell proliferation and nitric oxide production assays, contained carbohydrate, phosphate (except for one fraction) and amino groups. On the basis of amino group analysis, which revealed that POS contained amino groups and GPL did not, POS is not a cleavage product of mycobacterial GPL, as is the case with GPI and IPG in mammalian cells. However, there were interesting similarities in biological activity between GPL and POS.

Serological studies were undertaken to characterise mycobacterial POS both structurally and immunologically. Although POS exhibit biological activity in systems where mammalian IPG are also active, there are clearly structural differences between POS and IPG because some antibodies that bind IPG do not bind POS. POS are immunogenic: administration of complete Freund's adjuvant induced an anti-POS antibody response in rabbits (but not in mice) and sera from tuberculosis patients contained significantly higher levels of anti-POS antibody than healthy controls. POS is proliferogenic for human peripheral blood mononuclear cells *in vitro*, in particular, POS activates human B cells *in vitro* causing a greater increase in B cell CD25 expression than T cell CD25 expression.

Thus the material described has some interesting immunological properties, although its role in the pathogenesis of tuberculosis remains to be determined. The growth factors IL-2 and insulin have both been shown to signal using IPG cleaved from GPI, it is therefore intriguing that mycobacteria appear to contain a homologue of an immunologically relevant mammalian second messenger.

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List of figures and tables

List of abbreviations used

ACTH	Adrenocorticotrophic hormone
AIDS	Acquired immunodeficiency syndrome
AraLAM	Arabinose capped lipoarabinomannan
ATP	Adenosime triphosphate
β2-m	β2 microglobulin
BCG	Bacillus Calmette Geurin
BMBR	Boehringer Mannheim blocking reagent
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CD	Cluster of differention antigen
CFA	Complete Freund's adjuvant
CPM	Counts per minute
Cy5	Cyanine 5
DAG	Diacyl glycerol
DMEM	Dulbeco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPM	Disintegrations per minute
DTH	Delayed type hypersensitivity
EDTA	Ethylene diamine tetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme linked immunosorbent assay
FACS	Fluorescence activated cell sorting
FAS-1	Fatty acid synthase 1
FasL	Fas ligand
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
GPI	Glycosylphosphatidyl inositol
GPI-PLC	Glycosylphosphatidyl inositol phospholipase C
GPI-PLD	Glycosylphosphatidyl inositol phospholipase D
GPL	Glycophospholipid

HBSS	Hank's balanced salts solution
HEK	Human embryonic kidney
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
HPTLC	High performance thin layer chromatography
HRPO	Horse radish peroxidase
hsp	Heat shock protein
IFA	Incomplete Freund's adjuvant
IFNγ	Interferon gamma
Ig	Immunoglobulin
IGF	Insulin like growth factor
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IPF	Idiopathic pulmonary fibrosis
IPG	Inositol phosphoglycan
IPP	Isopentenyl pyrophosphate
KO	Knockout
LAM	Lipoarabinomannan
LAMP-1	Lysosome associated membrane protein 1
LM	Lipomannan
LPS	Lipopolysaccharide
ManLAM	Mannose capped lipoarabinomannan
MEP	Monoethyl phosphate
mfVSG	Membrane form variant surface glycoprotein
MHC	Major histocompatibility complex
MR	Mannose receptor
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
NAD^+	Nicotinamide adenine dinucleotide (oxidised form)
NADH	Nicotinamide adenine dinucleotide (reduced form)
NF-κB	Nuclear factor kappa B
NGF	Nerve growth factor
NKT	Natural killer T lymphocyte

NMR	Nuclear magnetic resonance
NO	Nitric oxide
NOS	Nitric oxide synthase
O.D.	Optical density
PA	Phosphatidic acid
PAD	Pulsed amperometric detection
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PC	Phosphatidyl choline
PDC	Pyruvate dehydrogenase complex
PDH	Pyruvate dehydrogenase
PE	Phosphatidyl ethanolamine
PE	Phycoerythrin
PerCP	Peridinin chorophyll protein
PHA-P	Phytohaemagglutinin P
PI	Phosphatidyl inositol
PI-GAM	Phosphoinositide capped glyceroarabinomannan
PIM	Phosphatidyl inositol mannoside
PI-PLC	Phosphatidyl inositol phospholipase C
POS	Phospho-oligosaccharide
PPD	Purified protein derivative
Rf	Retardation factor
RNI	Reactive nitrogen intermediates
ROI	Reactive oxygen intermediates
RPMI	Rosewell Park Memorial Institute
RT-PCR	Reverse transcription polymerase chain reaction
SDS	Sodium dodecyl sulphate
STF	Soluble tuberculosis factor
sTNFR	Soluble tumour necrosis factor receptor
sVSG	Soluble variant surface glycoprotein
TAP	Transporter associated with antigen processing
TCR	T cell receptor
TGF	Transforming growth factor

Th	T helper lymphocyte
TLC	Thin layer chromatography
TLR	Toll like receptor
TMB	3,3',5,5'-tetramethylbenzidine
TNFα	Tumour necrosis factor alpha
TNFR	Tumour necrosis factor receptor
TRAF2	Tumour necrosis factor receptor associated factor 2
TUBag	Tuberculosis antigen
TUNEL	Terminal deoxynucleotidyl nick end labelling
VSG	Variant surface glycoprotein

Chapter 1

Introduction

The global threat of tuberculosis

Tuberculosis (TB) is a chronic disease of humans, usually (although not always) of the lung, caused by the organism *Mycobacterium tuberculosis*. TB is one of the most important diseases to affect mankind. It is estimated that two billion people, or one third of the world's population, are infected with *M. tuberculosis* and that in 1998 there were 8 million new clinical cases and 1.5 million deaths due to the disease (WHO global heath report, 1999). TB now may kill as many as three million people a year, making it one of the most significant causes of premature preventable death worldwide. Deaths from tuberculosis are particularly frequent in the developing world where access to healthcare is limited.

Effective anti-tuberculous drugs have been available for almost half a century and a vaccine for even longer, yet prevention and treatment of tuberculosis still pose a significant problem to clinicians. An important reason for the current failure to control tuberculosis is the fact that even the best available treatment must be continued for six months. For reasons of logistics and cost, this treatment regimen is not a realistic proposition in many developing countries, where most deaths due to tuberculosis occur. Nor, indeed, is this an option in inner cities of the developed world, where tuberculosis distribution closely parallels the distribution of poverty (Spence *et al.* 1993). The problem is that the patients feel well after a few weeks and stop taking their anti-mycobacterial drugs. If the treatment regimen is stopped prematurely, however, there is a great risk that the patient will relapse back into a state of active disease (Balasubramanian *et al.* 1990).

1.1 Immunity to mycobacteria and *M. tuberculosis*

Despite the virulent nature of *M. tuberculosis* and its obvious success as a human pathogen, the majority (around 90%) of HIV negative *M. tuberculosis* infected individuals remain well and never go on to develop active disease (Kochi 1991). Of the 5 to 15% that develop active disease half do so within three years of infection and half do so three years or more after infection. These two distinct clinical syndromes are respectively known as primary and post-primary tuberculosis. Primary tuberculosis is disease immediately following

infection, whereas post-primary tuberculosis is re-activation of latent infection (Stead 1967).

1.1.2 Protection versus immunopathology

The reasons for the necessity of a six-month treatment regimen and that 90% of *M. tuberculosis* infected individuals do not develop disease are interrelated. Most tuberculosis patients in fact have a necrotising pattern of immune response to *M. tuberculosis* analogous to that first described by Koch in guinea pigs (Koch 1891). Koch observed that 4-6 weeks after infection in guinea pigs intradermal challenge with either whole organisms or culture filtrate resulted in necrosis both locally and in the original tuberculous lesion; this pattern of response became known as the Koch phenomenon. A similar phenomenon also occurs in humans: the tuberculin test is frequently necrotic in tuberculosis patients or people who have been tuberculous (Rook *et al.* 2001).

It is now well established that a Koch phenomenon pattern of response does not constitute optimal protective immunity to *M. tuberculosis.* The situation appears to be quite the reverse. It is possible in animal models to induce a state of "Koch-like" responsiveness prior to immunisation: such animals are then *more* susceptible to the disease than unimmunised controls when infected *via* the intramuscular or intratracheal routes (Wilson *et al.* 1940, Hernandez-Pando *et al.* 1997a). If infected into the skin, the result is the same as that observed by Koch, the lesion produced undergoes necrosis and falls off. In the human, whilst a disease-afflicted individual exhibits a Koch-like, necrotising response to *M. tuberculosis* an infected, healthy individual mounts a protective immune response. In other words, tuberculosis is an immunopathological disease due to the inappropriate (i.e. necrotic) pattern of response to antigens of *M. tuberculosis.* An individual in whom re-activation of latent infection occurs must therefore be switching from a protective immune response to a destructive immune response. The precise nature of or reasons for this immunological "switch" remain unknown.

In at least one trial, the differing immune responses in humans were reflected to some degree in the size of a tuberculin skin test site. This study recruited around 70 000 people in Northern Malawi and tuberculin tests were performed on the subjects. Follow-up demonstrated that the relationship between the diameter of induration at the skin test site

and risk of subsequently developing tuberculosis was J shaped. For a skin test diameter of 1-10mm individuals were protected against tuberculosis, but a skin test diameter of greater than 10mm predicted a greater chance of developing tuberculosis than for those who were tuberculin negative (Fine *et al.* 1994). Interestingly, the difference in the immune response is also manifest in hyporesponsiveness to common mycobacterial antigens shared between all mycobacterial species with apparent preservation of the response to *M. tuberculosis* specific antigens. Responses to common mycobacterial antigens in healthy, *M. tuberculosis* exposed individuals are preserved (Kardjito *et al.* 1986).

The role of TNFα in immunity to tuberculosis and the necrosis of tuberculous lesions

Exactly what constitutes protective immunity to tuberculosis is not yet known, but several factors are known to play an important role. One of these is the inflammatory cytokine, tumour necrosis factor alpha (TNFα). In mice, treatment with anti TNFα antibodies led to widespread dissemination of BCG infection (Kindler *et al.* 1989). Also, disablement of the 55-kDa TNFα receptor (TNFR), either by treatment with neutralising antibodies or by gene knockout, led to rapid death from *M tuberculosis* infection (Flynn *et al.* 1995). These effects may occur because of the essential role of TNFα in granuloma formation and macrophage activation. In mice infected via the intratracheal route there is an increased level of TNFα at day 3 and a second peak that coincides with mature granuloma formation, in the third week of infection (Hernandez-Pando *et al.* 1997b).

Many of the symptoms of tuberculosis, however, are attributable to TNFα toxicity. TNFα may cause fever and weight loss, such as that seen in tuberculosis. This is supported by the observation that thalidomide, which shortens the half life of TNFα mRNA (Moreira *et al.* 1993), provides symptomatic relief and weight gain when administered to tuberculosis patients (Kaplan 1994).

Thus the role of TNFα appears to be paradoxical: one the one hand, TNFα is essential for protection; on the other it seems to be responsible for the pathology of tuberculosis. The resolution of these apparently contradictory observations may lie in the balance of type 1 and type 2 cytokines produced as part of the strong cell-mediated immune response to *M. tuberculosis*. The original Th1/Th2 scheme arose to categorise CD4+ helper T cells by secreted cytokine profiles (Mosmann *et al.* 1986). A Th1 response is characterised by the

secretion of interferon gamma (IFNγ), a Th2 response by interleukin 4 (IL-4). The terms "type 1" and "type 2" are used to describe the entire pattern of cytokine release, not only that from CD4+ cells, because CD8+ T cells, monocyte/macrophages, B cells, other antigen presenting cells and even stromal cells all contribute to the overall pattern of response. A type 1 response is dominated by IFNγ, IL-2, IL-15 and IL-12 whereas a type 2 response is dominated by IL-4, 5, 6 and 13 (Salgame *et al.* 1991, Clerici and Shearer 1994).

The cell-mediated immune response in tuberculosis

There is overwhelming evidence for the importance of the cell-mediated immune response in tuberculosis. In murine models of tuberculosis, increased numbers of both CD4+ and CD8+ T cells accumulate in the lungs and the draining lymph nodes 2 to 4 weeks after infection, and many of these cells display an activated phenotype (Feng *et al.* 1999, Serbina *et al.* 2000). T cell hybridomas generated from mice 10 to 14 days after infection with *M. tuberculosis* responded strongly to mycobacterial antigen by proliferation and IFNγ secretion, and splenic T cell populations adoptively transferred at this time gave protection from infection (Orme 1987, Orme *et al.* 1992). Furthermore, a wide variety of models using knockout (KO) mice in which genes important to cellular immunity are absent suggest which components are involved in immunity to tuberculosis.

Mice that lack CD4+ αβ T cells altogether (achieved by disruption of the MHC class II genes or the TCR) are extremely sensitive to tuberculosis (Mogues *et al.* 2001) and are even susceptible to the non pathogen *M. bovis* BCG (Ladel *et al.* 1995a). Similarly, mice treated with anti CD4 antibody are also more susceptible to the disease and in a model of latency anti CD4 treatment triggered reactivation of disease (Scanga *et al.* 2000). CD8+ T cells also play a significant role, as shown by the increased sensitivity of mice that lack this cellular subset through KO of a variety of different genes (Flynn *et al.* 1992, Sousa *et al.* 2000, Mogues *et al.* 2001).

CD4+ T cells mediate immunity through cytokine production (mainly IFNγ) and macrophage activation. Exactly how CD8+ T cells are protective is unclear. In mice CD8+ T cells are cytotoxic for infected monocyte/macrophages (Serbina *et al.* 2000), whilst human CD8+ T cells may actually kill intracellular and extracellular mycobacteria directly in a granulysin dependent manner (Stenger *et al.* 1998). On the other hand, macrophage

cytotoxicity mediated by CD4+ T cells did not result in the killing of intracellular *M. bovis* BCG organisms (Fazal *et al.* 1995). CD8+ T cells are able to respond to antigen derived from the endosomal/phagosomal compartment normally presented on MHC II which is "cross-presented" on MHC I molecules (Teitelbaum *et al.* 1999, Canaday *et al.* 1999). A major role of murine CD8+ T cells in experimental tuberculosis (possibly through cross-presentation) may be the production of IFNγ, especially early in infection (Tascon *et al.* 1998, Serbina and Flynn 1999).

The role of type 1 and type 2 responses in mice

Experiments involving the manipulation of immune responses according to the type 1/2 paradigm in the mouse suggest that a type 1 response is protective in tuberculosis. For example, mice in which the IFNγ gene is disrupted are very sensitive to tuberculosis and die rapidly from normally non fatal innoculae of *M tuberculosis* and *M. bovis* (Cooper *et al.* 1993, Flynn *et al.* 1993, Dalton *et al.* 1993). Similarly, mice lacking the IFNγ receptor gene rapidly succumb to *M. bovis* BCG infection (Kamijo *et al.* 1993). Disruption of the IL-18 gene also renders mice less resistant to tuberculosis, possibly because of the role of IL-18 in IFNγ induction (Sugawara *et al.* 1999). IL-12 is a powerful inducer of IFNγ expression: IL-12 knockout mice are more susceptible to tuberculosis (Wakeham *et al.* 1998), but the exact role of this cytokine varies between mouse strains.

Although the importance of a type 1 response can be readily demonstrated, mice with tuberculosis also express type 2 cytokines (Orme *et al.* 1993). The presence of this type 2 response is extremely damaging. An immune response that includes a type 2 component can be deliberately induced by immunisation before infection with a high dose (10^9 organisms) of a heat killed environmental mycobacterium, *M. vaccae.* In contrast, immunisation with 10^7 heat killed *M. vaccae* does not induce a type 2 component. Cultured splenocytes from mice that had been immunised with 10^9 *M. vaccae* made both IFNγ and IL-4 in response to PPD, whereas splenocytes from mice that had been immunised with 10^7 *M. vaccae* made only IFNγ in response to PPD (Hernandez-Pando and Rook 1994). The group of animals that were immunised with 10^9 *M. vaccae* exhibited marked TNFα sensitivity in a DTH site as measured by footpad swelling, whereas the animals that were immunised with 10^7 *M. vaccae* did not. The 10^9 *M. vaccae* group were much more

sensitive to intratracheal *M. tuberculosis* than unimmunised controls; the 10^7 *M. vaccae* group were protected (Hernandez-Pando *et al.* 1997a).

It seems, therefore, that in the mouse at least the effect of the cytokine TNFα depends upon the type 1/type 2 cytokine balance, a pure type 1 response being protective and a mixed type 1 and 2 response being detrimental. Further evidence for this comes from the Balb/c model of pulmonary tuberculosis, where the appearance of areas of necrosis in the lung is accompanied by the expression of IL-4 (by immunohistochemistry and RT-PCR) in the same parts of the lung (Hernandez-Pando *et al.* 1996). Of course the toxicity is extremely unlikely to be due to IL-4 directly, but rather correlates with the pattern of immune response that includes an IL-4 (or type 2) component. IL-4 knockout mice are not less susceptible to tuberculosis than wild type mice (North 1998), but such animals are still capable of making IL-13 which can substitute for many of the actions of IL-4 so therefore are not devoid of type 2 cytokine activity.

Evidence for the importance of type 1 and type 2 immune responses in human tuberculosis

Parallels exist between these experimental models in the mouse and the disease in humans. Five children have so far been described who had mutations in the IFNγ receptor resulting in the appearance of a premature stop codon (Jouanguy *et al.* 1996, Newport *et al.* 1996). In addition, four children with mutations in the IL-12 receptor, resulting in failure of receptor expression, have also been described (Altare *et al.* 1998). These children were all exquisitely sensitive even to atypical mycobacterial infections by organisms not normally pathogenic, underlining the importance of IFNγ in immunity to tuberculosis in humans. This group of patients was not reported to suffer from tuberculosis, presumably because their immunodeficiency was profound and they died before they encountered *M. tuberculosis*. Patients suffering from AIDS, in which there are profound deficiencies of CD4+ T cells, are also very susceptible to tuberculosis and this is one of the first secondary infections to be activated in HIV positive individuals (Hawken *et al.* 1993).

The above observations imply that in humans a type 1 immune response is protective in tuberculosis, but direct demonstration of protection by a type 1 response and the harmful nature of a mixed type 1 and type 2 response, as in the mouse, is not possible. However,

evidence that the same is the case in man comes from the study of tuberculosis patients and healthy contacts. These data indicate the presence of a switchover from a type 1 dominated response to one that includes a type 2 component.

Tuberculosis patients produce relatively more antibody and mount relatively weaker T cell responses to the 30kDa antigen of *M. tuberculosis* than healthy contacts, also the patients' cells release less IFNγ and more IL-10 in response to the 30kDa antigen (Torres *et al.* 1998). Expression of IL-4 in peripheral blood T cells from tuberculosis patients correlates with severity of disease and with cavitation of tuberculous lesions (Seah *et al.* 2000, van Crevel *et al.* 2000). Tuberculosis patients have *M. tuberculosis* specific IgE in their sera (Yong *et al.* 1989), which is dependent upon a Th2 response. Similarly *in vitro* production of IgG4, also largely type 2 cytokine dependent, is raised in tuberculosis patients (Wilsher *et al.* 1999).

The most recent evidence for the presence of a type 2 component to the immune response in clinical tuberculosis comes from a study in Japan, undertaken to examine whether or not *M. tuberculosis* can protect against asthma. The authors clearly show an increase in serum IgE levels and a reduction in the ratio of IFNγ to IL-4 made by CD4+ cells in the peripheral blood, confirming the observations described above (Suzuki *et al.* 2001). Moreover, the serum IgE level and the ratio of CD4+ cell IFNγ to IL-4 production correlated with the severity of tuberculosis, as measured by C reactive protein level and erythrocyte sedimentation rate. Interestingly, the authors concluded that *M. tuberculosis* could not protect against asthma because of the presence of a type 2 response, whereas the majority of infected but clinically well individuals in fact mount a pure type 1 response and therefore would not be expected to have raised serum IgE or enhanced IL-4 production, based on the evidence discussed above.

1.1.3 Cytokines and the Koch phenomenon

Taken together, the observations described above point to a clear combination of cytokines that are responsible for, or at least correlate with, the pathology of tuberculosis. It appears that it is the particular combination of type 1 + type 2 + TNFα leads to the necrotic pattern of response characteristic of tuberculosis, and that this may be the immunological state of a tuberculous human or a mouse immunised to be more susceptible to the disease (Rook and

Hernandez-Pando 1996). There is a close parallel between the fact that the Koch phenomenon accompanies progressive disease in guinea pigs and the harmful immunological state of mice that have received 10^9 *M. vaccae*. Whether or not these two experimental states are one and the same, and whether this is the same state as occurs in tuberculosis patients, is unknown at present, but it seems likely that this will turn out to be the case.

Some of the reasons for the toxicity of this cytokine combination are beginning to emerge. It is now known, for example, that some *M. tuberculosis* reactive T cells secrete IL-4 *in vitro* and that production of IL-4 causes expression of CD30 in the lymphocyte cultures. The CD30+ cells down regulate TNFR associated factor 2 (TRAF2) and are then sensitive to TNFα induced apoptosis, probably due to the role of TRAF2 in directing the response to TNFα towards the NF-κB mediated activation pathway (Seah and Rook 2001). These observations may provide one mechanism for the Th1 to Th2 switch that occurs in tuberculosis, as type 1 cells appear to be more susceptible than Th0 or type 2 cells to Fas mediated activation induced cell death after activation through the TCR/CD3 complex (Varadhachary and Salgame 1998). The experiments of Seah and Rook did not determine which cells present in their system were undergoing apoptosis. Whether this model system applies in other cell types is yet to be determined, but in active tuberculous granulomata vigorous necrosis is also occurring so further explanations will have to be sought in order to fully explain the immunopathology of the disease.

It is of interest to note that tuberculosis is not the only disease state in which the immunological combination of IL-4 plus TNFα, under the right circumstances, is harmful to the host. Animal models of parasite pathology induced by *Trichinoma spiralis* and *Schistosoma mansoni* show some important similarities. In *T. spiralis* intestinal infection in mice parasites are cleared after 18 days of infection, with significant accompanying gut pathology (Lawrence *et al.* 1998). TNFR1 KO mice successfully cleared the infection, but with a much lesser degree of tissue damage. IL-4 KO mice did not clear the infection and also exhibited little pathology. Therefore, IL-4 was required for protection but also regulated TNFα mediated pathology. In another murine model, the intense fibrotic response to *S. mansoni* infection, which is largely a consequence of the response to *Schistosoma* eggs, was prevented by prior immunisation with eggs and IL-12 (Wynn *et al.*

1995). This was associated with a striking increase in IFNγ production and a decrease in IL-4 production. TNFα production in the protected animals was actually increased, again showing the importance of the presence of IL-4 in regulating pathology mediated by TNFα.

1.1.4 The role of unconventional T cell subsets in immunity to mycobacteria

A number of T cell subpopulations that do not conform to the conventional dogma of αβTCR, CD4+, MHC II restriction or CD8+, MHC I restriction have been described. These cells are characterised either by unusual cell surface marker phenotype or by their interaction with unconventional antigen presentation molecules.

Natural killer T (NKT) cells and CD1 restricted T cells.

NKT cells are a subset of T lymphocytes that share some properties with natural killer cells. In particular they commonly bear the NK cell marker NK1.1, by which they often identified, along with co-expression of conventional T cell markers such as CD3 and CD4 (Yoshimoto *et al.* 1995, Schofield *et al.* 1999). However, NKT cells are a heterogeneous group of cells and may vary in their cell surface marker phenotype, at least partly in a tissue specific manner (Godfrey *et al.* 2000). The precise function of NKT cells is currently unknown, but it is clear that they are able to secrete large amounts of cytokines, including IL-4 and IFNγ, very rapidly after exposure to antigen (Yoshimoto *et al.* 1995). NKT cells express a relatively invariant T cell receptor repertoire, implying that what they recognise is highly conserved (Bendelac *et al.* 1997, Godfrey *et al.* 2000).

NKT cells (and some non NKT cells) recognise the MHC class I related β2-microglobulin associated CD1 family of antigen presenting molecules in humans (Porcelli *et al.* 1989). In mice NKT cells recognise CD1d, which is highly homologous to human CD1d (Bendelac *et al.* 1995, Park and Bendelac 2000). Although it has been suggested that mouse NKT cells recognise CD1 in the absence of foreign antigens (Bendelac *et al.* 1997), there is good evidence that human CD1b and CD1c can present lipid and glycolipid antigens from *M. tuberculosis* to T cells (Porcelli *et al.* 1992, Beckman *et al.* 1994, Sieling *et al.* 1995, Moody *et al.* 1999, Moody *et al.* 2000a, Moody *et al.* 2000b). These antigens include mycolic acids, lipoarabinomannan, glucose monomycolate and isoprenoid glycolipids, and endosomal processing of the antigens appears to be required (Sieling *et al.* 1995, Moody *et*

al. 1999). Uptake and processing of LAM for CD1 mediated presentation to T cells was mediated by the mannose receptor (Prigozy *et al.* 1997). Solution of the crystal structure of mouse CD1d1 (equivalent to human CD1d) revealed an antigen binding cleft that was narrower, deeper and less polar than previously described MHC class I and II antigen binding grooves (Zeng *et al.* 1997). It was subsequently shown that in mice a natural ligand for CD1 is the "self" glycolipid cellular glycosylphosphatidylinositol (GPI) (Joyce *et al.* 1998). Alterations in the acyl portion of the mycobacterial antigen GMM made no difference to CD1b restricted T cell proliferation, but even very subtle alterations in the hydrophilic end of the molecule did (Moody *et al.* 1997). It has been proposed on the basis of these findings that CD1 represents a lipid binding antigen presentation molecule of broad specificity (Park and Bendelac 2000).

The T cells used in the human studies above were generally αβ CD4- CD8- or CD8 + cells, not NKT cells, although there is evidence for the existence of CD4+, CD1 restricted T cells in human leprosy patients (Sieling *et al.* 2000). *In vivo*, murine NKT cells are involved in granulomatous responses to *M. tuberculosis* lipid and glycolipid fractions (Apostolou *et al.* 1999) and in IFNγ production induced by *M. bovis* BCG (Emoto *et al.* 1999). NKT cells are also involved in antibody responses to GPI anchored proteins from *Plasmodium falciparum* and *Trypanosoma brucei*, haptenated ovalbumin conjugated to mouse thy-1 GPI and free GPI from *Leishmania mexicana* (Schofield *et al.* 1999)

Despite the observations above, CD1d restricted T cells appear not to be necessary for immunity to *M. tuberculosis* in mice (Behar *et al.* 1999, Sousa *et al.* 2000). β2-microglobulin knockout mice are more susceptible to tuberculosis, but this is due to their deficiency in CD8 T cells (Flynn *et al.* 1992), although the degree of protection afforded by CD8 T cells appears to vary with the dose and route of administration of infection (Mogues *et al.* 2001). However, most of the mycobacterial lipids and glycolipids that are presented on CD1 in humans are presented on CD1b and c, not CD1d, the only version of CD1 equivalently expressed in mice. Moreover, CD1a, b and c are also abundantly expressed on dendritic cells in human tuberculoid leprosy legions, where a strong cell-mediated response is present (Sieling *et al.* 1999). αβ+ CD4- CD8- T cells from a leprosy patient have also been observed to be cytolytic for LAM-pulsed monocytes in a CD1b dependent manner (Sieling *et al.* 1995). A role for CD1 restricted T cells in human immunity to mycobacteria

is, therefore, not excluded. The recent description of a range of *CD1* genes in guinea pigs equivalent to *CD1b, c* and *e* genes in man (the *CD1e* gene does not appear to be transcribed in humans (Porcelli and Modlin 1999)) may generate a more productive model for studying the role of CD1 restricted T cells in tuberculosis (Dascher *et al.* 1999).

Gamma delta (γδ) T cells

γδ T cells are a subset of T lymphocytes that express an alternative version of the T cell receptor α and β chains (Bank *et al.* 1986). They are only a minority of all peripheral blood T cells, comprising approximately 1 to 5% of CD3+ cells (Kabelitz and Wesch 2001), but they are the majority of CD4- CD8- CD3+ cells in peripheral lymphoid organs in mice (Cron *et al.* 1988). This subset of T cells was originally reported to recognise both peptide antigens in mice (Born *et al.* 1990), and later reported to recognise non-peptide antigens in humans, on which most later work has focused (Pfeffer *et al.* 1992, Constant *et al.* 1994, Schoel *et al.* 1994, Wesch *et al.* 1997). γδ T cells appear to be able to recognise antigen in conjunction with classical MHC molecules (Matis *et al.* 1987) as well as non-classical MHC molecules such as Qa-1 in mice (Vidovic *et al.* 1989) and CD1 in humans (Porcelli *et al.* 1989). The major protein antigens recognised by γδ T cells in humans are probably the class I MHC related antigens MIC A and B (Hayday 2000). In addition, in humans, some mycobacterial γδ T cell antigens behave like superantigens in an MHC dependent manner (Pfeffer *et al.* 1992).

Many of the defined γδ T cell antigens are mycobacterial in origin, however the role of γδ T cells in immunity to mycobacteria is not clear. In one study that included several different knockout mice, mice lacking γδ T cell receptors were marginally more susceptible to infection than wild type controls using a low dose of inhaled bacilli (Mogues *et al.* 2001). In a previous study δ gene knockout mice were only more susceptible to tuberculosis if a very large challenge was given intravenously (Ladel *et al.* 1995b). Other authors postulate that γδ T cells are not essential for immunity but they do play a role in the organisation of granulomas, on the basis histological differences between the lungs γδ T cell deficient mice and wild type mice after aerosol infection (D'Souza *et al.* 1997).

Some properties of the mycobacterial ligands for human γδ T cells have been elucidated in more detail. A synthetic compound, monoethyl phosphate (MEP), causes proliferation of a

panel of γδ T cell clones that bear the Vγ2/Vδ2 TCR combination (Tanaka *et al.* 1994). MEP was observed to be similar to a natural mycobacterial antigen thought not to contain oligosaccharide, peptide or nucleotides on the basis of chemical treatments. The same mycobacterial material was found to act as an antigen for Vγ2/Vδ2 TCR bearing lymphocytes. Subsequently the ligand was reported by the same group to be isopentenyl pyrophosphate (IPP) (Tanaka *et al.* 1995). IPP was found to stimulate Vγ2/Vδ2 T cells but not Vγ1/Vδ1 T cells. The γδ T cell response to IPP required cell-cell contact but did not require antigen presentation (Morita *et al.* 1995). (As IPP is present in many prokaryotic and eukaryotic pathogens (Tanaka *et al.* 1996), these results indicate a role for γδ T cells in the early response to many pathogens, not just *M. tuberculosis*.) Another report partially characterised a low molecular weight antigen that was recognised by cells expressing a Vγ9/Vδ2 TCR (Schoel *et al.* 1994), an alternative nomenclature for the Vγ2/Vδ2 TCR (Bukowski *et al.* 1998). Like the compounds described above, a terminal phosphate was essential for activity. This compound, however, was thought to be carbohydrate in nature. A separate group almost simultaneously reported the partial structure of one of a group of four low molecular weight Vγ9/Vδ2 (Vγ2/Vδ2) TCR antigens purified from *M. tuberculosis*, named TUB antigens (Constant *et al.* 1994). The partially characterised antigen, TUBag4 contained phosphorylated thymidine with another, unidentified group linked to the phosphate. TUBag4 was not alkaline phosphatase sensitive, but other TUB antigens were.

The primary γδ T cell response to the γδ TCR antigen IPP (see above) was found to be dependent on the presence of αβ help derived from T cells responding to another, peptide antigen which could be substituted for by exogenous IL-2 (Wesch *et al.* 1997). The response to IPP is augmented by IL-15, a cytokine with similar properties to IL-2 (Garcia *et al.* 1998). This response has also been shown to be mediated through the TCRγ chain junctional region so IPP does not have the characteristics of a superantigen (Bukowski *et al.* 1998). Such observations are interesting because the Vγ9/Vδ2 (Vγ2/Vδ2) TCR subset of γδ T cells is the predominant γδ T cell subset in humans (Lanier *et al.* 1988, Parker *et al.* 1990), and human clinical studies show that this subset appears to play a role in tuberculosis. Responses of Vγ9/Vδ2 (Vγ2/Vδ2) T cells from the cerebrospinal fluid and blood of children with tuberculous meningitis and from the blood of children with primary tuberculosis to non-peptide antigens of *M. tuberculosis* were enhanced compared with

control subjects (Poccia *et al.* 1999, Dieli *et al.* 1999). These responses fell back to normal after anti-tuberculosis chemotherapy. Children with tuberculosis and healthy PPD positive children mounted greater Vγ9/Vδ2 (Vγ2/Vδ2) T cell proliferative responses to phosphoantigens than children who were PPD negative (Garcia *et al.* 1998). Again, γδ T cell responses were reduced after treatment.

γδ T cells isolated from human tuberculin positive donors have been reported to be cytotoxic to *M. tuberculosis* infected monocytes *in vitro* (Tsukaguchi *et al.* 1995). In the same study γδ T cells were equally cytolytic as CD4+ αβ T cells, and were a potent source of IFNγ, producing more IFNγ than the CD4+ αβ T cells on a per cell basis. γδ T cells proliferate rapidly in response to IPP, and their cytokine profile shows predominately IFNγ production (Garcia *et al.* 1997). Moreover, proliferative and IFNγ responses were markedly blunted in HIV positive individuals, who are very sensitive to tuberculosis. Addition of IL-12, which enhanced responses in normal donors, failed to restore responses in HIV positive individuals. These results indicate a potential role for human γδ T cells in the immune response to mycobacteria, but the exact nature of this role in immunity to mycobacteria and which antigens are responded to *in vivo* remain unknown. Interestingly, the TUBags are not restricted to *M. tuberculosis*, but are found in many mycobacterial species, however very low levels are found in the non-pathogenic *M. bovis* BCG compared with *M. tuberculosis* and opportunistic pathogens (Garcia *et al.* 1997).

1.1.5 Mycobacteria and macrophage interactions

The cell-mediated immune response against *M. tuberculosis* is directed against organisms that reside in the intracellular space, within macrophages. Several pathways are involved in the phagocytosis of mycobacteria including mannose and complement receptors (Schlesinger 1998, Kang and Schlesinger 1998). However it has been established for some time that, at least *in vitro*, mycobacteria can gain entry into many cell types that do not express these receptors so other pathways are likely to exist (Shepard 1958, Filley and Rook 1991).

Toll-like receptors

Once inside the macrophage, the mycobacteria cause the host cell to respond to their presence by the release of cytokines, including IL-1, IL-6, IL-12 and TNFα. Interaction between mycobacteria and Toll-like receptors (TLR) may be an important mechanism in inducing this response. TLRs are members of the IL-1 receptor family and are the mammalian equivalent of Toll (Medzhitov *et al.* 1997), a component of the innate immune system involved in anti fungal responses in Drosophila (Lemaitre *et al.* 1996).

The first human toll receptor to be described, TLR4, caused IL-1, IL-8 and B7.1 expression; NF-κB activation and IL-6 expression in the presence of IFNγ when expressed as a dominant active mutant in THP-1 human monocytes (Medzhitov *et al.* 1997). Subsequently two groups independently reported that TLR2 was able to confer lipopolysaccharide (LPS) sensitivity when transiently transfected into human embryonic kidney 293 (HEK 293) cells (Yang *et al.* 1998, Kirschning *et al.* 1998). It was also reported that TLR4 did not confer LPS sensitivity to HEK 293 cells. The role of TLR2 became contentious, however, when it was established that the C3H/HeJ LPS insensitive mouse had a defect that mapped to the TLR4 gene (Poltorak *et al.* 1998). Despite normal TLR2 expression this mouse strain was still hyporesponsive to LPS. It was later reported that TLR4 required a co-receptor, MD-2, to function effectively (Shimazu *et al.* 1999), providing a possible explanation for why transient transfection of HEK 293 cells with TLR4 did not confer LPS responsiveness.

In the wake of *in vitro* experiments into the function of TLR2 and TLR4 the study of KO mice for TLR2 and TLR4 revealed that TLR2 deficient mice were hyporesponsive to Gram positive cell wall components and TLR4 deficient mice were hyporesponsive to Gram negative cell wall components (Takeuchi *et al.* 1999). Interestingly, TLR4 deficient mice were also hyporesponsive to lipotechoic acid, but this did not appear to affect the overall response to Gram positive cell walls. These data pointed towards TLRs being pattern recognition receptors and have been backed up by further *in vitro* studies showing that TLR2 recognises a wide variety of different bacterial lipids and lipoproteins whereas TLR4 appears to be more specific for LPS (Janeway and Medzhitov 1999, Underhill *et al.* 1999a, Underhill *et al.* 1999b).

It has now been established that TLR4 can confer LPS responsiveness to CD14 expressing CHO cells, and in the same model TLR2 mediated NF-κB activation by araLAM from a fast growing, non-pathogenic mycobacterial species (Means *et al.* 1999a). TLR1 and TLR4 were unable to confer LAM sensitivity. The same group has showed that components of pathogenic mycobacteria activate NF-κB through both TLR2 and TLR4. A soluble, heat stable, proteinase resistant factor named soluble tuberculosis factor (STF) mediated activation *via* TLR2, whereas a heat sensitive, cell associated factor mediated activation *via* TLR4 (Means *et al.* 1999b). The authors showed that these as yet unidentified TLR ligands were distinct from LAM from these species, in other words TLR mediated activation is triggered by araLAM but not manLAM. In a more recent report Means *et al.* show that blocking TLR function, either by expression of double negative mutant TLRs or by expression of double negative mutant MyD88, the putative TLR associated signalling molecule blocked virtually all *M. tuberculosis* induced NF-κB activation and TNFα production (Means *et al.* 2001). The NO induction response to live *M. tuberculosis* was largely preserved, but iNOS promoter activity in response to STF was reduced.

Other components of mycobacteria, have also been shown to induce IL-12 production and iNOS promoter activity, as well as NF-κB activation, most potently the cell wall associated lipoprotein fraction (Brightbill *et al.* 1999). The effect on the IL-12 promoter was abrogated by transfection with a dominant negative TLR2 mutant, pointing to a role for this TLR in *M. tuberculosis* mediated IL-12 production. There may, therefore, be cellular responses to mycobacteria induced by TLRs other than NF-κB activation, which is normally measured.

1.1.6 Killing of mycobacteria

Reactive nitrogen and oxygen intermediates

In addition to causing cytokine release the intracellular *M. tuberculosis* cell may be exposed to a number of possible killing mechanisms. In the mouse both inhibition of growth and killing are readily demonstrated and appear to be mediated mainly by reactive nitrogen intermediates (RNI) derived from L-arginine rather than reactive oxygen intermediates (ROI), at least *in vitro* (Chan *et al.* 1992). In human cells, however, the same effects are more difficult to demonstrate (Rook *et al.* 1985, Rook *et al.* 1986). It has been reported

that alveolar macrophages were more effective than peripheral blood monocytes at inhibiting the growth of *M. tuberculosis* (Hirsch *et al.* 1994), although, strictly speaking, killing was not observed. Infection of human alveolar macrophages induces apoptosis, however, (Keane *et al.* 1997) and it may be that apoptosis of infected macrophages results in mycobactericidal effects (Oddo *et al.* 1998) (See later). Mycobactericidal effects have been observed in an *in vitro* culture system involving pre-culture of human macrophages with IFNγ and pre-culture of peripheral blood lymphocytes with *M. tuberculosis* lysate and IFNγ followed by co-culture of the prepared cells with live *M. tuberculosis* (Bonecini-Almeida *et al.* 1998).

The role of RNI and ROI in killing or controlling infection *in vivo* is more complicated. Some authors have replicated the observation that RNI are essential for control of *M. tuberculosis* proliferation by the use of NOS inhibitors as assessed by histology, bacterial burden and mortality (Chan *et al.* 1992, Chan *et al.* 1995) and by iNOS gene knockout (KO) (MacMicking *et al.* 1997). In addition, IFNγ KO mice, that are very susceptible to tuberculosis, make much less RNI in response to tuberculosis compared with littermates (Flynn *et al.* 1993). Inhibition of NO production using aminoguanidine in two murine models of latency also caused reactivation of tuberculosis with the features of acute disease (Flynn *et al.* 1998). However, other authors report iNOS KO mice showed no increase in proliferation of *M. tuberculosis* in the lungs until late in infection, but there was increased growth in the spleen (Adams *et al.* 1997). The same authors showed KO mice unable to make ROI had increased growth of *M. tuberculosis* in the lungs. The mechanism of action of NO in these cases is unclear as NO has signalling and second messenger functions that may be as important as its toxicity. Moreover, in the case of a model of *M. avium* infection, knocking out the iNOS gene actually improved the control of bacilliary growth in the spleen (Gomes *et al.* 1999). The authors speculate that this might be due to the fact that in the murine spleen NO can reach immunosuppressive levels, down regulating IFNγ production in response to antigen.

In humans the role of NO in immunity to *M. tuberculosis* remains unclear. It has been reported that freshly isolated and fixed alveolar macrophages from tuberculosis patients express more iNOS protein, NOS activity and possibly mRNA compared with cells from healthy volunteers (Nicholson *et al.* 1996). Furthermore alveolar macrophages derived

from patients with idiopathic pulmonary fibrosis (IPF) were able to kill *M. bovis* BCG in a NO dependent manner (Nozaki *et al.* 1997). (Cells from patients with lung cancer or pulmonary nodules did exhibit the same effect and the authors speculated that this might be due to exposure of the alveolar macrophages to high cytokine levels in the inflamed lung of IPF.) Interestingly, IPF is a condition which, like tuberculosis, appears to involve expression of both type 1 and type 2 cytokines, with the type 2 pattern of response predominating (Wallace *et al.* 1995). In addition, alveolar macrophage accessory cell function is altered in this condition (Furuie *et al.* 1997). The alveolar macrophage is the cell type known to inhibit the growth of *M. tuberculosis* more than peripheral blood monocytes, as discussed above (Hirsch *et al.* 1994).

If NO is made by human macrophages, it is never made in quantities as large as that made by murine macrophages. It is possible, therefore, that NO levels released by human macrophages stay in the optimal antimicrobial range. (Mice that are heterozygous at the *NOS2* allele retained enough NOS2 activity to have survival equivalent to the wild type group in one iNOS KO model (MacMicking *et al.* 1997).) There is not universal agreement about the role of RNI in immunity to *M. tuberculosis* in either mice or humans.

Apoptosis of macrophages

As described above, one of the functions of T cells in mycobacterial infection may be to kill infected macrophages. There is evidence that induction of apoptosis appears to reduce the viability of intracellular mycobacteria, whereas necrosis of macrophages does not (Molloy *et al.* 1994, Oddo *et al.* 1998). However, in one set of experiments CD8+ T cell lines killing infected macrophages *via* a granule dependent mechanism resulted in killing of mycobacteria but CD4- CD8- T cell lines killing *via* Fas/FasL interactions did not produce killing of mycobacteria (Stenger *et al.* 1998). Killing of mycobacteria in this system was found to be caused directly by the granule protein granulysin, which had a direct bactericidal effect on *M. tuberculosis*. Intracellular bacteria were protected from this effect of granulysin unless perforin was also present, presumably to act by allowing granulysin access to the macrophage cytoplasm. The mode of macrophage death was not specifically investigated in this system. Granule dependent killing is thought to induce necrosis by disrupting the target cell plasma membrane (Liu *et al.* 1995), but there is evidence that cytotoxic CD8+ T cell granules induce DNA breakdown as well as cytolysis (Nakajima and

Henkart 1994) In the experiments of Stenger *et al.* it was bacterial exposure to the CD8+ T cell granule derived protein granulysin rather than the mode of cell death that determined bacterial killing.

M. tuberculosis itself induces apoptosis *in vitro* of murine macrophages (Rojas *et al.* 1997) and human alveolar macrophages (Keane *et al.* 1997); such *in vitro* apoptosis induced by *M. tuberculosis* was mediated at least in part by TNFα and NO. Earlier work showed that not only monocyte like cells, but also other cell types were more susceptible to the cytotoxic effects of TNFα when infected with mycobacteria (Filley and Rook 1991). Granulomas from the lungs of a small sample of tuberculous subjects were also found to exhibit extensive macrophage apoptosis by in situ terminal transferase-mediated nick end labelling (TUNEL) of fragmented DNA. In contrast, in a single tissue sample of inactive disease, no apoptosis was observed (Keane *et al.* 1997). Apoptosis of human macrophages induced by *M. bovis* BCG or by *M. tuberculosis* H37Ra involved the down regulation of bcl-2 (an inhibitor of apoptosis) mRNA expression and protein (Klingler *et al.* 1997). Expression of the pro-apoptotic protein Bax, on the other hand, was unchanged, resulting in an imbalance in the normal pro and anti-apoptotic signals.

Treatment of human macrophages with ATP results in apoptosis with killing of intracellular *M. bovis* BCG (Molloy *et al.* 1994) and this killing is not dependent on ROI or RNI (Lammas *et al.* 1997). ATP induced killing of *M. tuberculosis* required the presence of active phospholipase D (Kusner and Adams 2000). The mycobactercidal effect of apoptosis induced by Fas/FasL interactions is more complex. In some *in vitro* systems Fas/FasL mediated apoptosis induced by CD4- CD8- T cells does not induce killing of *M. tuberculosis* (Stenger *et al.* 1997, Stenger *et al.* 1998). In another system where Fas/FasL interactions were induced by addition of soluble recombinant FasL killing of *M. tuberculosis* was observed (Oddo *et al.* 1998). Apoptosis induced by anti-Fas monoclonal antibody has also been reported not to kill *M. avium-intracellulare*, but hydrogen peroxide induced apoptosis did (Laochumroonvorapong *et al.* 1996).

It has been suggested that the function of apoptosis may be to kill heavily infected unresponsive macrophages and allow phagocytosis of bacilli by fresh macrophages (Fratazzi *et al.* 1999) as well as the direct mycobacterial killing discussed above. The

precise role of apoptosis in mycobacterial infection *in vivo* is not fully understood. Perforin, granzyme B and Fas appear not to be required for protection against *M. tuberculosis* in murine KO models (Laochumroonvorapong *et al.* 1997, Cooper *et al.* 1997). (Interestingly, apoptosis was still observed in the Fas KO mice.) However, in another model perforin KO mice died more rapidly from tuberculosis than did wild type mice, but this only became apparent after 20 weeks of infection (Sousa *et al.* 2000). The experiments of Laochumroonvorapong *et al.* and Cooper *et al.* did not continue for longer than 13 weeks and death of the animals was not examined, allowing for an explanation as to the differing results. Apoptosis may play a greater role, therefore, in the later stages of infection. The perforin KO mice of Sousa *et al.* were nevertheless more resistant to the disease than CD8, TAP or β2-m KO mice, indicating a greater role for CD8+ T cells than merely killing of infected macrophages. In humans, lung lobes involved in tuberculosis contain more apoptotic macrophages than non-involved lobes or normal lung samples (Klingler *et al.* 1997), although the significance of this observation is not yet known.

M. tuberculosis seems to induce apoptosis in some experimental systems (Keane *et al.* 1997, Klingler *et al.* 1997), yet be killed by apoptosis in other experimental systems (Molloy *et al.* 1994, Lammas *et al.* 1997, Kusner and Adams 2000). One explanation for this is that certain types of apoptosis are of benefit to the organism while other types are not. *M. tuberculosis* may therefore be preferentially inducing apoptosis *via* pathways that leave the organism unharmed.

1.1.7 Evasion of Killing mechanisms by mycobacteria

Mycobacteria have evolved several strategies to avoid being killed inside host cells. *M. tuberculosis* and other non pathogenic mycobacteria are taken up by the mannose receptor, a mechanism which bypasses the production of ROI and fails to promote phagosome maturation (Astarie-Dequeker *et al.* 1999). *M. tuberculosis* phagosomes incorporate LAMP-1 but not the vesicular proton ATPase thus inhibiting acidification of the phagosome and causing it to behave like the endosomal recycling pathway rather then the toxic phagolysosomal pathway (Sturgill-Koszycki *et al.* 1994, Xu *et al.* 1994, Sturgill-Koszycki *et al.* 1994).

M. tuberculosis containing phagosomes release vacuoles that contain lipoarabinomannan (LAM) which incorporate into the glycosylphosphatidylinositol (GPI) rich domains in the host cell membrane (Ilangumaran *et al.* 1995). Cleavage of macrophage GPI by GPI phospholipase D (GPI-PLD) may be involved in signalling and LAM can prevent their hydrolysis by inhibiting GPI-PLD (Dr. Kiajoo Puan, unpublished observations). These observations may be relevant to the failure of long-term mycobacterium infected macrophages to present antigen to CD4+ T cells (Pancholi *et al.* 1993). Viable *M. tuberculosis* can also inhibit complement-receptor mediated Ca^{2+} signalling which was associated with failure of phagosome-lysosome fusion and enhanced survival within human macrophages (Malik *et al.* 2000).

As described earlier, under certain conditions apoptosis has mycobactericidal effects. *M. tuberculosis* exhibits a number of strategies that may prevent apoptosis. One mechanism is the downregulation of Fas on infected macrophages (Oddo *et al.* 1998). Production of soluble TNF receptor 2 (sTNFR2) was induced by *M. tuberculosis* in an IL-10 dependent manner, and sTNFR2 opposed the apoptotic effect of TNF released by the infected macrophages (Balcewicz-Sablinska *et al.* 1998).

1.1.8 Summary

It can easily be seen that the interaction between *M. tuberculosis* and the host is a very complex one. *M. tuberculosis* possesses remarkable abilities to survive within host macrophages and avoid bacterial killing. At the same time, in the state of active disease, *M. tuberculosis* induces a strong immune response but this immune response itself mediates much or indeed all the tissue damage characteristic of tuberculosis. Although several correlates of this immunopathological response have been described, the precise nature of the change from a protective immune response to a destructive one remains an enigma. Much more work in this field is required in order to unsderstand how this damaging immune response arises and how it can be prevented, or how the immune system can be stimulated to become better at destroying *M. tuberculosis*.

1.2 Glycosylphosphatidylinositol molecules and inositol phosphoglycan second messengers

1.2.1 Glycosylphosphatidylinositol and inositol phosphoglycans in the immune system

As described above, the cell-mediated immune response is crucial in immunity to *M. tuberculosis* infection. Central to the cell-mediated immune response is the T lymphocyte. Following T cell receptor ligation by MHC and antigen, the cell expresses a number of receptors for growth factors essential for entry into the cell cycle and cell division. These receptors include those for IL-2, insulin and transferrin. One mechanism of insulin signalling are the inositol phosphoglycan (IPG) second messengers, derived from free membrane glycosylphosphatidylinositol (GPI) (Saltiel and Cuatrecasas 1986, Saltiel *et al.* 1986, Saltiel *et al.* 1987). This system is known to function in T lymphocytes upon activation (Gaulton 1991, Gaulton 1991). T cells rapidly up express the insulin receptor upon activation, and radiolabelled GPI was hydrolysed exactly in parallel with the increase in insulin receptor expression (Gaulton *et al.* 1988). The signalling mechanism of IL-2 is not fully understood, but the GPI/IPG system is strongly implicated (Eardley and Koshland 1991). Again rapid GPI hydrolysis (within 30 seconds) was detected after treatment of an IL-2 sensitive cell line with IL-2, indicating that IPG are generated rapidly in response to IL-2, as well as insulin.

1.2.2 Discovery and characterisation of GPI and IPG molecules

IPG second messengers are derived from free membrane GPI by the action of a GPI specific phospholipase D (GPI-PLD). Although the existence of free membrane GPI has been established for some time, GPI structures were first described as protein anchors.

GPI protein anchors

Early work pointing to the existence of a covalently linked lipid membrane anchor showed the release of alkaline phosphatase and acetylcholinesterase activity into the supernatant of cells in culture following treatment of the cells with PI-PLC from various bacterial sources including *Bacillus cereus* (Ikezawa *et al.* 1976) and *Staphylococcus aureus* (Low and

Finean 1977). Released alkaline phosphatase and acetylcholinesterase activity were unable to re-associate with phospholipid liposomes or partition into Triton X-114; nor could they re-associate with phosphatidylinositol (PI) (Low and Zilversmit 1980, Futerman *et al.* 1985). These observations indicated that cleavage of a covalent bond, and not non-specific disruption of the membrane, resulted in the appearance of enzyme activity in the supernatant.

Further evidence of a phospholipid covalently linked to protein involved work on the rat Thy-1 molecule. This showed that C terminal proteolysis fragments were isolated as aggregates and required detergent to be able to pass through Bio-Gel P10, implying the presence of lipid at the C terminus. Amino acid sequencing revealed an unidentified substance distal to what appeared to be the C terminal amino acid that reacted with ninhydrin and probably contained ethanolamine. This substance was found to be present in comparable, stoichiometric amounts in different proteloysis fragments. The C terminal proteolysis fragments were also found to be glucosamine containing, although the position of the glucosamine was not determined (Campbell *et al.* 1981). It was the trypanosome, however, that provided the model system for the complete structural characterisation of the first GPI membrane anchor.

Trypanosome GPI anchors

The protozoan parasite *Trypanosoma brucei brucei* is the causative organism of the cattle disease nagana and its close relatives *Trypanosoma brucei rhodesiense* and *Trypanosoma brucei gambiense* cause African sleeping sickness in man. The trypanosome provides an ideal organism in which to study GPI molecules because the surface of each cell is covered in a coat of 10^7 variant surface glycoprotein (VSG) molecules, each one anchored to the membrane by a GPI structure. The trypanosome is able to shed its entire surface protein coat and generate a new, immunologically distinct coat very rapidly thus avoiding the host immune response. This phenomenon generated great interest in the VSG molecule both as a model system of immune avoidance and with a view to finding a way of overcoming this antigenic variation. Early work on the VSG established the presence of ethanolamine in amide linkage at the C terminus of the protein and an associated novel carbohydrate (Holder and Cross 1981). There was no evidence of the presence of a lipid until it was

shown that trypanosomes contain an enzyme that cleaves the membrane binding form of the VSG molecule (mfVSG) to a water soluble form (sVSG) very rapidly during the protein purification process (Cardoso de Almeida and Turner 1983). The membrane binding form, mfVSG, is only obtained if this enzyme is inhibited during purification of the protein.

Subsequent to these discoveries the structure of the *T.b. brucei* VSG anchor was elucidated in more detail. The presence of ethanolamine and phosphate had already been determined. The glycan was shown to contain galactose, mannose and glucosamine. It was then shown that treatment of the lipidic form of the membrane anchor resulted in the release of diacyl glycerol (DAG) (the presence of which was confirmed by phospholipase A_2 digestion and acetolysis) and that nitrous acid deamination liberated phosphatidylinositol (PI) as a product. The partial primary structure of PI directly linked to a glucosamine residue *via* the inositol ring followed by mannose and galactose residues linked to the glucosamine in unknown conformation terminating in phosphoethanolamine was deduced and the term "glycosylphosphatidylinositol" was coined for the first time (Ferguson 1992).

The complete structure was finally solved by nuclear magnetic resonance (NMR) analysis of the C terminal glycopeptide (Ferguson *et al.* 1988). The C terminal glycopeptide is derived from the water soluble PI-PLC reaction product pronase treated (to remove all peptide except aspartate, the C teminus amino acid) followed by chemical removal of the aspartate and ethanolamine to yield the neutral glycan. Size fractionation of the neutral glycan had showed that it actually consisted of several different species each comprising an identical core structure composed of three mannose residues and two galactose residues. The variation in size was entirely due to the presence or absence of a further two galactose residues. The complete structure is shown in figure 1.1.

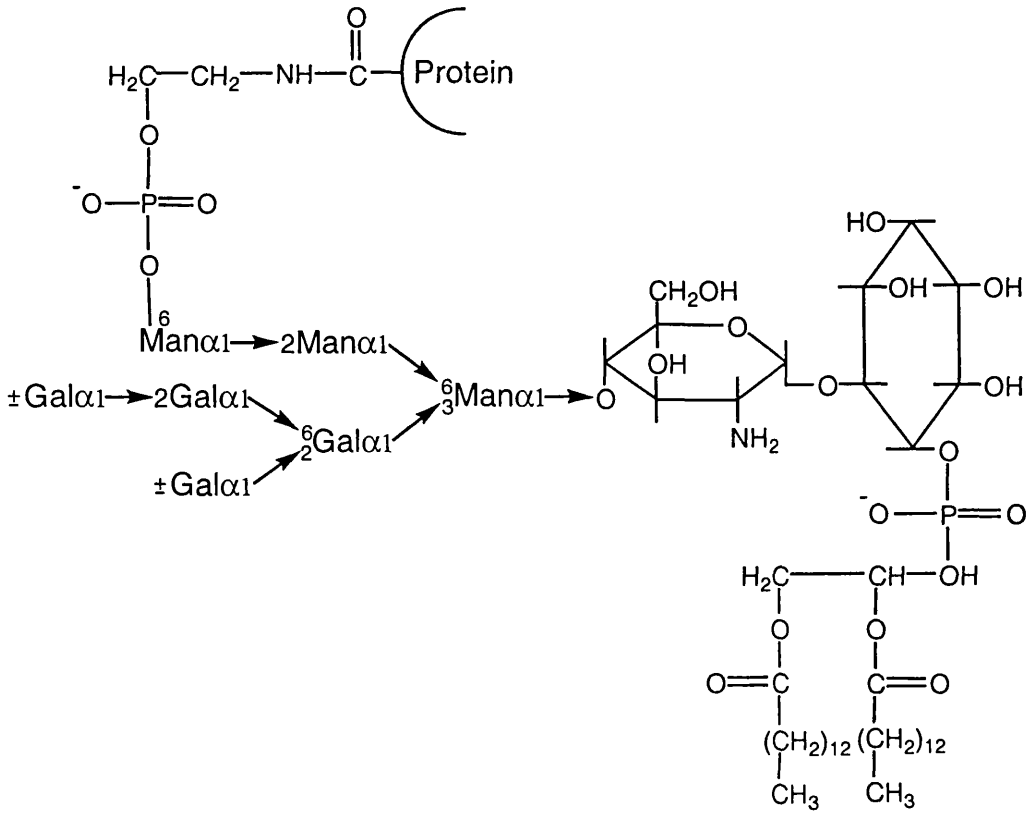


Figure 1.1. Primary structure of the trypanosome glycosylphosphatidylinositol variant surface glycoprotein anchor. The linkage between *myo*-inositol and glucosamine is in the α1→6 configuration. The ± symbol denotes that these particular galactose residues are variable in their presence within the GPI anchor. This figure is adapted from Ferguson 1992.

GPI anchor core structure

Once the VSG anchor structure was known, the same techniques were rapidly applied to other GPI structures. The next GPI structure to be solved was the Thy-1 anchor (Homans *et al.* 1988). The tri-mannose backbone of the Thy-1 anchor is identical to that of the VSG anchor but there is variation in the side chain structures. As a consequence of this structural similarity the following "core region" of GPI anchors was proposed:

NH_2-$(CH_2)_2$-PO_4-6Manα1$\rightarrow$2Manα1$\rightarrow$6Manα1$\rightarrow$4Glc$NH_2\alpha$1-6*myo*-inositol-1-PO_4-lipid

The identification of this core structure of GPI anchors allows the definition of putative GPI species according to the chemical properties that are predicted by the above structure, or variations upon it. In order to be defined as a GPI structure, the following properties should be demonstrated. The putative GPI should act as a substrate for (G)PI-PLC, contain a free amino group, be lipidic and contain phosphate. These properties overlap some extent, for instance if unknown material is able to act as a substrate for GPI-PLC this also implies the presence of lipid and phosphate.

1.2.3 Free membrane GPI molecules and IPG second messengers

There are now several complete structures known for GPI anchors. Such structural information regarding free membrane GPI from which the IPG family of second messengers is derived, however, is not so abundant. Larner *et al.* first presented evidence of a water-soluble non-peptide insulin mediator that inhibited cAMP protein kinase in the 1970s (Larner *et al.* 1974). Later partially purified fractions of the putative mediator were shown to activate pyruvate dehydrogenase and glycogen synthase phosphatase (Larner *et al.* 1979, Jarett and Seals 1979).

Evidence for a soluble, lipid derived, insulin second messenger

It was not until the mid 1980s, however, that experiments performed by Saltiel and co-workers in the 1980s identified the existence of low molecular weight modulators of cAMP phosphodiesterase activity that were derived from a hepatic plasma membrane glycolipid. This glycolipid had characteristics compatible with those of a GPI structure, being

radiolabelled by *myo*-inositol, glucosamine and myristic acid (Saltiel and Cuatrecasas 1986, Saltiel *et al.* 1986). Hydrolysis of the glycolipid was stimulated by insulin, resulting in modulator release that was mimicked by exogenously added bacterial PI-PLC. This observation led to the hypothesis that insulin activated a PLC that in turn cleaved the glycolipid generating an insulin second messenger. Very shortly afterwards Mato *et al.* confirmed the observations of Saltiel *et al.* in H35 hepatoma cells (Mato *et al.* 1987a). Further characterization of the glycolipid by this group showed that the glycolipid did not contain *myo*-inositol, but later work showed that it contained *chiro*-inositol, glucosamine, galactose and phosphate (Mato *et al.* 1987b).

Further evidence of the involvement of GPI and IPG in the signal transduction of insulin has followed the original observations of Saltiel, Mato and co-workers. In a system utilizing K562 erythroleukaemia cells that were unable to synthesize GPI tyrosine phosphorylation and activation of the pathway leading to MAP kinase occurred in response to insulin treatment but glycogen synthesis did not (Lazar *et al.* 1994). This indicated that IPG are responsible for the metabolic, not mitogenic effects of insulin. However, it has also been shown that IPG can mimic the effect of insulin on protein tyrosine phosphorylation in adipocytes (Alemany *et al.* 1987). Interestingly, a mutant T cell line that selectively lacks GPI protein anchors, but not insulin sensitive GPI has been described (Avila *et al.* 1992). This cell line was defective in TCR mediated signalling, but exhibited GPI turnover in response to insulin identical to that of the parent GPI competent cell line. This observation provides evidence for a different biosynthetic pathway of GPI protein anchors and free membrane GPI.

There are other experiments indicating that IPG play an important role in insulin signaling. Antibodies raised against coffee bean α-galactosidase treated trypanosome sVSG were found to cross react with insulin generated IPG mediators purified from BC_3H1 cells. The anti IPG antibodies made in this way cross-reacted between mannose containing sVSG glycan and mammalian IPG (Jones and Varela-Nieto 1998). The same antibodies were able to partially block the effects of IPG both on intact BC_3H1 cells and on cell free enzyme assays (Romero *et al.* 1990). Additionally, Type II diabetic GK rats have a defect in the synthesis of a *chiro*-inositol containing IPG; these rats display normal insulin mediated hexose transport but have a profound defect in glycerol-3-phosphate acyl transferase

activity. This defect is corrected by addition of exogenous *chiro*-inositol containing IPG (Farese *et al.* 1994). These lines of evidence indicate further the role of IPG in mediating the metabolic actions of insulin.

IPG are released outside the cell

Another important conclusion suggested on the basis of the results of Romero *et al* was that IPG are released outside the cell and therefore must exert their effects by being translocated across the plasma membrane into the cytoplasm (Romero *et al.* 1990). This gives rise to the possibility of a paracrine action for IPG utilizing hormones. Several other lines of evidence support this conclusion. Active mediators are released into the medium of cell cultures and when added back mimic hormone treatment (Eardley and Koshland 1991), PI-PLC was able to mimic insulin induced mediator release when added to intact cells (Saltiel and Sorbara-Cazan 1987). Insulin sensitive GPI has been repoted to be located on the outer leaflet of the plasma membrane (Alvarez *et al.* 1988). Free GPI distinct from GPI protein anchor on the basis of lipid variation (diacylglycerol as opposed to alkyl-acyl glycerol) the insulin sensitivity of which was not determined has also been described at the cell surface of human and murine cells (Singh *et al.* 1996).

These observations raise the question of how, if IPG are released outside the cell, they are able to act within the cytoplasm. IPG are unlikely to act on cell surface receptors and transduce a further signal as they are active when added to a variety of different enzymes in cell free assay systems, as well as being active in many cell based assays. IPG are, therefore, true second messengers. A specific IPG transport system has been identified (Alvarez *et al.* 1991), but complete characterisation of this system has not been achieved.

GPI specific phospholipases in mammalian tissues

In fact mammalian tissues do not contain any PLC activity that is able to act upon a glycan containing phosphatidylinositol species. Shortly after the discovery and characterization of GPI protein anchors in mammalian tissues, several different groups reported the existence of a GPI phospholipase D in the plasma of a number of species (Davitz *et al.* 1987, Low and Prasad 1988). The cleavage site was determined by the demonstration of phosphatidic acid as opposed to diacylglycerol as a reaction product. Mammalian GPI-PLD was shown

not to act upon PI, phosphatidylethanolamine or phosphatidylcholine and was identified as specific for GPI by the structural requirement of phosphatidylinositol-glucosamine in the substrate. The enzyme was originally isolated from plasma but was also shown to be present in bovine brain, murine islets of Langerhans and canine islets of Langerhans (Hoener *et al.* 1990, Metz *et al.* 1991). Interestingly, GPI-PLD has been reported to be secreted from a pancreatic β cell line in response to the same stimuli as for insulin secretion (Deeg and Verchere 1997). In humans GPI-PLD is expressed in liver and there appears to be only one copy of the gene (Schofield and Rademacher 2000).

The PI-PLC mentioned above in defining the characteristics of a GPI is usually derived from bacteria, either *Bacillus thuringenesis* or *Staphylococcus aureus*. PI-PLC derived from these sources will act upon anything containing PI, irrespective of whether there is a glycan present or not, hence giving the result originally obtained by Salteil (Saltiel and Cuatrecasas 1986, Saltiel *et al.* 1986, Saltiel *et al.* 1987). GPI-PLC is derived from the trypanosome and is not found in mammalian tissues.

Although the search for a mammalian GPI specific phospholipase was prompted by the discovery of mammalian GPI linked proteins, GPI-PLD has been identified as a candidate to act upon free membrane GPI, as opposed to protein linked GPI, and generate bio-active IPG (Jones *et al.* 1997). Whether or not GPI-PLD has any physiological role in releasing GPI linked proteins from the cell surface in mammals is unknown. Certainly there is enough enzyme present in plasma to cause the release of all GPI linked proteins within a few seconds; yet *in vivo* this does not occur: the enzyme appears to be inactive in the circulation.

Other growth factors and cytokines that signal using IPG

Although insulin is the most extensively characterised hormone to act *via* the GPI/IPG signaling system, many other cytokines, growth factors and hormones are also known, or at least suspected to signal using GPI/IPG. IGF-1 (Leon *et al.* 1995), NGF (Represa *et al.* 1991), EGF (Clemente *et al.* 1995), TGF-β1 (Vivien *et al.* 1993), IL-2 (Eardley and Koshland 1991) and ACTH (Cozza *et al.* 1988) have all been shown to cause rapid hydrolysis of radiolabelled GPI. How similar in structure the respective IPGs produced are, however, is not known.

1.2.4 Purification and activity of IPG from mammalian tissues

IPG may be purified from mammalian tissues by a variety of different means, but the method most extensively used in recent years in our collaborators' laboratory is based upon that described by Nestler *et al* which utilises anion exchange chromatography. IPG are eluted from the anion exchange resin column at 10mM HCl, pH 2.0 and at 50mM HCl, pH 1.3 (Nestler *et al.* 1991). These two fractions have been named IPG-P (pH 2.0) and IPG-A (pH 1.3) respectively, after their biological activities. IPG-P stimulates the activity of the enzyme pyruvate dehydrogenase (PDH) phosphatase, which dephosphorylates and in doing so activates PDH. PDH catalyses conversion of pyruvate derived from glucose into acetyl Co-A, which in turn enters the citric acid cycle. IPG-P, therefore, acts to enhance glycolysis and up-regulate the utilisation of glucose for entry into the citric acid cycle. IPG-P also stimulates glycogen synthase phosphorylase, activating glycogen synthase and promoting glycogenesis. IPG-A inhibits the activity of cAMP dependent protein kinase, and drives lipogenesis in adipocytes. The mediators first described by Mato, Salteil, Larner and their respective co-workers may be divided into these two IPG fractions. It has been proposed that IPG-P contains D-*chiro*-inositol, galactosamine and mannose; and IPG-A contains *myo* and *chiro*-inositol, glucosamine, galactose and phosphate groups (Jones and Varela-Nieto 1998).

1.2.5 Structure of IPG

Despite the fact that the first observations in this field were made 15 years ago, there are still no complete structures known for IPG second messenger molecules or their precursor GPI molecules. This may be due in part to the scarcity of material, and possibly due to complex microheterogeneity, which appears to be the rule rather than the exception for glycan structures. HPLC data from our collaborators' laboratory have shown that IPG preparations are indeed a complex mixture of closely related species. Moreover, GPI purified from T lymphocytes could be resolved into four separate bands by high performance TLC and that IL-2 selectively stimulated the hydrolysis of two out of these four bands (Gaulton 1991). Either the various different growth factors and cytokines that signal using IPG second messengers are able to selectively cleave different GPI molecules to produce IPGs of varying structure and activity in a hormone specific and perhaps also a

tissue specific manner, or selection is at the level of GPI biosynthesis. Some GPI structures may not be susceptible to GPI-PLD cleavage, explaining the results obtained by Gaulton (Gaulton 1991). Direct evidence for either of these possibilities is lacking at present.

One additional structural property that has come to light in our collaborators' laboratory is that IPG may contain or require the presence of divalent cations for activity (Rademacher and Caro 1997). IPG-P type isolated from human liver contains manganese and zinc ions and can substitute for manganese in the activation of PDH phosphatase, a manganese dependent enzyme. IPG-P can also stimulate activity in the absence of calcium, whereas without IPG-P both calcium and manganese are required. IPG-P appears to prime PDH phosphatase by making it more sensitive to the effects of calcium. Moreover, removal of metal ions by pretreatment with dithizone abolishes activity in the PDH phosphatase assay. However, although these observations suggest that IPG require metal cations, they do not directly show that the cations are an integral part of the IPG structure, the metal ions may co-purify with IPG. This is unlikely given that IPG are purified by anion exchange, but there are other data in the literature that may or may not agree with those described above, depending upon the interpretation. Chiefly, the observations described by Jones *et al.* using purified rat liver GPI treated with GPI-PLD *in vitro* in an apparently divalent cation free system yielded a bioactive product, as measured by cAMP dependent protein kinase inhibition and stimulation of cell proliferation within the chicken embryo cochleovestibular ganglion (Jones *et al.* 1997). Several possibilities exist to explain this result. Either the GPI used for these experiments already contained divalent cations when it was purified, or the GPI-PLD functions as a metal transferase as well as a phospholipase and contained divalent cations prior to use in the experiment. Alternatively divalent cations may be required for the PDH phosphatase assay but not for the assays used by Jones *et al.* Either way, some ambiguity remains concerning the presence of metal ions as a structural requirement for IPG. Free membrane GPI has never been subject to metal analysis within the published literature.

1.2.6 Studying the role of GPI derived IPG second messengers in the immune system

Unfortunately, no good models of immunocytes deficient in GPI molecules appear to exist for experimental use. The clonal blood disorder, paroxysmal nocturnal haemoglobinburia (PNH), is characterised by acquired mutations in the *PIG-A* gene resulting in defective GPI synthesis. T cells from PNH patients are deficient in TCR responses induced with anti CD3 monoclonal antibody (Romagnoli and Bron 1999), but there are abnormalities in PNH leucocytes whether these cells are capable of synthesising GPI or not (Ware *et al.* 1998). This indicates that there may be other defects, as yet unknown, that exist to explain the abnormal responses of PNH cells. A mutant of the Jurkat cell line that lacks the *PIG-A* gene exists, but this transformed cell line does not require IL-2 to grow anyway so may have yet other mutations that render GPI mediated signalling redundant (Bastisch *et al.* 2000). A T cell line that lacks protein linked GPI but has insulin sensitive GPI was defective in activation through the T cell receptor (Avila *et al.* 1992), so perhaps both protein linked and free membrane GPI are required for proper T cell function. This cell line must have a defect distal to the addition of glucosamine onto PI as this step is common to all GPI structures known in mammalian cells (Jones and Varela-Nieto 1998), but this may occur at such a place that a subset of free membrane GPI is also not synthesised.

1.3 IPG and mycobacteria

Mycobacteria are well known to contain glycosylated PI species and have been shown to contain phospholipase C and phospholipase D activity, although not with glycan specificity (Johansen *et al.* 1996). Interestingly PLD activity appeared to be confined to pathogenic species. The existence of a GPI with the glycan structural core of *myo*-inositol-α6-1-$GlcNH_2$-α4-trimannose as defined above has not been reported in mycobacteria.

1.3.1 Mycobacterial glycolipids and the mycobacterial cell envelope

One of the major classes of phosphatidylinositol based carbohydrates in mycobacteria are the phosphatidylinositol mannosides (PIMs) and the biosynthetically related lipomannan (LM) and lipoarabinomannan (LAM). These molecules are found within the mycobacterial cell envelope. The mycobacterial cell envelope is a highly complex structure and confers upon mycobacteria many of their unique and remarkable properties, such as acid fastness and the ability to readily survive within host phagocytes. The current model of the mycobacterial cell envelope is based upon that originally proposed by Minnikin (Minnikin 1982). The outer layer of the mycobacterial cell envelope is formed by a capsule comprised mainly of polysaccharide along with proteins which may be in the process of being secreted and a mixture of lipids. The surface exposed lipids vary between species, examples for *M. tuberculosis* include phenolic glycolipid, lipo-oligosaccharide and PIM (Ortalo-Magne *et al.* 1996). Dimycolyl trehalose, or cord factor, was found deeper in the capsule. The exposed surface of three stains of *M. tuberculosis* appeared mainly to consist of arabinan, mannan and phenolic glycolipid by monoclonal antibody staining (Ozanne *et al.* 1996).

Deep to the capsule of mycobacteria lies a covalently linked cell wall skeleton consisting of a crystalline layer of mycolates anchored to arabinogalactan which, in turn, is attached *via* a disaccharide linker to peptidoglycan present at the inner aspect of the cell wall skeleton unit (Daffe and Draper 1998). The crystalline highly impermeable layer of mycolates, interdigitated with an outer layer of phospholipids and glycolipids that are not covalently bound to the cell wall skeleton, is thought to be fundamental to the properties of the

mycobacterial cell wall (Daffe and Draper 1998, Baulard *et al.* 1999). Non-covalently associated with the cell wall skeleton is a host of polypeptides and other small molecules.

Forming the boundary of the cell cytoplasm is the cell membrane, consisting of phospholipids: mainly phosphatidylethanolamine, phosphatidylserine, phosphatidyl-glycerol, phosphatidylinositol and cardiolipin (Brennan and Ballou 1967, Jackson *et al.* 2000). Phosphatidylinositol is an essential phospholipid component of mycobacteria, as shown by the disruption of the gene for PI synthase in *M. smegmatis*, which resulted in a marked deficiency in PI, PIM, triacylated PIM and lysoPIman$_2$ associated with a decrease in cell viability (Jackson *et al.* 2000).

The cell envelope is the target of many of the anti-mycobacterial drugs. Ethambutol inhibits arabinogalactan biosynthesis by preventing the polymerisation of D-arabinofuranose derived from β-D-arabinofuranosyl-1-monophosphodecaprenol into the structure (Wolucka *et al.* 1994, Mikusova *et al.* 1995). Polymerisation of arabinose into LAM was also inhibited to a lesser degree. Isoniazid inhibits the inhA gene product, an enzyme involved in mycolic acid biosynthesis (Quemard *et al.* 1995). Pyrazimamide inhibits the eukaryotic like fatty acid synthase 1 (FAS1) of *M. tuberculosis*, also inhibiting cell envelope fatty acid biosynthesis (Zimhony *et al.* 2000).

Glycosylated phosphatidylinositols of mycobacteria

The major GPI like structures found within mycobacteria are PIM, LM and LAM. LM and LAM are based on phosphatidylinositol (Hunter and Brennan 1990) with a large mannan segment. Linked to the mannan segment of LAM at the non-reducing end of the molecule is the arabinan moiety (Chatterjee *et al.* 1991a), which is further mannose capped (manLAM) in some species (Chatterjee *et al.* 1992a) to form a mature species of variable molecular weight, around an average of 17 kDa (Chatterjee *et al.* 1991b). The fatty acids of LAM are mainly palmitate and tuberculosterate (Hunter and Brennan 1990, Leopold and Fischer 1993). LAM from *M. tuberculosis* H37Rv and *M. bovis* BCG associated with the cell membrane and more superficial within the cell envelope may be purified separately and shown to have varying degrees of mannosylation on the arabinnan segment, and varying acylation (Nigou *et al.* 1997, Gilleron *et al.* 2000). Parietal LAM from *M. bovis* BCG (LAM derived from mycobacteria without cell disruption) was found to have a novel fatty

acid, 12-O-[methoxypropanoyl]-12-hydroxystearate, as part of the lyso-phosphatidylinositol moiety (Nigou *et al.* 1997). Interestingly, this form of LAM was markedly more efficient at stimulating cytokine production from dendritic cells than cellular LAM, (LAM obtained from disrupted cells) although it constitutes only a small proportion of total LAM. The same group have shown the presence of similar LAM, a 12-O-[methoxypropanoyl]-12-hydroxystearate containing lyso-PI-arabinomannan, in *M. tuberculosis* H37Rv (Gilleron *et al.* 2000).

PIM is synthesised by a mannosyl transferase activity from phosphatidyl-*myo*-inositol and GDP-mannose in *M. phlei* and *M. tuberculosis* (Brennan and Ballou 1967, Brennan and Ballou 1968). It is now known that LM and LAM are based on a PIM structure with further mannose residues attached to the C-6 position of *myo*-inositol to form an $\alpha 1 \rightarrow 6$ linked backbone with $\alpha 1 \rightarrow 2$ mannose branches (Chatterjee *et al.* 1992b). The $\alpha 1 \rightarrow 6$ linkages of mycobacterial mannan are synthesised using β-mannosylphosphodecaprenol as the mannose donor (Yokoyama and Ballou 1989). Linear LM (from which mature LM and LAM are biosynthesised) is extended by further mannosylation of PIM at the C-6 position by the same mannose donor, although the relationship, if any, between the mannan described by Yokoyama & Ballou and LM is not known (Besra *et al.* 1997).

Further evidence of the relationship between PIM, LM and LAM comes from the structural analysis of the acylation patterns of the phosphatidylinositol portion of the molecules. PIM has been known for a long time to be present in multiply acylated forms (Brennan and Ballou 1968). The nature of some of the acyl groups in PIM from *M. tuberculosis* has subsequently been investigated further and evidence that LM and LAM are derived from tri-acylated PIM presented (Khoo *et al.* 1995a). Recent detailed structural characterisation of the phosphatidylinositol end of LM and LAM from *M. bovis* BCG has revealed that two, three or four acyl groups may be present. The phosphatidyl inositol may contain diacyl glycerol or be in the lyso form; additional points of attachment of acyl groups are at position 6 of the mannose residue linked to position 2 of *myo*-inositol and at position 3 of *myo*-inositol itself (Gilleron *et al.* 1999, Nigou *et al.* 1999). LAM contains on average three fatty acid esterifications per molecule, consistent with its derivation from triacylated PIM (Vercellone *et al.* 1998). The core structure of mycobacterial PIM, the PI anchor of

LM/LAM is shown in figure 1.2 and a schematic partial structure of LAM is shown in figure 1.3.

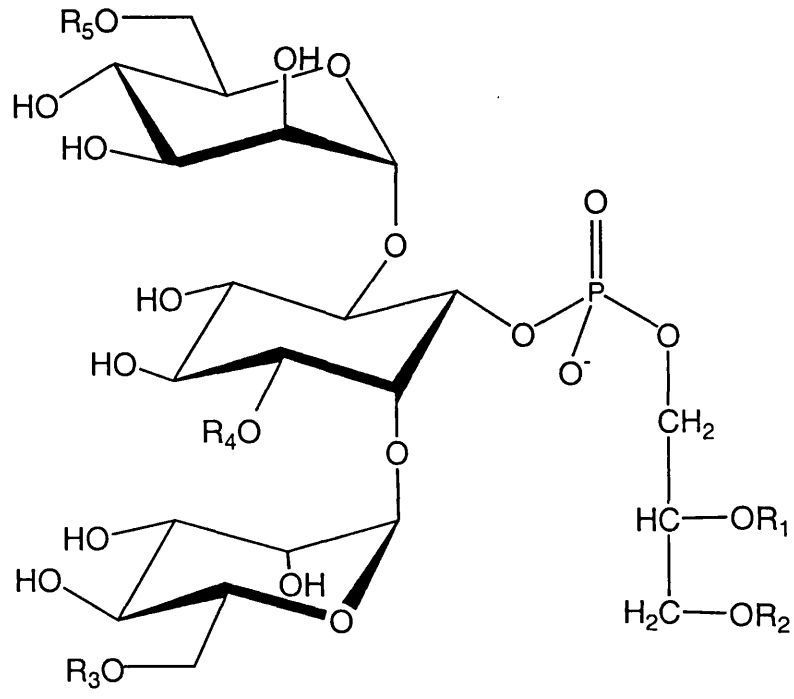


Figure 1.2. Core structure of the lipidic portion of mycobacterial PIM, LM and LAM. This figure represents the core structure of PIM_2, upon which the higher PIMs, LM and LAM are based. R_1, R_2, R_3 and R_4 are the potential sites of acylation of the molecule. The most abundant fatty acids found in these structures are palmitic acid and tuberculosteric acid. R_5 is the point of attachment of the mannan portion of LM and LAM, in $\alpha 1 \rightarrow 6$ configuration. The mannopyranosyl backbone is substituted at position C2 by single D-mannopyranosyl units. The single D-mannopyranose residues of PIM_2 are in the linkage $\alpha 1 \rightarrow 2$ and $\alpha 1 \rightarrow 6$, respectively. This figure is adapted from structures published by Besra *et al.* 1997 and Nigou *et al.* 1999.

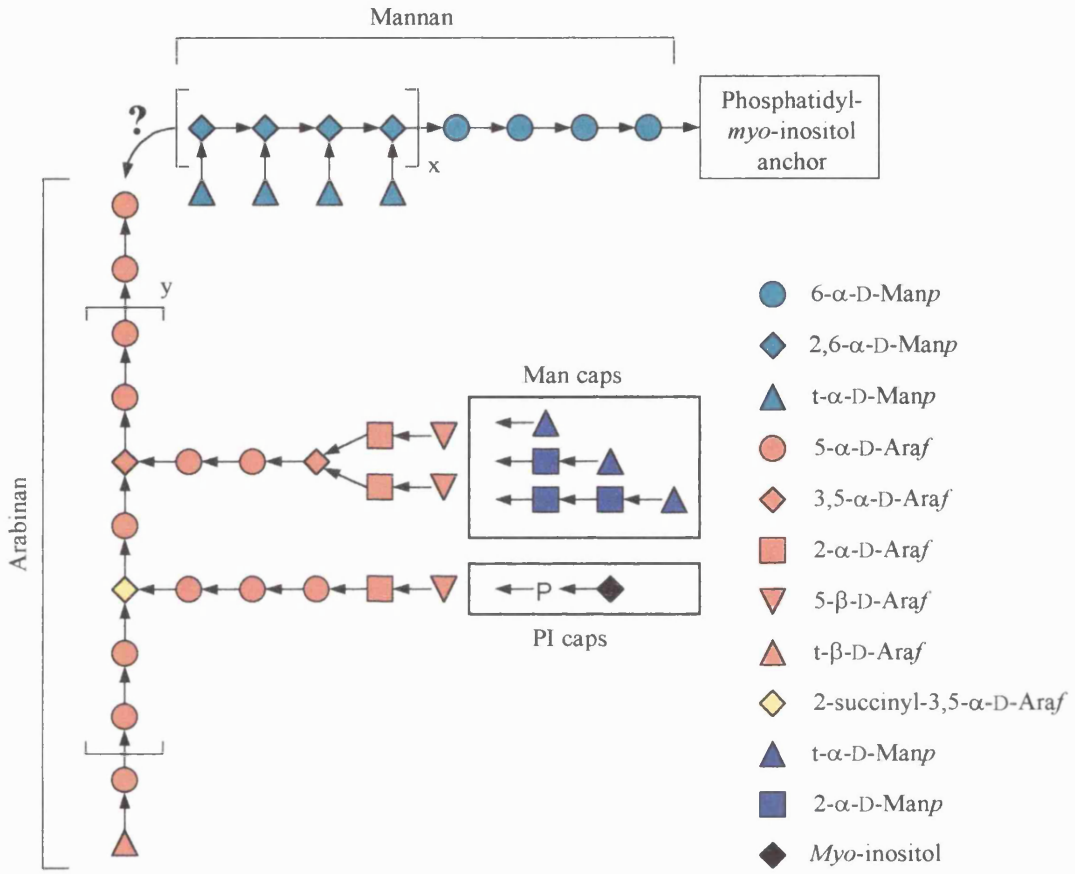


Figure 1.3. Schematic partial structure of mycobacterial lipoarabinomannan. LAM and LM are based upon the phosphatidylinositol anchor shown in figure 1.2. In this figure, green represents the mannan portion and orange represents the arabinan portion of the molecule. Sugar linkages are shown in the key; *p* = pyranose form, *f* = furanose form. The precise nature of the link between the mannan segment and the arabinan segment is unknown. The lengths of the mannan and arabinan chains are variable, this is indicated by x and y in the figure. Mannose capping, which occurs in slow growing species such as *M. tuberculosis*, *M. bovis* BCG and *M. leprae* is represented in blue, this form of LAM is referred to as manLAM. LAM that is not capped by mannose residues, araLAM, either has no capping or may be capped with phosphoinositol, shown in black. AraLAM is found in fast growing species, such as *M. smegmatis* and the unidentified fast growing species originally believed to be *M. tuberculosis* H37Ra. This figure is reproduced from Vercellone *et al.* 1998.

Puzo's group have speculated that the variation in acylation has implications for antigen presentation to T cells by CD1. The entrance to the antigen binding groove of mouse CD1d1 is only 6-7Å wide (Zeng *et al.* 1997), and the possibility that additional acyl groups may interfere with mycobacterial glycolipid access to the groove by steric hindrance was entertained (Nigou *et al.* 1999). It should be pointed out, however, that the crystal structure of CD1 from which the size of the groove entrance was derived was solved for mouse CD1d1, whereas in fact LAM is presented on human CD1b (Sieling *et al.* 1995), the groove size of which has not been formally established.

The significance of LAM in the pathogenesis of tuberculosis

Mycobacterial LAM has been found to have very significant immunomodulatory properties. PIM, LM and LAM from *M. tuberculosis* all induced mRNA for several different cytokines and suppressed antigen induced T cell proliferation (Barnes *et al.* 1992). Deacylation of LAM abrogated this response, prompting the hypothesis that the phosphatidyl inositol portion of the molecule that is required for this activity. This is not a universal finding, however. Parietal LAM from *M. smegmatis* that is not acylated and displays phosphoinositide capping at the non-reducing arabinan terminus (PI-GAM) was a potent inducer of TNFα from the human THP-1 monocyte/macrophage cell line (Gilleron *et al.* 1997). The acylated LAM fraction from *M. smegmatis* that does not contain PI-GAM was inactive in the TNFα release assay.

Many other observations also suggest that chemically distinct LAM structures from different strains of mycobacteria vary in their capacity to induce cytokine release from macrophages. In general, it seems that mannose capped LAM (manLAM) from slow growing mycobacterial species induces little TNFα release, whereas uncapped LAM (araLAM) from fast growing mycobacterial species (such as the PI-GAM described above) causes potent induction of TNFα release (Barnes *et al.* 1992, Roach *et al.* 1993, Roach *et al.* 1995, Khoo *et al.* 1995b). Expression of LAM that induces little TNFα production is not a determinant of pathogenesis, as originally suggested (Adams *et al.* 1993), because it is now established that the pathogenic strains *M. leprae*, *M. tuberculosis* Erdman and H37Rv; the less pathogenic *M. tuberculosis* H37Ra and the non-pathogenic *M. bovis* BCG all contain manLAM (Khoo *et al.* 1995b). On the other hand, LAM from a fast growing species, *Mycobacterium* sp. (previously erroneously identified as *M. tuberculosis* H37Ra)

was of the araLAM variety. TNFα is important in granuloma formation, which helps to prevent bacterial dissemination; additionally TNFα can induce apoptosis, which in turn has mycobactericidal effects, at least under some conditions (see above). It can be seen, therefore, why minimising the release of TNFα would be of benefit to an invading microorganism.

In addition to the effects of LAM on cytokine production, there are other effects mediated by LAM that may be significant in mycobacterial interaction with host phagocytes, and survival therein. LAM can act as a scavenger of toxic oxygen free radicals and can block the effects of IFNγ on macrophage activation in both murine and human cells (Sibley *et al.* 1988, Chan *et al.* 1991). The mannose cap of manLAM coated microspheres preferentially mediated binding to macrophage mannose receptors (MR) compared with araLAM, and manLAM from the more virulent strains *M. tuberculosis* Erdman and H37Rv mediated uptake of microspheres and organisms more efficiently than manLAM from *M. tuberculosis* H37Ra, also *via* the MR (Schlesinger 1993, Schlesinger *et al.* 1994, Schlesinger *et al.* 1996). However, more recent results indicate that the MR does not in fact discriminate between pathogenic and non-pathogenic mycobacteria (Astarie-Dequeker *et al.* 1999). Nevertheless, phagocytosis mediated by the MR did fail to elicit ROI generation and phagosome maturation, providing a mechanism for protected entry into macrophages. Therefore, although entry to host phagocytes is not a property purely of pathogenic mycobacteria, it may represent a process assisting in pathogenesis.

1.3.2 Potential structures from which IPG activity may be derived

It can be easily seen that mycobacteria possess several structures that are candidates to form a precursor of IPG-like molecules. PIM_2 or the higher PIMs, if they were removed from their lipid tail by the action of a phospholipase, could form a small inositol and phosphate containing oligosaccharide. Such molecules might be derived directly from LAM itself by the action of a phospholipase accompanied by removal of a large portion of the sugar. In addition, the phosphoinositide caps of araLAM from fast growing mycobacteria (Khoo *et al.* 1995b), if they were removed enzymatically or otherwise along with an oligosaccharide represent potential IPG-like structures. It is of note that the structural requirements for material to be active in some of the conventional assays used to look for IPG biological activity are quite diverse. For example, poly-phosphorylated galactose is strikingly active

in the PDH phosphatase activation assay (Dr. S Kunjara, unpublished data). IPG-like material from mycobacteria need not necessarily be derived from a precursor glycolipid as it is in mammals. Molecular modelling studies suggest that many combinations of oligosaccharides (usually comprising four carbohydrate residues) can adopt the right conformation to bind a divalent cation and theoretically have the potential to exhibit IPG-like biological activity (Dr. A Jaques, unpublished data).

1.3.3 Previous studies on mycobacteria and iPG activity

The existence of material within mycobacteria from which IPG-like structures might be derived and the presence of phospholipases in mycobacteria prompted an examination for the presence of IPG-like biological activity in mycobacteria in our laboratory and that of our collaborators. The mycobacterial species *M. tuberculosis* H37Rv, *M. tuberculosis* H37Ra and *M. vaccae* R877R were all subject to the same extraction process as that used in our collaborators' laboratory for the extraction of mammalian IPG, a modification of that described by Larner and co-workers (Nestler *et al.* 1991). *M. vaccae* R887R was included in the study so as to able to compare any such activity from a non-pathogen with that from a pathogen and because of interest in the use of this organism as an immunotherapy for tuberculosis. All three species were found to contain IPG-like activity that co-purified exactly with mammalian IPG (Rook *et al.* 2000). The biological assays performed to verify this included stimulation of PDH phosphatase activity, stimulation of lipogenesis in isolated rat adiopocytes, inhibition of cAMP dependent protein kinase, proliferation of the human epidermal growth factor receptor transfected 3T3 fibroblast cell line (3T3 EGFr) and proliferation of the Y1 adrenal carcinoma cell line.

Some of the biological activities of the mycobacterial IPG are shown in figure 1.4 and table 1.1. Because several important cytokines make use of the GPI/IPG signalling system, it was postulated that the presence of active IPG or IPG homologues within mycobacteria may disrupt the function of the host cells inside which the mycobacteria reside or the functions of other cells involved in the inflammatory or immune response. We have previously observed that, in addition to the activities described above and presented in table 1.1, certain IPG fractions from mycobacteria were able to markedly modulate the concentration of TNFα required to achieve 50% killing of L929 fibroblasts (figure 1.5). Interestingly, the A type IPG fraction from *M. vaccae* R877R primarily enhanced the

toxicity of TNFα and the P type fraction of *M. tuberculosis* primarily attenuated TNFα toxicity. This is in keeping with many of the properties of *M. tuberculosis* and its various components described above, namely that *M. tuberculosis*, although causing significant cytokine mediated tissue damage and having the ability to kill infected cells, also seeks to prevent cell death to aid its own intracellular survival. The precise significance of these observations is not known at this time.

Subsequent work in our laboratory has shown that IPG from *M. tuberculosis* is a powerful stimulus for nitric oxide (NO) production from interferon-γ activated murine macrophages and that NO release was not due to contamination by LPS or LAM (although it was found that mycobacterial IPG itself stimulates the limulus assay for LPS). It was also shown that "blank" IPG purifications, where no starting cells are used, did not yield activity (Dr. KiaJoo Puan, PhD thesis 2000).

	Milliunits/gram of bacterial cells in extraction	
	PDH phosphatase	*Lipogenesis*
M. vaccae 10mM HCl fraction	160	420 ± 18 (n=3)
M. vaccae 50mM HCl fraction	120	220 ± 76 (n=3)
M. tuberculosis 10mM HCl fraction	650	No activity
M. tuberculosis 50mM HCl fraction	inhibitory	No activity
Human liver 10mM HCl fraction	1960	No activity
Human liver 50mM HCl fraction	600	2460 ± 231 (n=3)

Table 1.1. IPG-like activity derived from mycobacteria. Mycobacterial cells were subject to the same procedure as mammalian tissues for isolation of IPG (a full description of the methods for isolation of active material and for the activity assays is given in chapter 3). IPG mediators are isolated from anion exchange resin at 10mM HCl (referred to as IPG-P) and at 50mM HCl (referred to as IPG-A). A unit is defined as the activity causing a 50% increase the basal level of the assay system. The 50mM HCl fraction of *M. tuberculosis* inhibited the activity of PDH phosphatase. In this pilot experiment, neither *M. tuberculosis* fraction exhibited lipogenic activity. Subsequent experiments showed that these fractions are active in the lipogenesis assay. Data from IPG from human are shown as a positive control. Normally the mammalian 10mM HCl fraction is not active in the lipogenesis assay. Data for the lipogenesis assay (a cell culture based assay system) are the mean of triplicate assays ± one standard deviation of the mean. PDH phosphatase activation assays (a cell free purified enzyme assay) are single data points. These experiments were performed by Dr E. Bayley, Dr H. Caro and Dr S. Kunjara.

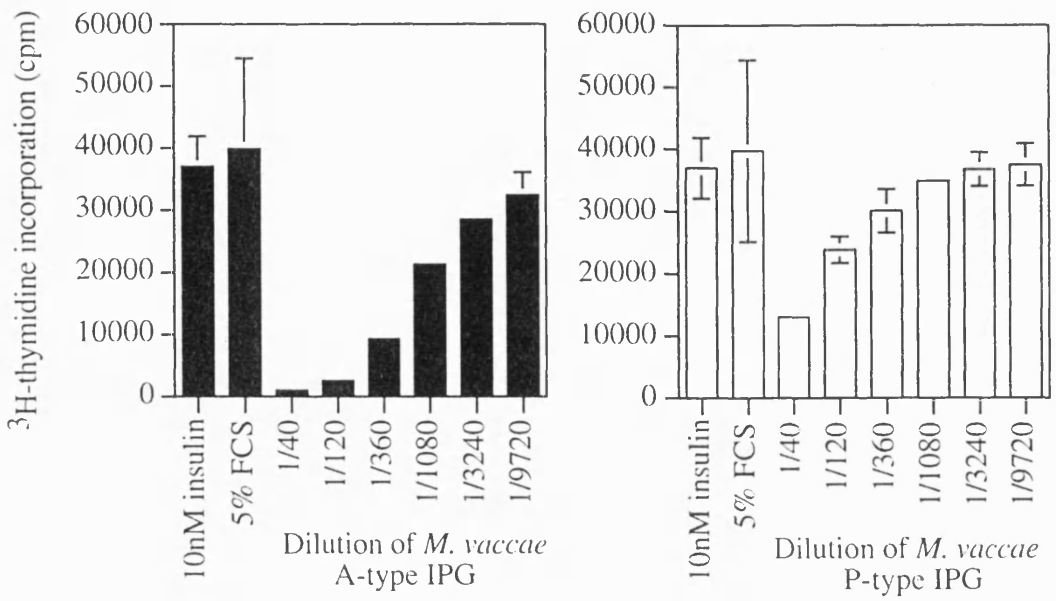


Figure 1.4. IPG activity derived from *M. vaccae* inhibits the proliferation of Y1 murine adrenal carcinoma cells. Mycobacterial cells were subject to the same procedure as mammalian tissues for isolation of IPG (a full description of the methods for isolation of active material and for the activity assays is given in chapter 3). IPG mediators are isolated from anion exchange resin at 10mM HCl (referred to as IPG-P) and at 50mM HCl (referred to as IPG-A). IPG-like mediator was diluted from a stock solution where 10µl corresponds to the amount of material purified from 1 gram of bacterial cells. Material from *M. vaccae* inhibited the proliferation of Y1 cells in a dose dependent manner, as measured by tritiated thymidine incorporation. Data are the mean of triplicate assays ± one standard deviation of the mean. Mycobacteria derived material was referred to as IPG at the time this experiment was performed in our laboratory so the same nomenclature is used here. However, this is not technically correct as the material identified from mycobacteria was identified as a biological activity, not as a structure. This experiment was performed by Dr E. Bayley.

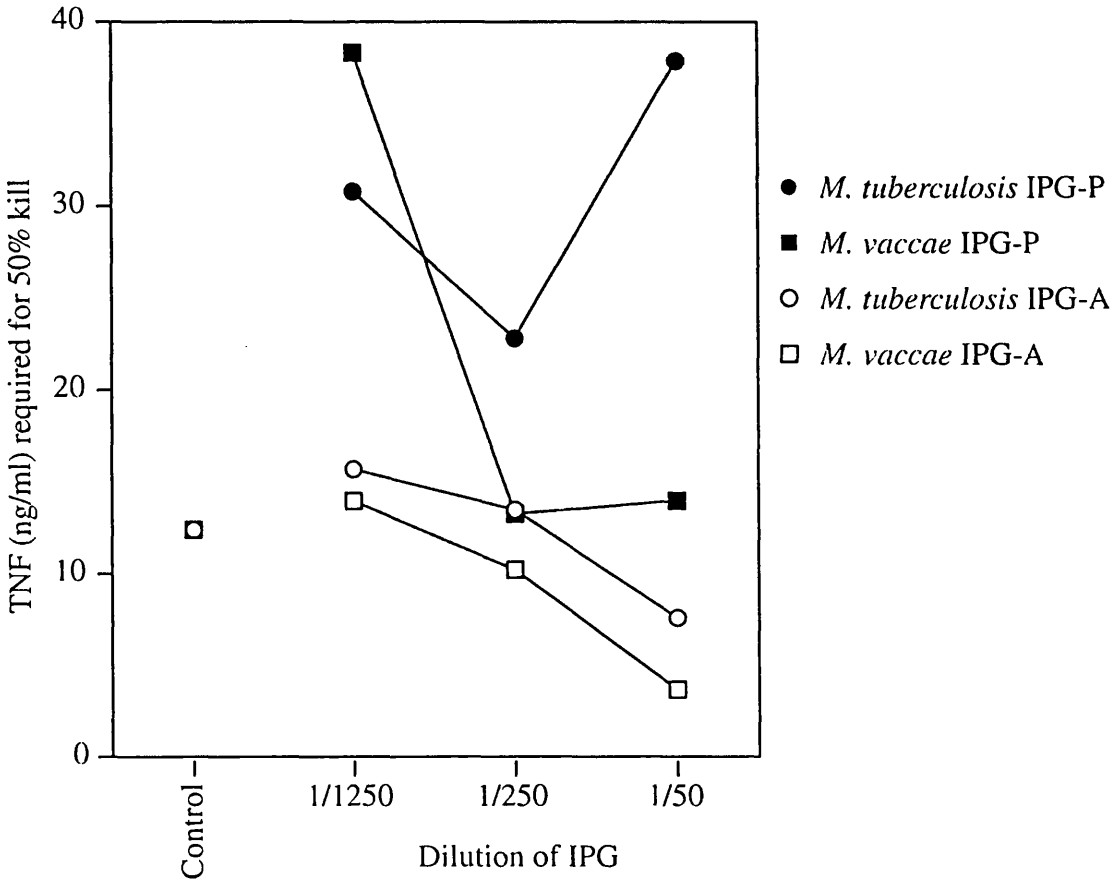


Figure 1.5. The effect of mycobacterial IPG on the toxicity of TNFα to the L929 TNF sensitive murine fibroblast cell line. A TNFα-sensitive clone of L929 cells were plated at $2x10^4$ cells per well in 96 well plates and allowed to adhere. The culture medium was replaced with 50μl of IPG at the dilutions indicated (IPG were obtained and diluted as described in the legend of figure 1.4) and a further 50μl of seven serial dilutions of recombinant murine TNFα was added. After incubation for 46 hours the cells were fixed with ethanol and stained with crystal violet to stain cell associated protein. Crystal violet remaining after washing with water was solubilised in 33% acetic acid and absorbance read at 570nm with reference at 405nm. Cell survival was calculated relative to controls without TNFα and plotted against TNFα concentration. The TNFα concentration giving 50% survival was determined by interpolation and it is these values shown in this figure. IPG samples without TNFα did not significantly alter cell survival. *M. tuberculosis* IPG-P inhibited TNFα toxicity at all concentrations tested, and *M. tuberculosis* IPG-A enhanced TNFα toxicity only at the highest concentration. *M. vaccae* IPG-P inhibited TNFα toxicity only at the lowest concentration and *M. vaccae* IPG-A enhanced TNFα toxicity in a dose dependent manner. This experiment was performed by Dr. E. Bayley.

1.4 The aims of this thesis

Whether or not mycobacteria contain a GPI homologue that is related to the IPG activity purified from the species described above was not examined. If such a glycolipid does exist in mycobacteria then further characterisation both of the glycolipid and of the IPG-like material is required to elucidate whether or not they have the same relationship as exists in mammalian cells. Secondly, while many of the effects of mycobacterial IPG-like material on macrophage functions have been studied, nothing is known about effects on lymphocytes. This is an attractive area of study because 1) the cell-mediated immune response is very important in immunity to *M. tuberculosis* and 2) T lymphocytes appear to make use of the GPI/IPG signalling system in response to some very important cytokines, IL-2 and insulin, both of which are essential T cell growth factors for the activation of resting cells.

Therefore, the aims of the present work are:

1. To examine mycobacterial cells (*M. tuberculosis* H37Rv, H37Ra and *M. vaccae* R877R) for the presence, or absence, of a GPI-like glycolipid.

2. Any identified glycolipid will be characterised using simple biochemical methods similar to those used in the characterisation of mammalian free membrane GPI and GPI anchors to establish what similarity it has to known properties of mammalian GPI. The biological activity of such material will also be investigated.

3. To confirm the presence of active IPG-like material in mycobacteria (*M. tuberculosis* H37Rv, H37Ra and *M. vaccae* R877R) and attempt preliminary characterisation of this material to determine its relationship, if any, to a putative mycobacterial GPI.

4. To address the question of whether or not IPG-like material from mycobacteria is immunogenic, using animal models and serum samples from tuberculosis patients and healthy controls.

5. The potential existence of anti-IPG immune responses in animals may be exploited to generate antibodies against mycobacterial IPG-like material. Such reagents may have uses in further *in vitro* studies of the effects of mycobacterial IPG-like material, and in addition may be helpful in addressing the degree of similarity between mycobacterial IPG-like material and mammalian IPG.

6. To investigate the effects of mycobacterial IPG-like material on human lymphocytes using an *in vitro* model based on peripheral blood mononuclear cells (PBMCs), which are easy to obtain, measuring variables such as proliferation and cell surface marker expression. These studies will focus on human T cells as they are abundant in PBMCs and play a very significant role in immunity to *M. tuberculosis*.

The implications of experimental findings will be discussed with particular reference to the relevance of mycobacterial GPI or IPG-like material to immunity to tuberculosis.

Chapter 2

Isolation and Partial Characterisation of A Glycophospholipid from *Mycobacterium tuberculosis* and *Mycobacterium vaccae*

2.1 Introduction

Although structures that contain a glycan and phosphatidylinositol are found within mycobacteria, mycobacterial cells have never been subject to the procedures described in the literature for the isolation of free membrane GPI. IPG-like activity derived from mycobacteria has already been documented but whether or not this material is derived from a lipid is unknown. The aim of this chapter was to apply the published GPI isolation procedures to mycobacterial cells. The glycophospholipid(s) isolated have been investigated into their compatibility with the known properties of previously described GPI structures. These are: the presence of sugar residues, phosphate, free amino groups and inositol. Also the sensitivity to cleavage by PI specific and GPI specific enzymes and biological activity has been addressed. Mycobacterial glycophospholipid has been abbreviated to GPL throughout.

2.2 Materials

Tissue culture plastics and ELISA plates were obtained from Nunc (Life Technologies, Paisley, UK). Tissue culture media and additives were obtained from Gibco (Life Technologies, Paisley, UK). Recombinant murine interferon gamma (rmIFNγ) was supplied by Pharmingen (San Diego, CA, USA) and *E. coli* O127:B8 LPS was obtained from Sigma (Poole, Dorset, UK). [9,10(n)- ^{3}H]-palmitate, D-[2-^{3}H]-glycerol, *myo*-[2-^{3}H]-inositol, D-[2-^{3}H]-mannose, D-[6-^{3}H]-galactose and D-[6-^{3}H]-glucosamine were obtained from Amersham radiochemicals (Buckinghamshire, UK). Glycosylphosphatidylinositol phospholipase C (GPI-PLC) derived from *Trypanosoma brucei* and phosphatidylinositol-phospholipase C (PI-PLC) derived from *Bacillus thuringenesis*, expressed in *B. subtilis* were obtained from Oxford Glycosystems (Oxford, UK). Glycosylphosphatidylinositol phospholipase D (GPI-PLD) derived from bovine plasma was obtained from Boeringher Mannheim (Lewes, East Sussex, UK). Thick walled glass reaction vials for phosphorous assay and enzyme treatments were obtained from Pierce (Chester, Cheshire, UK). 20 x 20 cm silica gel 60 glass backed 250μm thickness thin layer chromatography (TLC) plates and 20 x 20 silica gel 60 aluminium backed 250μm thickness high performance thin layer chromatography (HPTLC) plates were obtained from Whatman (Maidstone, Kent, UK).

Silica gel 60 for column chromatography, particle size 40-63μm was obtained from Merck (Poole, Dorset, UK). Recombinant human IL-12 ELISA standard, human IL-12 p40 capture and biotinylated detection antibodies were purchased as a kit from R&D systems (Abingdon, Oxfordshire, UK). Streptavidin-HRPO was obtained from Dako (Glostrup, Denmark). Pyruvate dehydrogenase complex (PDC) and pyruvate dehydrogenase phosphatase were purified from bovine heart by Dr. Kunjara in the laboratory. ATP (disodium salt) and NAD^+ were obtained from Roche Diagnostics Ltd (Lewes, East Sussex, UK). Organic solvents, acids, alkalis and salts for buffers and mycobacterial culture media were obtained from BDH, AnalaR grade (Poole, Dorset, UK). Teflon filters for organic solvents were obtained from Millipore (Watford, Hertfordshire, UK). Liquid scintillation counting was performed using Ultimagold™ obtained from Packard Bioscience (Groningen, Holland). All other chemicals were obtained from Sigma (Poole, Dorset, UK). All glassware used was soaked in 4M nitric acid at least overnight and rinsed in deionised water before use.

2.3 Methods

2.3.1 Culture of mycobacteria

The mycobacterial strains *M. vaccae* R877R, *M. tuberculosis* H37Ra and *M. tuberculosis* H37Rv were present and growing in our laboratory. When necessary, mycobacteria were stored at -20°C on Lowenstien Jensen (LJ) slopes and passaged at least once on LJ medium when removed from storage. For the experiments described, mycobacteria were grown on non-antigenic Sauton's medium, either solid or liquid (3% glycerol, 45mM asparagine, 9.5mM citrate, 8.6mM K_2HPO_4, 1mM $MgSO_4.7H_2O$, 0.19mM ammonium ferric citrate, 1% glucose, with or without 1% agar) at pH 6.2, 32°C (*M. vaccae* R877R) or pH 6.8, 37°C (*M. tuberculosis* H37Ra and H37Rv). All mycobacterial strains were passaged on Sauton's medium at least twice before use.

2.3.2 Extraction and purification of glycophospholipid from mycobacteria

GPL was purified according to the methods previously described for the purification of GPI from mammalian cells and tissues (Caro *et al.* 1993). Typical starting masses were

between five and 20g wet weight of mycobacterial cells. Mycobacterial cells were harvested when they had reached confluent growth on Sauton's slopes. The cells were disrupted by ultrasonication using a 100-Watt ultrasonicator, VibraCell™ model (Sonics and Materials, Inc., Danbury, Connecticut, USA) in 0.9% saline for 30 minutes. The lipid fraction was recovered by centrifugation at 29 500g in a Beckman J2-21M/E Ultracentrifuge (Rotor JA-20, Beckman Instruments Inc, Palo Alto) for 90 minutes and the supernatant was discarded.

The lipid fraction was solublised in solvent I (chloroform/methanol/HCl, 1:2:0.02 v/v/v) using 4ml of solvent per gram of organisms by brief ultrasonication followed by mechanical stirring for 30 minutes. Additional chloroform (3ml per gram of organisms) and 0.1M KCl (3ml per gram of organisms) were added with vigorous stirring for 30 minutes. The resulting two phases were separated by centrifugation at 1000rpm in a Digifuge^{GL} (Heraeus Instruments, Brentwood, Essex, UK) for 5 minutes. The upper, aqueous phase was re-extracted with chloroform (3ml per gram of organisms), the two phases separated again by centrifugation and the aqueous phase was discarded. The final separation of the chloroform phase from the remainder of the aqueous phase was achieved using a separating funnel.

Silica gel 60 (activated at 110°C, 1 hour, approximately 0.5g silica per gram of organisms) was added to the chloroform extract with stirring for 30 minutes. After adsorption of the lipids the silica was transferred to a chromatography column and eluted with two bed volumes of solvent II (chloroform/methanol/HCl, 300:50:3 v/v/v), until the cell wall pigments were seen to have moved through the column. The solvent II fraction was discarded and the remaining lipids were eluted from the column with two bed volumes of methanol, until the lipids were seen to have moved off the silica leaving it white, and dried *in vacuo.*

Dried material was resuspended in solvent III (chloroform/acetone/methanol/acetic acid/water 50:20:10:10:5 v/v/v/v/v) and loaded onto a second silica chromatography column (containing the same amount of silica as the first). The column was eluted with two bed volumes of solvent III, until the remaining unwanted material was removed (seen as a brown discolouration moving through the column). The solvent III fraction was

discarded, the column was finally eluted with two bed volumes of methanol and the eluate dried *in vacuo.*

For application to silica TLC plates dried material was resuspended in chloroform/methanol (1:1 v/v). 1 plate per 4-5g of organisms was used. The plates were also loaded with phosphatidic acid (PA) and phosphatidylcholine (PC) standards and developed in solvent IV (chloroform/methanol/water/ammonia 9:6:0.5:0.35 v/v/v/v). Plates were allowed to dry fully and the lipids were visualised under iodine vapour. The glycolipids were scraped off, extracted three times with methanol (3mls per plate), filtered, and dried *in vacuo.* Material was stored at -20°C until use. For some applications (see section 2.4.4) the material was further purified by butanol/water phase separation. The material was dissolved in 8 to 10ml water saturated butanol. 4 to 5 ml water was added, mixed vigorously, and separated by centrifugation at 1500rpm for five minutes in a Centaur 1 MSE. The upper (butanol) phase was collected and dried *in vacuo*, the water phase discarded.

2.3.3 Metabolic labelling of mycobacterial glycophospholipid

Mycobacteria were cultured in 250ml Erlenmeyer flasks in an orbital incubator at 100 rpm, 32°C. Pilot experiments were conducted in liquid Sauton's medium free of glycerol containing 10^6dpm/ml [2-^{3}H]-glycerol (specific activity 1.1Ci/mmol). [2-^{3}H]-glycerol uptake into the cells was demonstrated by washing the cells free of [^{3}H]-glycerol then dissolving the cells in 0.5ml of hydroxy-hyamine and 0.5ml methanol. The resulting liquid was added to an equal volume of scintillant for counting in a Beckman LS 5000CE liquid scintillation counter (Beckman Instruments Inc, Palo Alto).

For metabolic labelling, 2g *M. vaccae* R877R cells were grown in 25ml liquid Sauton's medium containing 0.5mCi [2-^{3}H] glycerol (specific activity 1.1 Ci/mmol), [9,10(n)- ^{3}H]-palmitate (specific activity 52.0 Ci/mmol) or *myo*-[2-^{3}H]-inositol (specific activity 18.0 Ci/mmol). For experiments that included [^{3}H]-glycerol, medium free of unlabelled glycerol was used. After ten minutes and 24 hours of culture in the presence of the radiolabelled substrate, 10ml of the culture volume was withdrawn and separated from excess radiolabelled substrate by washing the organisms in 10ml ultrapure water and centrifuging at 10 000 rpm for ten minutes in a Beckman J2-21M/E Ultracentrifuge, rotor JA-20. GPL was then extracted by the method described above. Counts were performed on a sample

(usually one percent) of the material present in the solvent I extract of the pellet (reflecting the counts present in the total membranes), eluates from each silica chromatography column and after the TLC (the final product). Labelling with 0.5mCi D-[2-^{3}H]-mannose (specific activity 16.5 Ci/mmol), D-[6-^{3}H]-galactose (specific activity 32.0 Ci/mmol) and D-[6-^{3}H]-glucosamine (specific activity 32.0 Ci/mmol) was performed for three hours under otherwise identical conditions. Samples were taken for counting after each extraction stage as described above. All counts were performed using a Beckman LS 5000CE liquid scintillation counter.

2.3.4 Inorganic phosphate assay

Inorganic phosphate was determined indirectly by incubation with 70% perchloric acid at 180°C to release phosphorous from phosphate. Reaction of phosphorous with molybdate produces coloured phosphomolybdate which allows spetrophotometric determination with a peak absorbance at 830nm (Bartlett 1958). 10μl of standards (20, 10, 5, 2.5, 1.25 and 0μM KH_2PO_4, or phosphatidylinositol at the concentrations indicated in the text) and unknown sample were dried *in vacuo* in conical 1ml reaction vials. 90μl of 70% perchloric acid was added and the vials heated to 180°C for 45 minutes. Vials were allowed to cool to room temperature. 600μl water, 50μl 5% ammonium molybdate and 50μl 10% ascorbic acid were added. Vials were heated to 95°C for 15 minutes, allowed to cool to room temperature, made up to 1ml and read in an LKB Biochrom Ultrospec II spectrophotometer (LKB Biochrom Ltd, Cambridge, UK) at 830nm.

2.3.5 Glycophospholipid digestion by phospholipases

Mycobacterial GPL was subject to treatment by GPI-phospholipase C (GPI-PLC), GPI-phospholipase D (GPI-PLD) and PI-phospholipase C (PI-PLC).

Treatment by GPI-PLC/D took place in 50μl 0.075% nonidet P-40, 75mM NaCl, 20 mM tris maleate, pH 7 at 37°C for 90 minutes. Enzyme concentrations were 0.5U/ml GPI-PLC and 0.1U/ml GPI-PLD respectively, where one unit of activity is defined as the amount of enzyme that will hydrolyse one pmole of GPI (acetylcholinesterase anchor) in one minute at pH 7.4, 37°C. Negative controls were comprised of GPL only, enzyme only and reaction buffer only. The reaction was stopped by the addition of 750μl chloroform/methanol (1:2

v/v). 250μl 0.1M HCl was added to form two phases, the samples were mixed vigorously by vortex and the phases separated by low speed centrifugation for one minute. The upper (aqueous/methanol) phase was withdrawn into a separate tube and both phases were dried *in vacuo*.

Treatment by PI-PLC took place in 50μl 0.05% triton X-100, 25mM tris acetate pH 7.4 at 37°C overnight. The enzyme concentration was 1U/ml, where one unit is defined as the amount of enzyme that will hydrolyse one μmole phosphatidylinositol per minute at pH 7.5 and 37°C. The reaction was stopped by the addition of 600μl water saturated butan-1-ol followed by 300μl water. The samples were mixed vigorously by vortex and the phases were separated by centifugation. The upper butanol phase was withdrawn into a separate tube and both phases were dried *in vacuo*.

2.3.6 Analytical thin layer chromatography (TLC)

TLC for the analysis of GPI and phospholipase reaction products was performed using high performance TLC plates (HPTLC). The material to be analysed is loaded onto plates in a band in methanolic solution and allowed to dry. The plates were run in solvent V (chloroform/methanol/0.25% KCl 5:4:1 v/v/v), solvent III or solvent IV (see section 2.3.2). After running the plates were allowed to dry. For the development of colour, plates were sprayed with 1% anisaldehyde in acetic acid with 2% concentrated sulphuric acid and heated. To detect radiolabelled material, the plates were sprayed with en3Hance and autoradiographed for 5 to 10 days at -80°C, after which the films were developed according to standard protocols.

2.3.7 J774 cells and nitrite assays (Griess reaction)

The J774 murine monocyte/macrophage cell line was originally derived from a female BALB/c mouse and is readily adherent and phagocytic (Ralph and Nakoinz 1975). J774 cells were maintained in DMEM (with sodium pyruvate, 1000mg/L glucose, 2mM L-glutamine and pyroxidine) supplemented with 50U/ml penicillin, 50μg/ml streptomycin and 10% FBS (Gibco) at 37°C in a humidified atmosphere containing 5% CO_2. Cells were grown until confluent (every three to four days) and then scrapped and split one in six to one in ten.

Nitric oxide production may be assayed indirectly by measuring the derived nitrite, which is indicative of the nitric oxide formed (Marletta *et al.* 1988). For nitrite assays cells were plated in 96 well plates at 10^5 cells per well in 50µl of DMEM supplemented with penicillin/streptomycin only. Cells were left for two to four hours to adhere, after which experimental additions were made. IFNγ, LPS and GPI or enzyme treated samples were added to a final volume of 125µl. Final concentrations were typically IFNγ 5-6ng/ml, LPS 20ng/ml and GPI or reaction products as indicated in the text. Native GPL was solubilised first in a small volume of methanol (0.5% of final tissue culture medium volume) and very well mixed immediately prior to addition. Nitrite production over 24 hours was determined by withdrawing 100µl of tissue culture supernatant and reacting with an equal volume of the Griess reagent (1% sulfanilamide w/v, 0.1% naphthylethylenediamine dihydrochloride w/v and 2.5% orthophosphoric acid v/v) (Ding *et al.* 1988) for 10 minutes in the dark at room temperature followed by optical density measurement at 570nm. Reference standards of 100, 50, 25 12.5, 6.25, 3.125, 1.5625 and 0µM nitrite were made by dilution of 1mM $NaNO_2$ in unsupplemented tissue culture medium.

2.3.8 PDH phosphatase activation assay

The activation of pyruvate dehydrogenase (PDH) phosphatase provides a measure of P-type IPG activity and has been used extensively for the detection of P-type mediators derived from mammalian tissue. The principle of the assay is illustrated in figure 2.1. Active PDH phosphatase from bovine heart was inactivated by incubation with ATP, which generates the phosphorylated form of the enzyme. P type IPG activates PDH phosphatase, converting the inactive phosphorylated form of PDH into the active non-phosphorylated form. Active PDH then catalyses the conversion of pyruvate and NAD^+ into acetyl Co A and NADH, the latter was measured by spectrophotometry. Thus the activation of PDH was determined by measuring the rate of production of the reduced form of NAD^+, NADH.

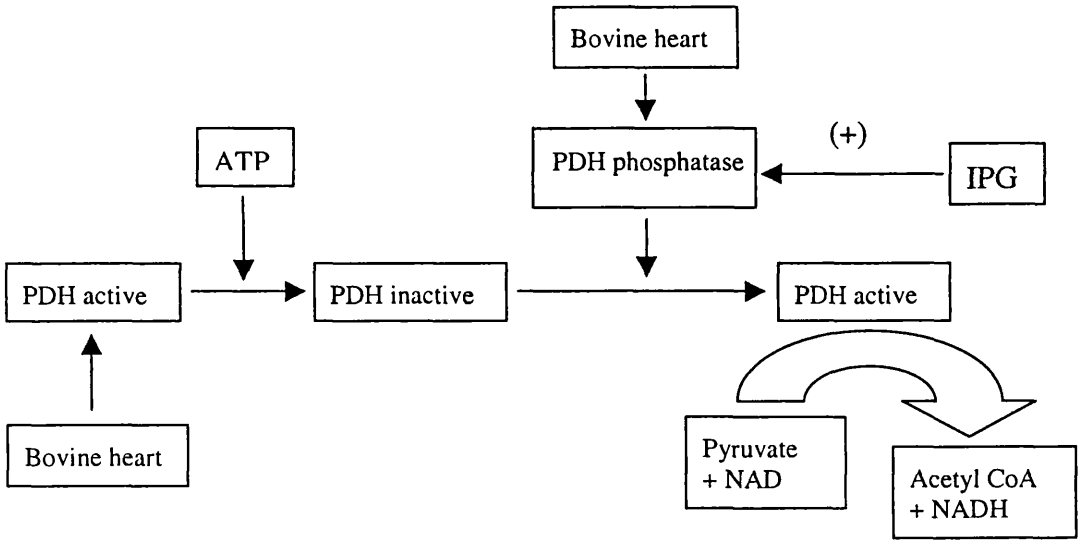


Figure 2.1. Schematic representation of the PDH phosphatase assay.

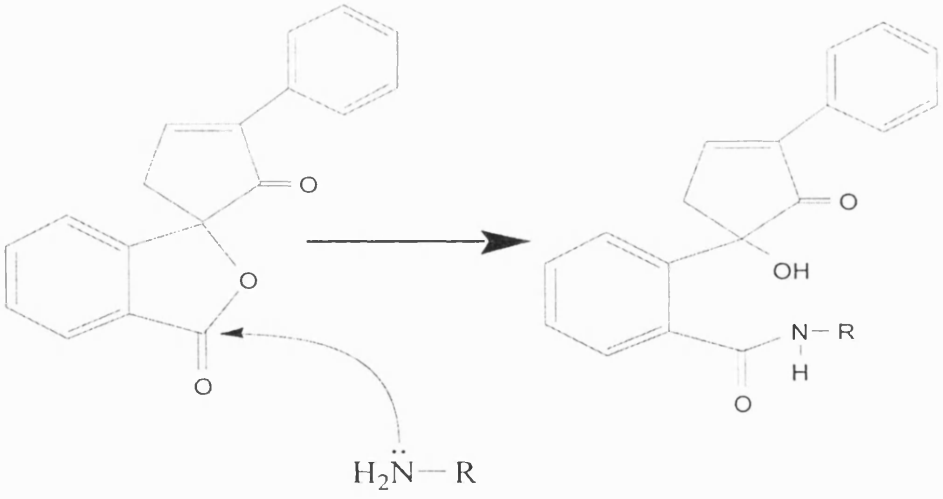
The pyruvate dehydrogenase complex (PDC), a preparation of active (non-phosphorylated) PDH and the soluble PDH phosphatase were isolated from bovine heart mitochondria. These enzymes were prepared by Dr. Kunjara as described by Lilley *et al* (Lilley *et al.* 1992). PDC and PDH phosphatase were stored at -80°C until use. The initial activity of PDH was 20 units/ml (one unit of enzyme produces 1μmol NADH per minute). This value was reduced to less than one percent of the original value after inactivation with ATP. PDH was inactivated by incubating 0.1ml of PDC with 0.4ml PDC inactivation mixture containing 20mM potassium phosphate buffer at pH 7.0, 1mM ATP, 1mM DTT, 0.1mM EGTA and 1mM magnesium chloride and incubating at 30°C for 15 minutes.

For the activity assay, a sample (50μl) of inactivated PDC was pre-incubated at 30°C with 200μl of PDH phosphatase assay mixture containing 1mg/ml fat-free BSA, 10mM magnesium chloride, 0.1mM calcium chloride and 1mM dithiothretiol in 20mM potassium phosphate buffer, pH 7.0, for three minutes. 10-20μl IPG solution (or other test solution as indicated) and 10μl of the PDH phosphatase were then added and the incubation was continued for a further three minutes. The reaction was then stopped by addition of 135μl of 300mM sodium fluoride. The activated PDH activity was then determined spectrophotometrically by measuring the rate of NADH production as follows. A volume

of 200µl of the stopped reaction was added to 1ml of reaction mixture containing 50mM potassium phosphate at pH 8.0, 2.5mM of the oxidised form of NAD, 0.2mM TPP, 0.13mM coenzyme A, 0.32mM DTT and 2mM sodium pyruvate. The production of NADH was followed for seven minutes using absorbance at 340nm.

2.3.9 Fluorescamine reaction for free primary amino groups

The fluorescamine reagent allows the quantitation of free primary amino groups by derivatisation to produce a fluorescent product (Udenfriend *et al.* 1972). This reagent has previously been applied to the study of glycan structures containing amino groups (Caro *et al.* 1997). Fluorescamine undergoes the following reaction:



Standards of 100, 75, 50, 25 and 0µM glucosamine were used in 1ml of reaction buffer (200mM sodium borate, 0.05% Nonidet P-40, pH 9). 1ml of fluorescamine 3mg/10ml (approximately 1mM) in dry acetone was added to the samples and standards. Samples were incubated at 60°C for 30 minutes and analysed using a Jenway 6200 fluorimeter (Jenway Ltd, Dunmow, Essex, UK) with an excitation wavelength of 390nm and a detection wavelength of 480nm.

2.3.10 Culture of THP-1 cells and IL-12 production assay

The THP-1 human monocyte/macrophage cell line was grown in RPMI 1640 supplemented with 10% fetal calf serum, 50U/ml penicillin, 50μg/ml streptomycin and 2mM L-glutamine. Cells were maintained in a humidified atmosphere containing 5% CO_2 at 37°C. Prior to use the cells were further differentiated by overnight culture with dimethyl sulfoxide (DMSO). When the cell had achieved approximately 80% confluency in a culture volume of 20ml, the culture volume was made up to 40ml with 1.2% DMSO and incubated for 24 hours to allow differentiation to take place. After differentiation cells were centrifuged at 1100rpm for four minutes using a Digifuge^{GL} (Heraeus Instruments, Brentwood, Essex, UK) to remove the DMSO containing medium and made up to 4.2 x 10^6 cells per ml in RPMI supplemented with 20% FCS and otherwise as above. 50μl of cell suspension per well was plated into a 96 well tissue culture plate and mycobacterial GPL was added at the concentrations indicated in the text up to a final volume of 125μl. The assay was performed in triplicate sets of culture wells. Prior to addition GPL was solubilised in a small volume of methanol, 0.5% of the final tissue culture volume, and mixed well after addition of the culture medium. The positive control was LPS at 20ng/ml and the negative control was tissue culture medium only. After incubation for 18 hours the supernatants were collected, centrifuged to remove any remaining cells and assayed for IL-12 p40 by ELISA.

IL-12 p40 capture antibody was coated onto a Maxisorp ELISA plate at 2.5pg/ml in 0.1M sodium bicarbonate (100μl per well) overnight at 4°C. The plate was washed once with 200μl PBS/0.05% tween 20 and blocked with 200μl PBS/2%BSA for one hour at 37°C. The plate was washed again three times with 200μl PBS/0.05% tween 20. In order to increase the sensitivity of the assay, 100pg/ml IL-12 p40 was added to every sample in the ELISA. 100μl of the pre-treated samples was added to each well of the ELISA plate. Each tissue culture well was assayed as a single ELISA well, and human IL-12 p40 standards of 4000, 2000, 1000, 500, 250, 125, 62.5 and 31.25pg/ml in duplicate wells were included on each ELISA plate. The plate was incubated with the samples and standards for two hours at 37°C, washed three times with 200μl PBS/0.05% tween 20 and 100μl per well of biotinylated anti IL-12 p40 detection antibody diluted 1 in 300 in PBS/2%BSA was added. The plate was incubated for one and a half hours at 37°C with the detection antibody and then washed three times with 200μl PBS/0.05% tween 20. 100μl strepavidin-HRPO diluted

in PBS/2%BSA per well was added and incubated for one hour at 37°C. Finally the plate was washed three times with 200µl PBS/0.05% tween 20 and 100µl of 3,3',5,5'-tetramethylbenzidine (TMB) liquid substrate system was added. Optical density was measured at 630nm. Upon analysis of the data the 100pg/ml IL-12 added for the assay was subtracted.

2.4 Results

2.4.1 M. tuberculosis *H37Ra, H37Rv and* M. vaccae *R877R contain a glycophospholipid with approximately the same Rf as mammalian GPI*

Upon application of the extraction method described above to *M. tuberculosis* H37 Ra, H37Rv and *M. vaccae* R877R cells, a band was observed under iodine vapour on the TLC plates of the final stage that migrated with an Rf of approximately 0.2 to 0.25 in solvent IV, between the PA and PC standards. Although this is less than the Rf of previously described GPI structures (Mato *et al.* 1987a), the position of this material with respect to the PA and PC standards was the same as for mammalian GPI(Caro *et al.* 1993). Iodine stained TLC plates are faint and best seen with the naked eye. The TLC mobility of GPL from *M. vaccae* R877R in solvent IV is shown in figure 2.2 after development on a HPTLC plate and staining with anisaldehyde, alongside samples from earlier in the purification procedure and authentic standards of phosphatidylethanolamine (PE), PC, PA and diacylglycerol (DAG). The TLC mobility of mycobacterial GPL, however, was different in solvent III, showing some mobility in this solvent whereas previously described GPI species from several sources do not (Mato *et al.* 1987a, Caro *et al.* 1993, Villar *et al.* 1998). This is shown along with HPTLC of *M. vaccae* R877R lipids before all silica chromatography steps in figure 2.3a. Figure 2.3b shows GPL in relation to authentic standards of PC, PE and DAG, again developed in solvent III.

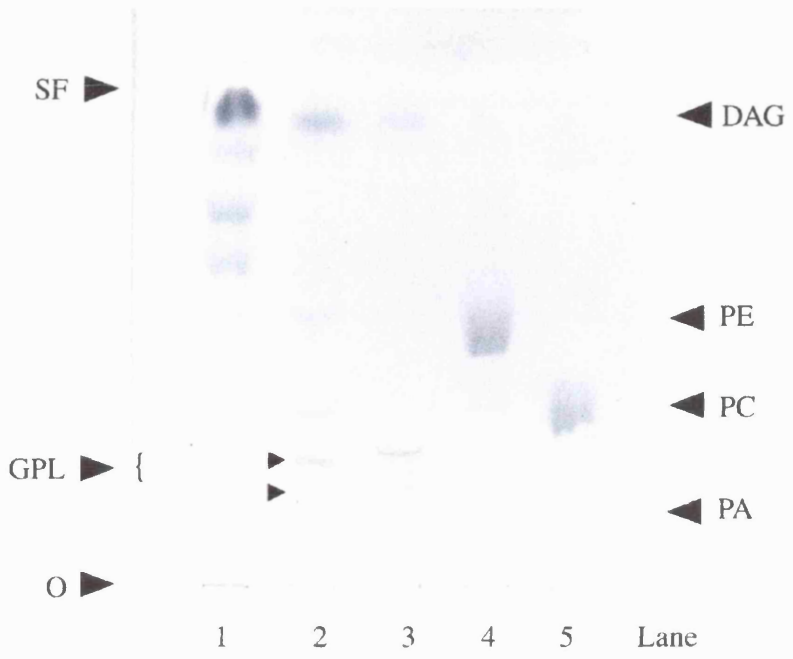


Figure 2.2. Anisaldehyde staining of lipids and GPL from *M. vaccae* R877R after development by HPTLC in a basic solvent system (solvent IV). *M. vaccae* R877R cells were subject to the GPL isolation procedure as described. Lane 1 shows lipids from *M. vaccae* purified up to the point of chloroform/aqueous phase separation, immediately before the first silica chromatography step. Lane 2 shows the same extract after the acidic solvent (solvent III) silica chromatography step. The products from these previous steps were dried and applied to a HPTLC plate along with previously purified GPL from *M. vaccae* R877R (GPL, lane 3) and standards of phosphatidylethanolamine (PE, lane 4), phosphatidylcholine (PC, lane 5), PA and DAG (not shown). GPL from *M. vaccae* R877R resolves into two bands (small arrows) in this solvent with Rfs of approximately 0.25 and 0.2. These Rf values are slightly lower than those previously published, however the position with respect to PA and PC is the same. The lipids were visualised using anisaldehyde and heat. The resulting green colour (which is not faithfully reproduced in this figure) shows the presence of sugar residues. O, origin; SF, solvent front; GPL, glycophospholipid.

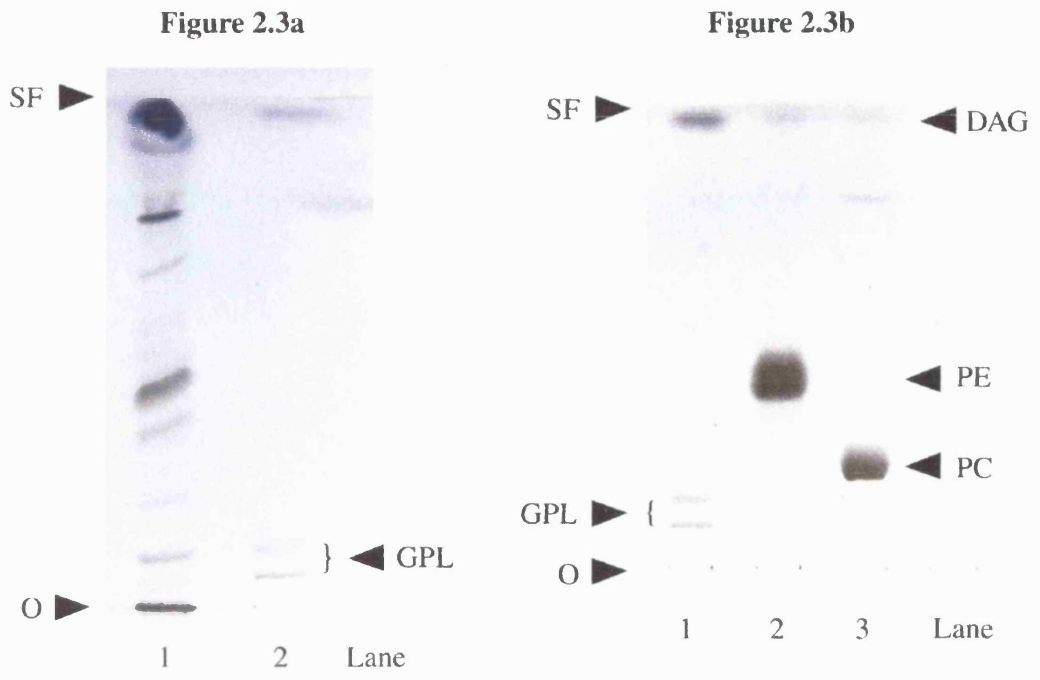


Figure 2.3. Anisaldehyde staining of lipids and GPL from *M. vaccae* R877R after development by HPTLC in an acidic solvent system (solvent III). *M. vaccae* R877R cells were subject to the GPL isolation procedure as described. Figure 2.3a lane 1 shows lipids from *M. vaccae* purified up to the point of chloroform/aqueous phase separation, immediately before the first silica chromatography step. The eluate from this column was dried and applied to a HPTLC plate along with previously purified GPL from *M. vaccae* R877R as a standard (lane 2). Figure 2.3b shows purified GPL from *M. vaccae* R877R (GPL, lane 1) in relation to authentic standards of phosphatidylethanolamine (PE, lane 2), phosphatidylcholine (PC, lane 3) and diacylglycerol (DAG, not shown) developed in solvent III. GPL from *M. vaccae* R877R resolves into two distinct bands with Rfs of approximately 0.07 and 0.1 in solvent III, in distinction to mammalian GPI, which has not been reported to exhibit any mobility in this solvent. Lipids were visualised using anisaldehyde followed by heat. The resulting green colour (which, as in figure 2.2, is not faithfully reproduced in this figure) shows the presence of sugar residues. O, origin; SF, solvent front; GPL, glycophospholipid.

2.4.2 Metabolic labelling of glycophospholipid from M. vaccae *R877R*

GPL from *M. vaccae* R877R was radiolabelled with different substrates to provide labelled material for enzyme digestion, to provide compositional information and to investigate the abundance of GPL within mycobacterial cells.

M. vaccae R877R cells were cultured with radiolabelled glycerol or palmitate to label the lipid part of the molecule. Initial metabolic labelling attempts using in the region of 10^6dpm/ml [^{3}H] glycerol (usually sufficient to label mammalian material) gave counts so low as not to be measurable in the final product. [^{3}H]-glycerol was being taken up into the cells, however, as measured by counting a hydroxy-hyamine digestion of the cells after separation from the residual radioactive substrate. The level of radioactivity in the hydroxy-hyamine solution was out-competed to approximately 1% of its value by a large excess (3%) of unlabelled glycerol (figure 2.4). Of a 10ml cell suspension containing 10^7 dpm (10^6 dpm/ml) only 180 000, or about 2% of the total radioactivity was found inside the cells. For the purposes of metabolic labelling, therefore, 0.5mCi in 25ml, equivalent to approximately 0.7 x 10^8 dpm/ml was included in the culture medium.

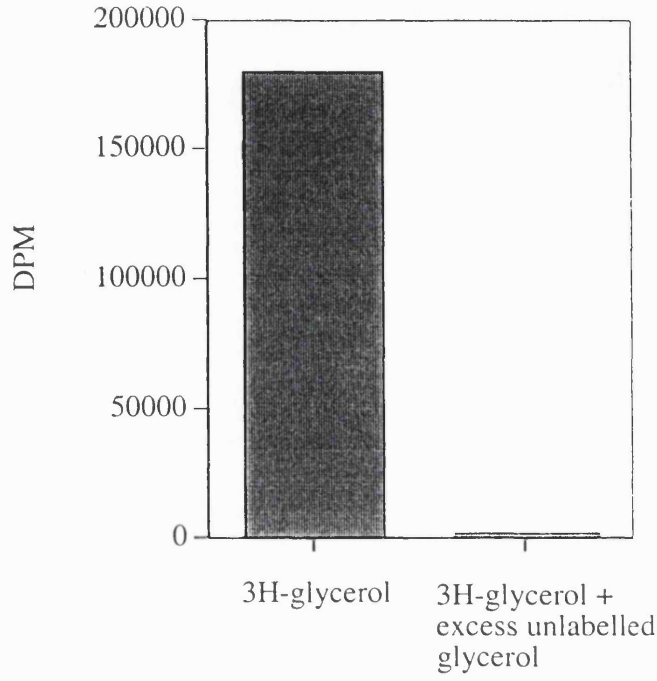


Figure 2.4. [^{3}H]-glycerol is taken up by *M. vaccae* R877R. *M. vaccae* R877R cells were cultured in medium with and without glycerol containing 10^6dpm/ml [3-H]-glycerol. After 24 hours 10ml was removed, the cells were washed and centrifuged free of excess [3-H]-glycerol and were lysed with 0.5ml hydroxy hyamine and 0.5ml methanol. The resultant solution was mixed with 1ml scintillant for liquid scintillation counting. The presence of a large excess of cold glycerol (3% v/v) inhibited the uptake of [3-H]-glycerol by 99%. Values shown are disintegrations per minute (dpm).

In order to establish the kinetics of labelling of GPL in *M. vaccae* R877R, cultures were set up and cells harvested at two time points, after ten minutes and after 24 hours. Samples were taken for counting after each major stage of the extraction. Although the total amount of label incorporation was greater after 24 hours (figure 2.5a), it was noted that when expressed as a proportion of the counts in the membrane, GPL was actually relatively more labelled after only ten minutes of incubation than it was at 24 hours (figure 2.5b). This result suggested that GPL was turning over and incorporating new label at a rate that was faster than the average rate of turnover of the rest of the membrane considered as a whole. This was suggestive of GPL having a physiological function in signalling or homeostasis rather then being a structural component of the cell envelope. In keeping with this is the parallel observation that GPL comprises only a small proportion of the total membrane, certainly less than 1%.

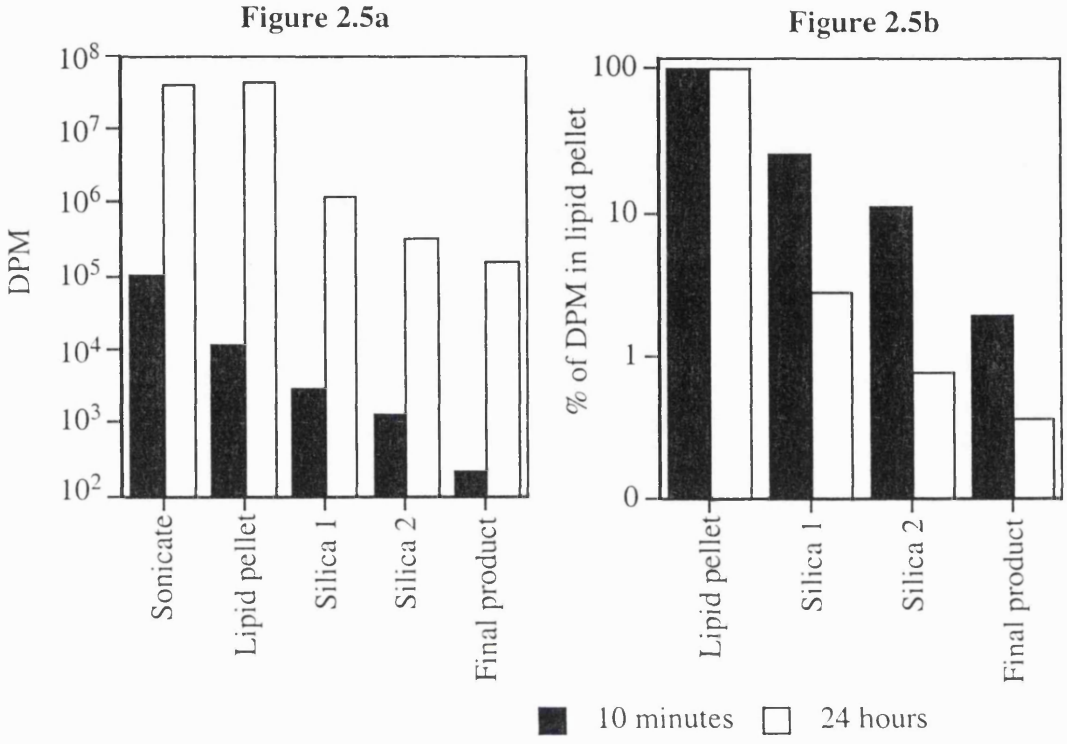


Figure 2.5. Relative incorporation of [^{3}H]-glycerol into GPL from *M. vaccae* R877R after ten minutes and 24 hours. *M. vaccae* R877R cells were innoculated into glycerol free Sauton's medium containing 0.5mCi [^{3}H]-glycerol in 25ml (approximately 0.7 x 10^8dpm/ml). Ten minutes after inoculation, then again at 24 hours, 10ml cell suspension was withdrawn and GPL extracted as described. An aliquot of 1% of the total volume was removed for liquid scintillation counting after each of the major stages of the extraction (except at the final stage when the entire product was counted) performed after ten minutes (filled columns) and after 24 hours (open columns). At the final stage of the extraction, the entire iodine visualised GPL band was scraped and counted. Total label incorporation, figure 2.5a, was much greater (at least two and a half log decades) after 24 hours than after ten minutes. Values shown in figure 2.5a are disintegrations per minute (dpm). Expressed as the percentage of radioactivity present in the lipid pellet remaining after each subsequent extraction stage, figure 2.5b, the proportion of label incorporated into GPL was greater after ten minutes than after 24 hours.

These observations were both repeated with the same qualitative result, however the actual numbers involved were very variable with the relative incorporation at both ten minutes and 24 hours varying significantly between experiments (table 2.1). As GPL is purified by a long complex procedure that takes at least two days, it is very difficult to minimise the variation between different extractions. This makes repeating these experiments and comparing the results from different occasions problematic. The estimation of the proportion of the total lipid fraction that is comprised of GPL, however, was performed many times and was mostly between 0.1 and 1% of the total lipid. Analysis of the data derived using [^{3}H]-glycerol as a substrate for 24 hours showed that the mean proportion of the total lipid fraction that is GPL was 0.46%, standard deviation 0.34 (n = 3). It was also observed that as the DPM found in the total lipid fraction was inversely correlated with the percentage of the lipid fraction DPM that were present in GPL (figure 2.6), perhaps indicating that faster growth of bacteria (inferred from the DPM in the lipid fraction, but not specifically measured) gave less specificity of GPL labelling with [^{3}H]-glycerol. This is a tentative conclusion, however, as growth rate was not specifically determined in these experiments.

Confirmation that the labelled material was indeed the glycophospholipid of interest was obtained using aliquots of [^{3}H]-glycerol labelled material developed in solvent V on an HPTLC plate. Duplicate bands on an HPTLC plate were visualised by autoradiography or anisaldehyde staining and the bands visualised by both methods migrated exactly the same distance (figure 2.7).

Label	Lipid fraction DPM	GPL DPM	% of lipid fraction
Glycerol (1) (10 mins)	11.9×10^3	221	1.86%
Glycerol (1) (24 hours)	44.1×10^6	158×10^3	0.36%
Glycerol (2) (10 mins)	1.40×10^6	49.7×10^3	3.55%
Glycerol (2) (24 hours)	26.6×10^6	133×10^3	0.91%
Palmitate (10 mins)	49.2×10^6	9793	0.020%
Palmitate (24 hours)	53.2×10^6	164×10^3	0.31%
Inositol (10 mins)	46.8×10^3	265	0.57%
Inositol (24 hours)	2.51×10^6	16.6×10^3	0.66%
Glycerol (24 hours)	67.0×10^6	69.3×10^3	0.10%
Glucose (18 hours)	2.89×10^6	6658	0.23%

Table 2.1. Incorporation of various radioactive labels into GPL from *M. vaccae* R877R. *M. vaccae* R877R cells were inoculated into glycerol free Sauton's medium containing 0.5mCi of the label indicated in 25ml (approximately 0.7×10^8dpm/ml). Ten minutes after inoculation, then again at 24 hours, 10ml cell suspension was withdrawn and GPL extracted as described. The amount of radioactivity present at any given extraction stage was determined by counting an aliquot (usually 1%) of the available material. Lipid fraction DPM refers to the total radioactivity present in the membrane pellet, counted after full emulsification in solvent. GPL DPM is the amount of radioactivity present in the final product after purification. The percentage of radioactivity in the lipid pellet that remains in the final product is calculated from these two figures.

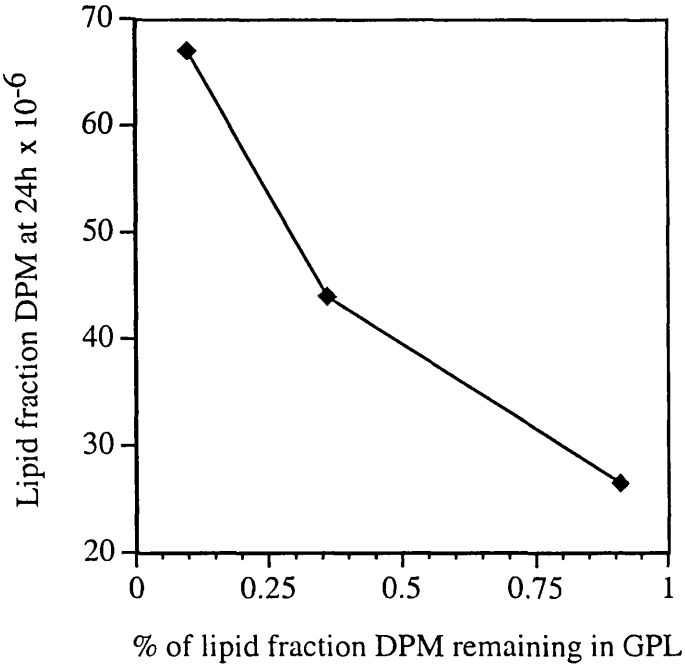


Figure 2.6. Specificity of labelling of GPL from *M. vaccae* R877R with the amount of radioactivity present in the total lipid fraction at 24 hours. *M. vaccae* R877R cells were labelled with [^{3}H]-glycerol as described and after 24 hours GPL was extracted. Total lipid fraction DPM and the percentage of lipid fraction DPM remaining in the final product (GPL) were determined as described above for three separate experiments. This figure shows a schematic relationship between these variables as each point represents a different experiment. The total DPM in the lipid fraction displayed an inverse relationship with the percentage of the lipid fraction DPM remaining in the final product.

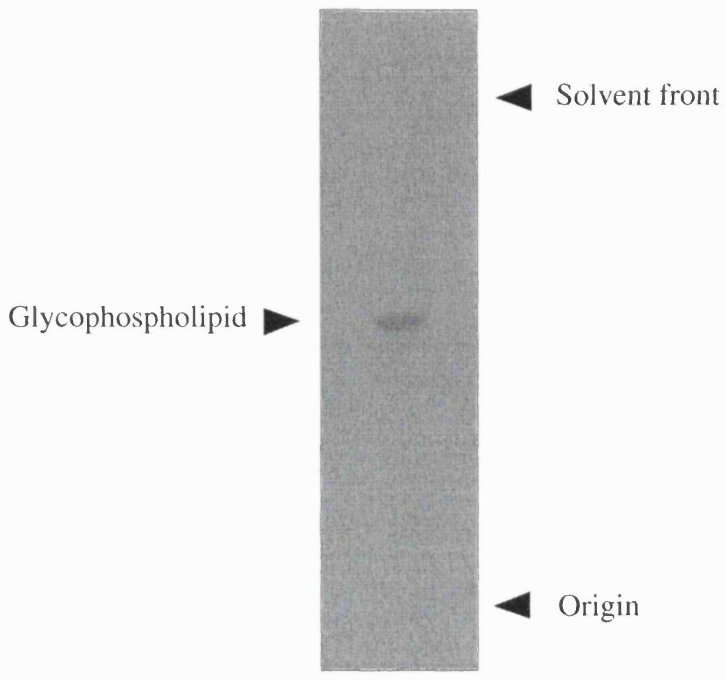


Figure 2.7. Autoradiograph of GPL from *M. vaccae* R877R radiolabelled with [^{3}H]-glycerol. *M. vaccae* R877R cells were radiolabelled with [^{3}H]-glycerol and GPL was extractd as described. Radiolabelled GPL was run on an HPTLC plate in solvent V and autoradiographed at -80°C for 5 days. The position of an anisaldehyde stained GPL standard run on the same HPTLC plate is marked: Glycophospholipid. The anisaldehyde stained material and the radiolabelled material migrated to exactly the same position on the TLC plate.

Labelling of GPL from *M. vaccae* R877R with other substrates for ten minutes and 24 hours did not give the same result as was observed with glycerol (figure 2.8). Two substrates, one that incorporates into the lipid part of the molecule, [^{3}H]-palmitate; and one that incorporates into the glycan part, [^{3}H]-inositol, both failed to show an increase between the relative proportion of the lipid fraction labelled at ten minutes and at 24 hours or showed increased labelling at 24 hours. As the relative pool sizes and kinetics of these substrates unlabelled within the cells prior to addition of labelled substrate was not determined, the significance of the data obtained using [^{3}H]-glycerol as a substrate remains unknown.

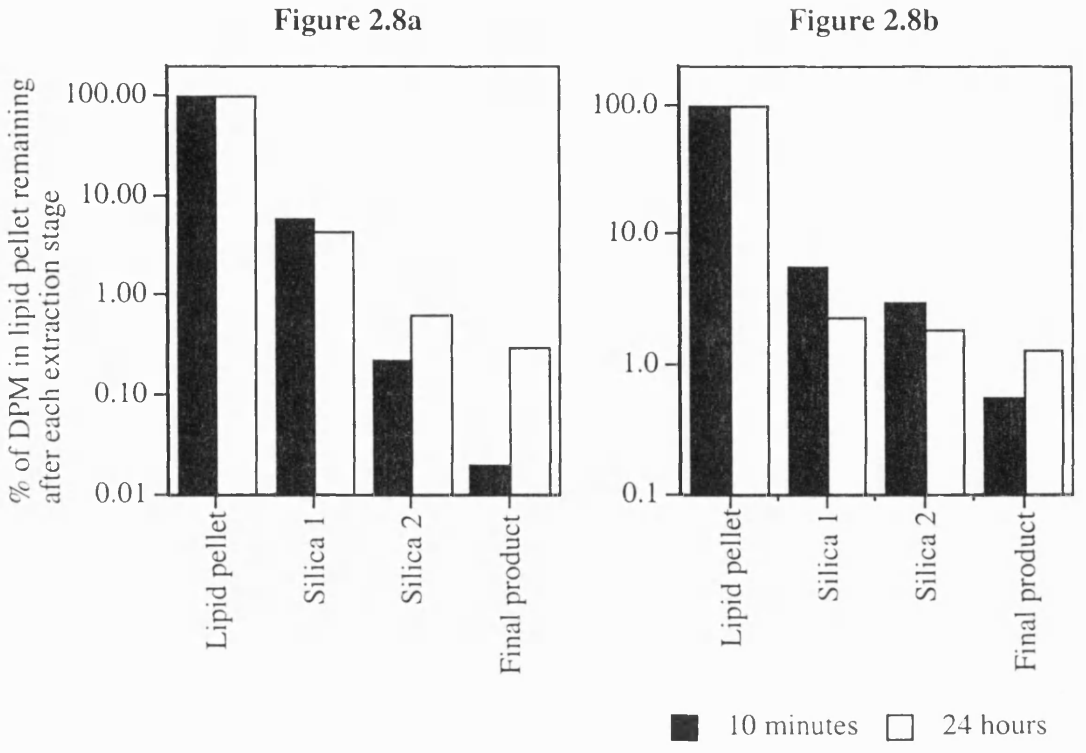


Figure 2.8. Relative incorporation of [^{3}H]-palmitate and [^{3}H]-inositol into GPL from *M. vaccae* R877R represented as the percentage of total counts in the lipid fraction remaining thereafter at ten minutes and 24 hours. *M. vaccae* R877R cells were inoculated into liquid Sauton's medium containing 0.5 mCi [^{3}H]-palmitate (figure 2.8a) or 0.5 mCi [^{3}H]-inositol (figure 2.8b) in 25 ml (approximately 0.7 x 10^8dpm/ml). Ten minutes after inoculation, then again at 24 hours, 10ml cell suspension was withdrawn and GPL extracted as described. An aliquot of 1% of the total volume was removed for liquid scintillation counting after each of the major stages of the extraction, except at the final stage, when the entire product was counted. These counts are shown as the percentage of the radioactivity that was determined to be in the lipid pellet after each subsequent stage of the extraction performed after ten minutes (filled columns) and after 24 hours (open columns).

More information regarding the composition of the glycan component of GPL was obtained by labelling with [^{3}H]-mannose, [^{3}H]-galactose and [^{3}H]-glucosamine. *M. vaccae* R877R cells were cultured for three hours in the presence of these substrates. GPL from *M. vaccae* R877R was found to incorporate mannose and galactose, but did not incorporate glucosamine (figure 2.9).

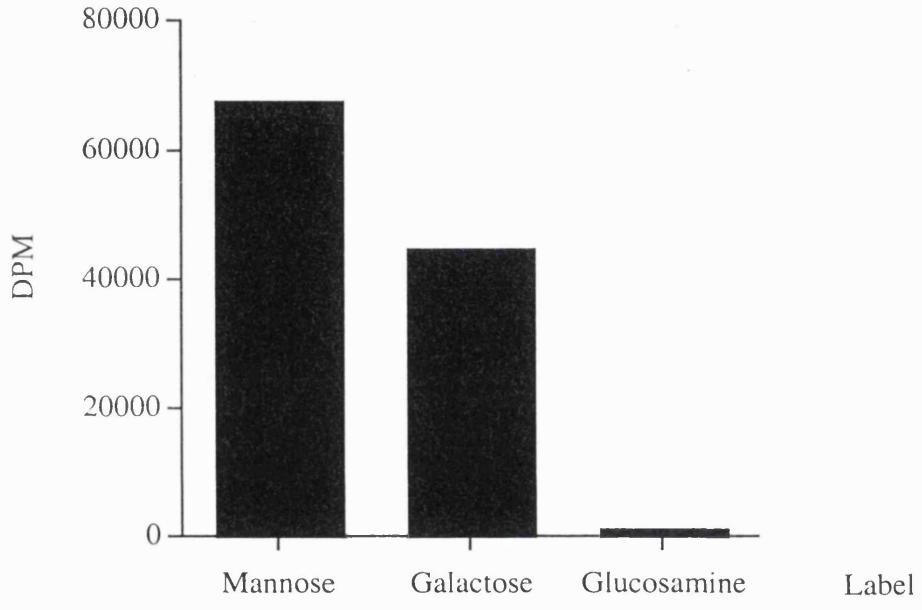


Figure 2.9. Relative incorporation of [^{3}H]-mannose, [^{3}H]-galactose and [^{3}H]-glucosamine into GPL from *M. vaccae* R877R. *M. vaccae* R877R cells were inoculated into Sauton's medium containing 0.5mCi [^{3}H]-mannose, [^{3}H]-galactose or [^{3}H]-glucosamine in 25ml (approximately 0.7 x 10^8dpm/ml). After three hours of incubation GPL was extracted from each of the cultures as described. An aliquot of 1% of the total volume was removed for liquid scintillation counting after each of the major stages of the extraction. The values plotted are the disintegrations per minute present in the final product from the three extractions.

2.4.3 Glycophospholipid from M. vaccae and M. tuberculosis contains phosphate

GPL extracted from *M. vaccae* R877R and *M. tuberculosis* H37Ra were examined for the presence of phosphate by treatment with 70% perchloric acid followed by reaction with molybdate, spectrophotometric determination at 830nm and reference to potassium phosphate standards. Using this method the respective phosphate content of GPL was 40 pmol/g of organisms for *M. vaccae* R877R and 94 pmol/g of organisms for *M. tuberculosis* H37Ra.

Potassium phosphate may not provide a true standard as not all of the phosphate present in GPL may be available to take part in the reaction with molybdate. A known amount of PI was 70% perchloric acid treated and assayed with reference to potassium phosphate standards to provide a more realistic standard. Only one hundredth of the total phosphorous present in PI was found to be available for the reaction. The possibility that GPL was acting as an inhibitor of the reaction was investigated by addition of equal amounts of GPL to PI standards (the method of standard additions). Figure 2.10 shows the slope of the standard curve was unaffected by addition of an equal amount of the unknown GPL to each standard, therefore GPL did not affect the sensitivity of the molybdate reaction.

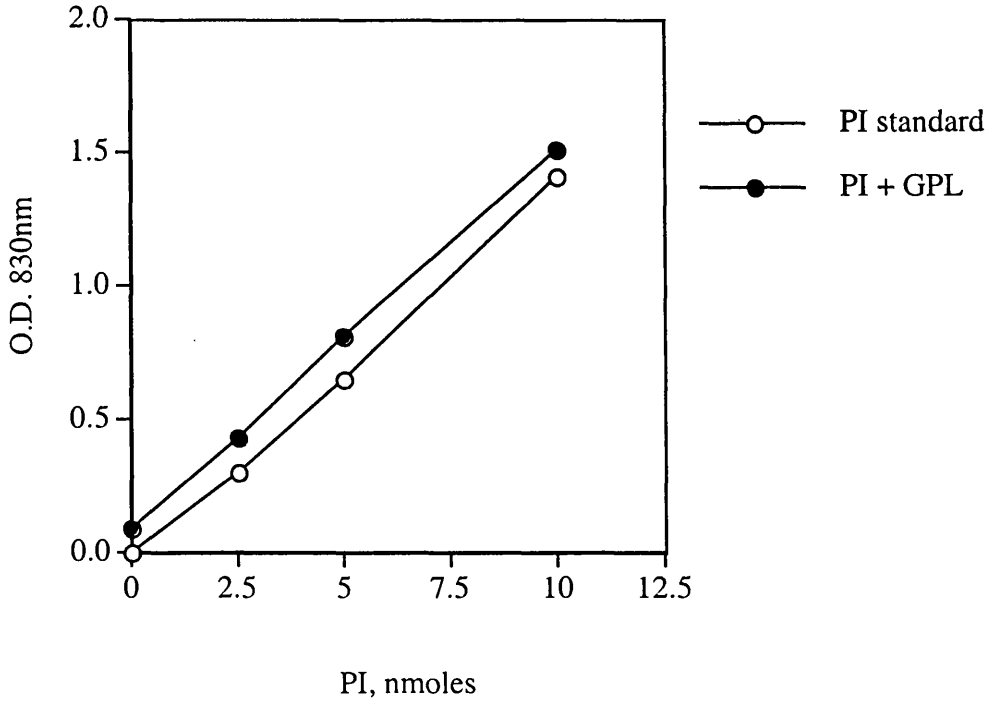


Figure 2.10. Method of standard additions for phosphate determination on GPL from *M. tuberculosis* H37Ra. GPL from *M. tuberculosis* H37Ra was purified and reacted with molybdate as described. GPL was added to each of the points on the standard curve in equal amounts (closed circles) and assayed in parallel to identical standards with no GPL (open circles). After 70% perchloric acid treatment and reaction with molybdate phosphorous was determined as phosphomolybdate by measurement of optical density at 830nm. The slope of the PI standard curve was $y = 0.142x$ and the slope of the PI standards plus GPL was $y = 0.142x + 0.121$. Therefore the two slopes are the same, indicating that the presence of GPL does not affect the sensitivity of the assay.

This assay, therefore, may not display maximum sensitivity when applied to mycobacterial GPL. The decrease in sensitivity seen when PI is used instead of inorganic phosphate implies that this reaction is better applied to the determination of phosphomonoesters, not phosphodiesters, as are present in inositol phospholipids. These data therefore also may imply that the phosphate present in mycobacterial GPL examined is present as phosphodiester not phosphomonoester. The lack of sensitivity of this assay is therefore probably due to unavailability of phosphate derived from the phosphodiester of GPL to take part in the reaction, not due to GPL inhibiting the reaction. Final phosphate determinations for *M. tuberculosis* H37Ra and *M. vaccae* R877R are shown in table 2.2.

Organism	**Phosphate content**
M. tuberculosis H37Ra	9.4nmol/g
M. vaccae R877R	4nmol/g

Table 2.2. Phosphate content of mycobacterial GPL. Mycobacterial GPL was treated with 70% perchloric acid and reacted with molybdate as described to determine phosphate content. Phosphomolybdate was measured by absorbance at 830nm and phosphate determination performed by comparison with phosphatidylinositol standards. Phosphate content is described as the amount of phosphate per gram of starting material in the original extraction.

2.4.4 Glycophospholipid digestion by phospholipases

Hydrolysis of a GPI structure by a GPI specific phospholipase should produce, upon phase separation, an aqueous soluble inositol phosphoglycan (IPG) species and, in the organic fraction, diacyl/alkylacyl glycerol in the case of a PLC or phosphatidic acid in the case of a PLD (Hooper and Turner 1992). The uncleaved GPL should partition into the organic phase. Therefore, in principle, examination of the organic phase will reveal disappearance of GPL, and examination of the aqueous phase will reveal the presence of a glycan of any kind, only if GPL was sensitive to the enzyme.

Phosphatidylinositol specific phospholipases

In order to show the presence of phosphatidylinositol in GPL, material from *M. vaccae* R877R metabolically labelled molecule with 3H-glycerol (see section 2.4.2) was treated with PI-PLC overnight. The reaction was stopped by phase separation in water and water saturated butanol; the upper, organic phase was applied to an HPTLC plate and developed in solvent V. Autoradiography of the developed plate shows that almost all the labelled GPL was hydrolysed over this incubation and a band appeared which co-migrated with the same Rf as diacylglycerol, the expected PI-PLC reaction product (figure 2.11). This demonstrated that all or most radiolabelled GPL from *M. vaccae* R877R contained phosphatidylinositol as the lipid moiety. This also showed that the [^{3}H]-glycerol was labelling the lipid portion of the molecule.

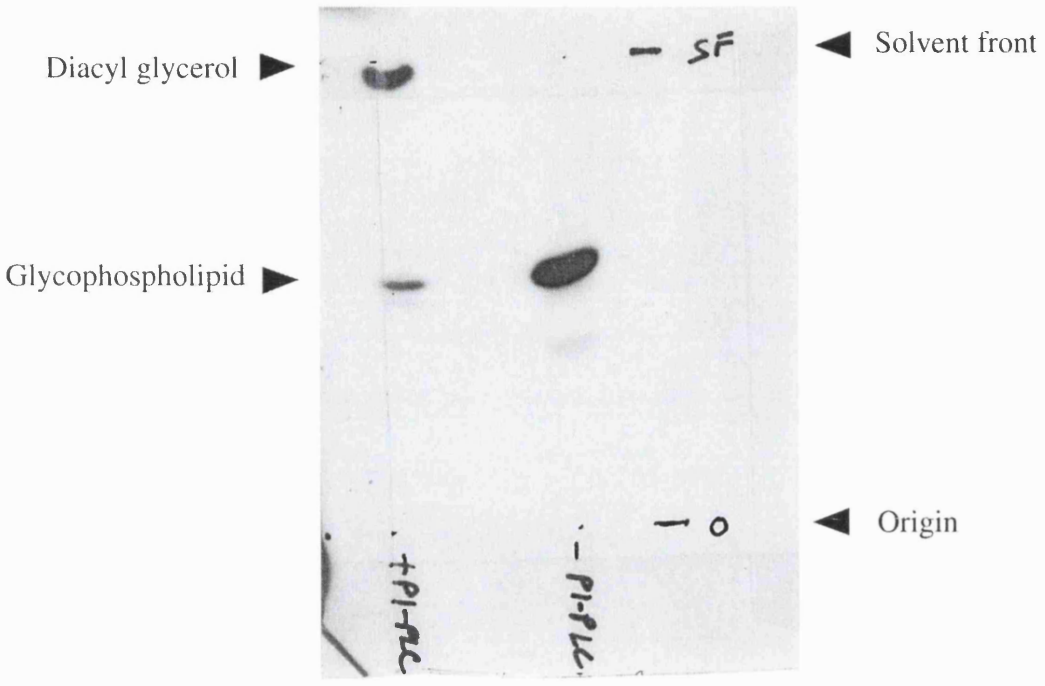


Figure 2.11. Autoradiograph of PI-PLC hydrolysed GPL from *M. vaccae*. [^{3}H]-glycerol labelled GPL from *M. vaccae* R877R was incubated with 0.05U of PI-PLC or without enzyme overnight at 37°C. After incubation, the samples were butanol/water phase separated and the butanol phase was applied to an HPTLC plate. The plate was developed in solvent V, sprayed with en3Hance and autoradiographed at -80°C for ten days. The labelled GPL was almost completely PI-PLC sensitive. The enzyme treated sample showed a band that migrated with the solvent, compatible with it being diacyl glycerol, the expected PI-PLC reaction product. +PI-PLC: GPL with PI-PLC. –PI-PLC: GPL incubated with identical buffer except without the enzyme.

Radiolabelled material from *M. tuberculosis* was not available so susceptibility to PI-PLC was examined by bioactivity of the aqueous phase of PI-PLC treated GPL from *M. tuberculosis* H37Ra in the PDH phosphatase activation assay. GPL from *M. vaccae* R877R was also PI-PLC treated as this material was known to be PI-PLC sensitive, and to determine whether the reaction product affects PDH phosphatase activation. GPL was incubated overnight with and without PI-PLC as described and the reaction stopped by butanol/water phase separation. Examination of the aqueous phases of these enzyme reactions and the respective controls for induction of PDH phosphatase activity, however, showed that the only requirement for activity was the presence of GPL, not GPL and the enzyme (figure 2.12). The data shown for *M. tuberculosis* H37Ra included a butanol/water extraction step prior to the enzyme treatment of which only the butanol phase was treated. This showed that the activity present was not due to a water soluble contaminant that was being carried over from the stock GPL into the aqueous phase of the reaction products. GPL itself, therefore, is active in this assay. This would be dependent upon native GPL not partioning entirely into the butanol phase after the reaction, which was subsequently shown using [^{3}H]-mannose labelled material from *M. vaccae* R877R.

[^{3}H]-mannose labelled GPL from *M. vaccae* R877R was phase partitioned using water and butanol. An aliquot of each phase was counted then each phase was dried *in vacuo* and subject to a further round of butanol/water phase separation and counting. GPL initially separated into butanol and water in proportions 80% and 20% respectively. The GPL that partitioned into butanol then separated 90% into butanol and 10% into water, whereas that which had partitioned into water gave nearly the reverse, 20% separated into butanol and 80% into water (figure 2.13).

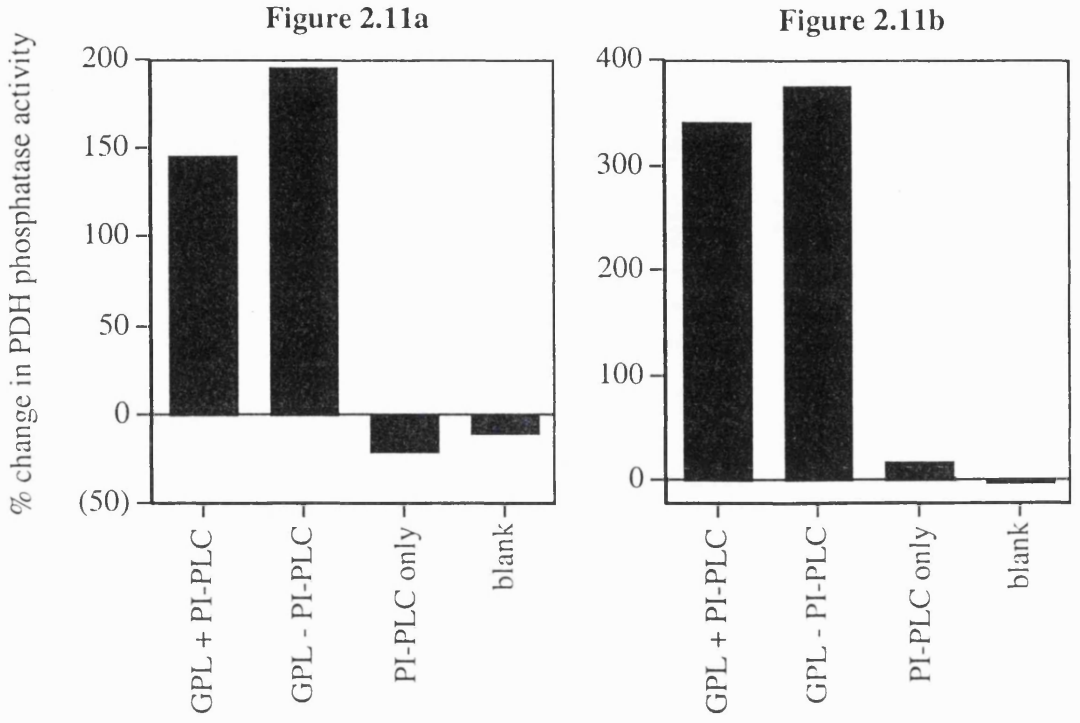


Figure 2.12. GPL from *M. tuberculosis* H37Ra and *M. vaccae* R877R activates PDH phosphatase. GPL equivalent to 500mg organism starting mass from *M. tuberculosis* H37Ra (figure 2.12a) and *M. vaccae* R877R (figure 2.12b) was incubated with 0.05U of PI-PLC overnight at 37°C, mock treatments comprising enzyme only, GPL only or buffer only. After incubation, the samples were butanol/water phase separated and the water phase was examined for the ability to activate PDH phosphatase. PDH phosphatase activation was determined by spetrophotometric measurement of the rate of NADH production by pyruvate dehydrogenase complex isolated from bovine heart mitochondria. Activity is expressed as the percentage increase (or decrease) in the rate of NADH production.

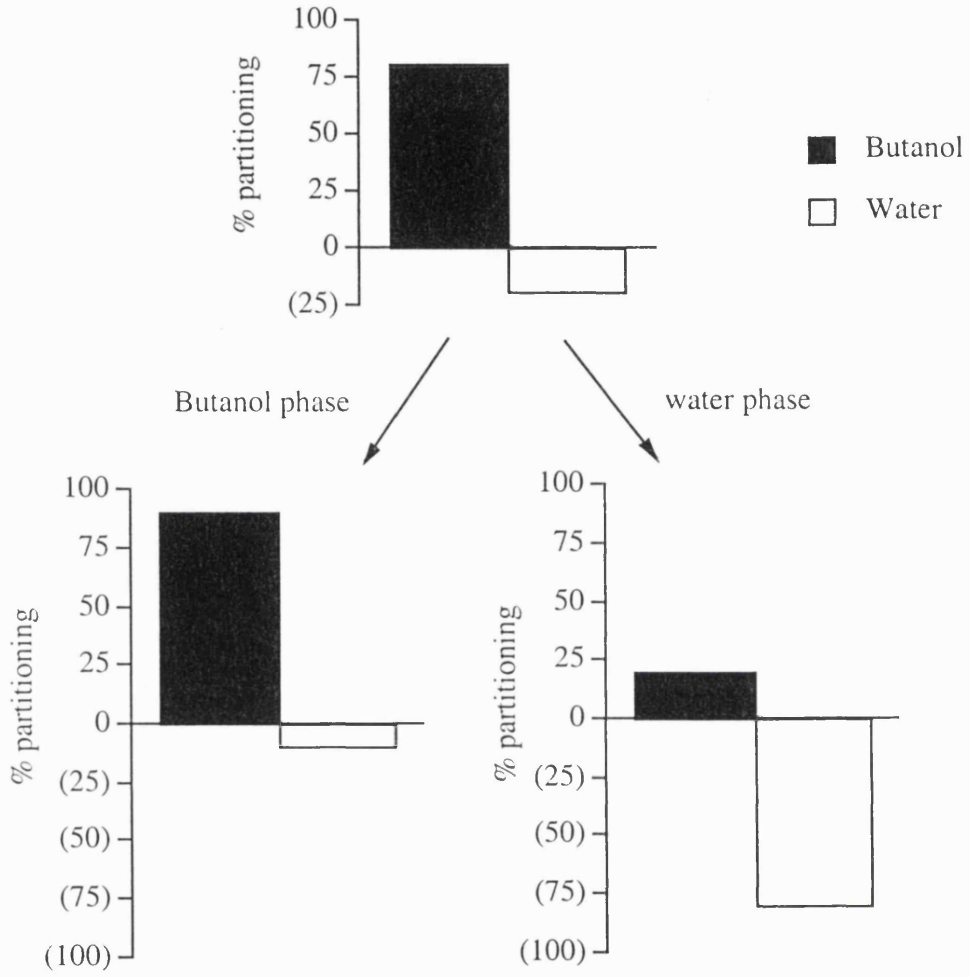


Figure 2.13. Phase partitioning of [^{3}H]-mannose labelled GPL from *M. vaccae* R877R. [^{3}H]-mannose labelled GPL was dissolved in 1ml of water saturated butanol and a further 1ml of water was added to phase partition the material. An aliquot of each phase was counted. Each phase was dried *in vacuo*, then separately subjected to the same procedure. Data plotted are the percentage of counts from each phase separation that move into the butanol (black bars) and water (light grey bars).

Having established that GPL itself is active in this assay and that it can partition into the water phase in butanol/water, interpretation of the results of the enzyme treatments becomes more complex. Figure 2.13 might show less activity after enzyme treatment in the case of *M. tuberculosis*, although the data are single points so the significance of the difference between the PI-PLC treated and untreated samples is not quantified. If GPL was available as a substrate for this enzyme, as strongly suspected from previous data, then the fact that the enzyme treated sample shows less activity than the enzyme untreated sample suggests that, in this assay, removal of the lipidic portion of the molecule may render it inactive, rather than the other way around. This phenomenon has been shown to be the case with some synthetic compounds in our collaborators' laboratory.

Glycosylphosphatidylinositol specific phospholipases

Having putatively established the presence of phosphatidylinositol, at least in the case of *M. vaccae* R877R GPL, material from both *M. vaccae* R877R and *M. tuberculosis* H37Rv were treated with GPI specific phospholipases. The reaction products separated by phase partitioning using chloroform, methanol and 0.1 M HCl and the upper, aqueous, phases were assayed for NO production activity from J774 cells. NO production induced by GPI-PLC and PLD treated GPL from *M. vaccae* R877R is shown in figure 2.14. Interestingly, the PLC product appeared to be toxic at high doses, whereas the PLD product was not. This result could be due to the toxicity of the cyclic phosphate generated as a PLC reaction product, or due to excessive activation of the cells. A repeat GPI-PLC treatment is shown in figure 2.15 and again the PLC reaction product was toxic at high doses. Although these representative experiments show variable NO production (perhaps this variation is due to the use of two different batches of rmIFNγ in these two particular experiments) there is dose dependent activity present in the aqueous phases of phase partitions from both GPI-PLC and PLD treatments over GPL incubated with no enzyme. (Figure 2.15 does not show a negative control due to shortage of available material at the time the experiment was performed.) These results were not due to contamination of the enzyme or reagent stock with LPS because a control experiment in which all the enzyme and buffer was added to J774 cells did not result in any NO production (data not shown).

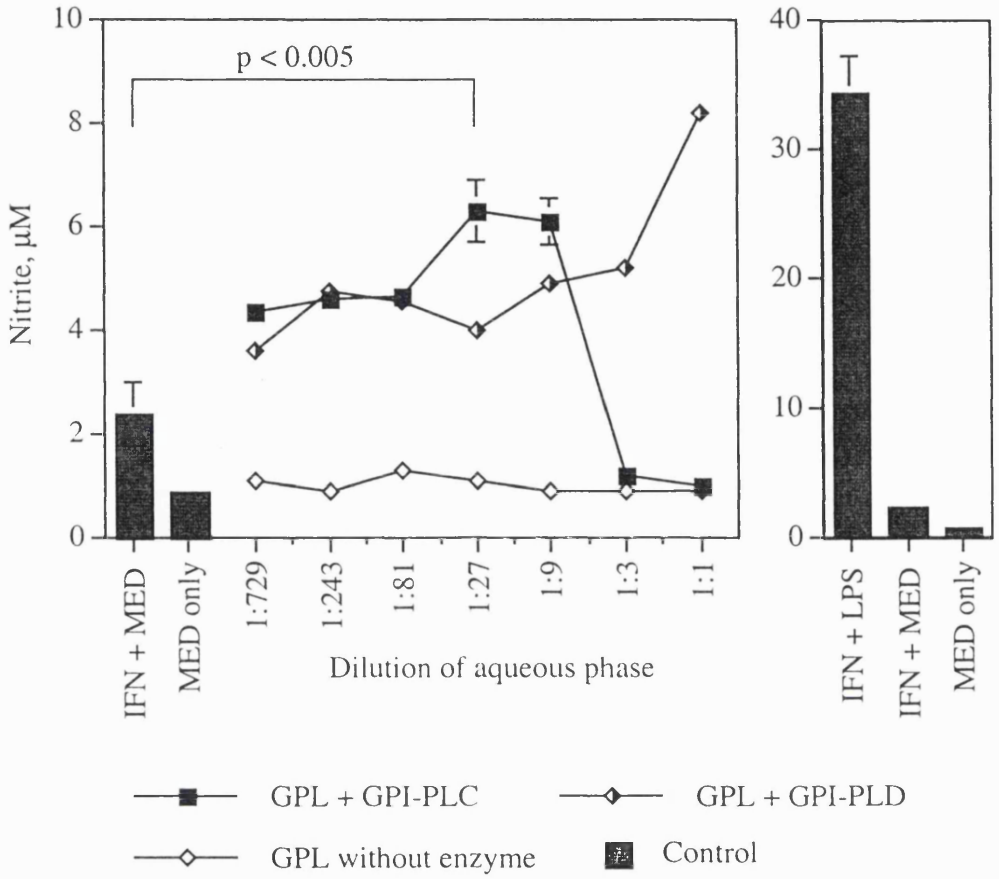


Figure 2.14. NO inducing activity of the aqueous fraction of GPI phospholipase treated GPL from *M vaccae* R877R. After incubation of 450mg equivalent organism starting mass of GPL with 0.025U of GPI-PLC, 0.005U of GPI-PLD or without enzyme for 90 minutes, the reaction was stopped by phase separation and the aqueous phase examined for NO inducing activity. The aqueous phase was dried, re-dissolved in 120µl culture medium, serially diluted 1 in 3 each time and added to IFNγ activated (5ng/ml) J774 cells. NO inducing activity was dose dependent and is expressed against the dilution of the aqueous phase where 1:1 represents the addition of 25µl of un-diluted solution to a final volume of 125µl per well. NO inducing activity was significant in the case of GPI-PLC treated samples ($p < 0.005$, student's t-test). (Statistical analysis was not performed upon the GPI-PLD data series as the standard deviation was not available.) NO inducing activity is represented as nitrite concentration in 100µl of tissue culture supernatant. The positive control was IFNγ activated cells with LPS (20ng/ml) which induces strong NO production. Negative controls were IFNγ activated cells only (IFN+MED) and cells with medium only (MED only) and are shown twice, relative to the experimental samples and the positive control. Values shown are the mean of triplicates +/- standard deviation in all cases except GPI-PLD treated GPL which is the mean of duplicates.

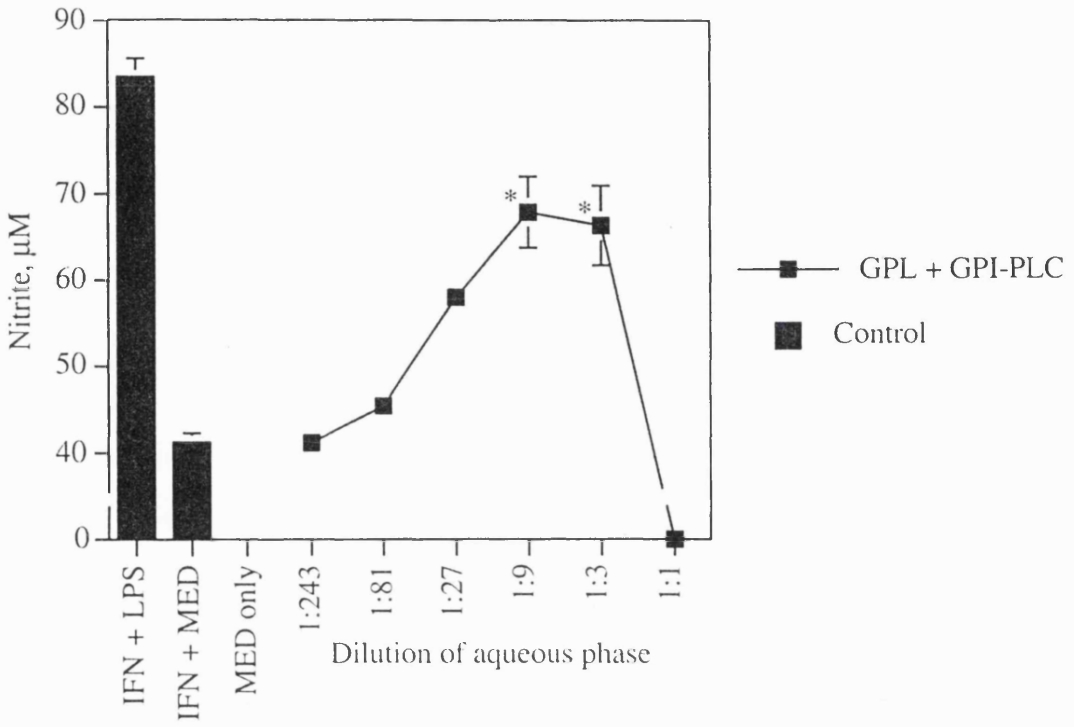


Figure 2.15. NO inducing activity of the aqueous fraction of GPI-PLC treated GPL from *M vaccae* R877R. After incubation of 450mg equivalent organism starting mass of GPL with 0.025U of GPI-PLC or without enzyme for 90 minutes, the reaction was stopped by phase separation and the aqueous phase examined for NO inducing activity. The aqueous phase was dried, re-dissolved in 120µl culture medium, serially diluted 1 in 3 each time and added to IFNγ activated (6ng/ml) J774 cells. NO inducing activity was significant (* = $p < 0.005$, student's t-test), dose dependent and is expressed against the dilution of the aqueous phase where 1:3 represents the addition of 25µl of the first dilution to a final volume of 125µl per well. Addition of the neat (1:1 dilution) aqueous phase was toxic to the cells in this experiment, as shown by less NO production in these cells than in the negative control. NO inducing activity is represented as nitrite concentration in 100µl of tissue culture supernatant. Positive and negative controls are the same as figure 2.14. Values shown are the mean of triplicates +/- standard deviation.

NO production induced by GPI-PLC treated GPL from *M. tuberculosis* H37Rv is shown in figure 2.16. As was observed with material derived from *M. vaccae* R877R, the highest doses were toxic to the cells. In this case, however, activity is also present in the sample not treated with GPI-PLC, and this activity achieved toxicity at the highest dose. This suggests that over-stimulation of the cells, rather than toxicity specifically due to a cyclic phosphate, was responsible for this effect. Although GPI derived from both mammalian tissue and the trypanosome does not partition into both phases (Hooper and Turner 1992), this result suggests that mycobacterial GPL does, as was shown to be the case with butanol/water as the solvents. Doubling the volumes used to stop the reaction (1.5 ml chloroform/methanol, 0.5 ml 0.1 M HCl) to minimize disturbance of the lower phase partially overcame this problem (figure 2.17), the activity of the GPI-PLC treated sample was significantly higher than the untreated sample, showing that GPL is sensitive to GPI-PLC. However, the untreated sample still showed significant activity. These data indicate that GPL does exhibit some solubility in both phases of the chloroform/methanol/water separation, as with water/butanol.

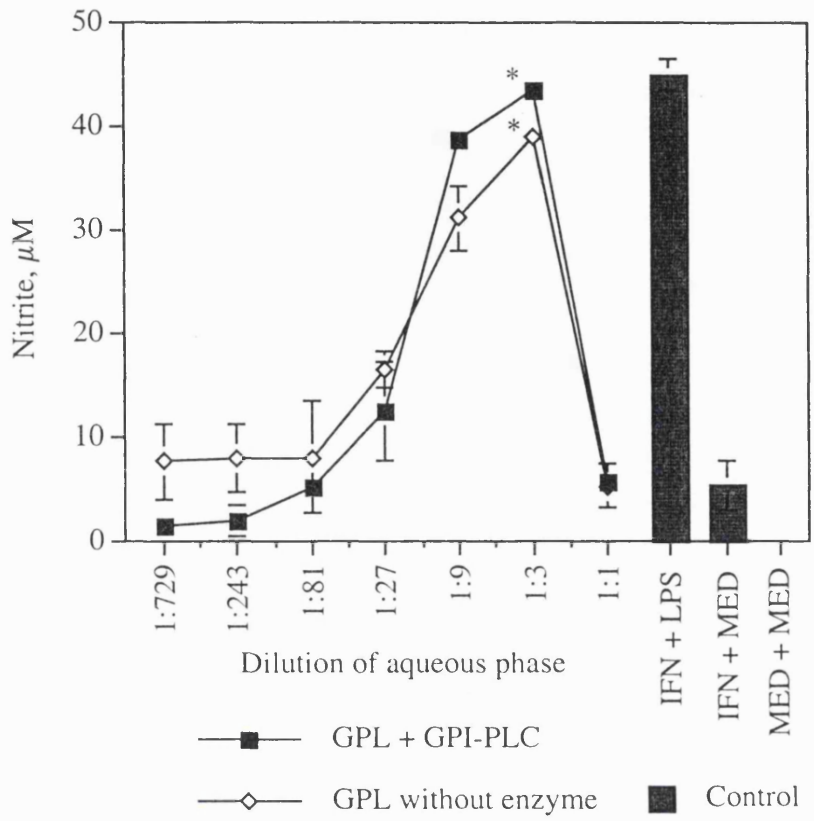


Figure 2.16. NO inducing activity of the aqueous fraction of GPI-PLC treated GPL from *M tuberculosis* H37Rv. After incubation of 450mg equivalent organism starting mass of GPL with 0.025U of GPI-PLC or without enzyme for 90 minutes, the reaction was stopped by phase separation and the aqueous phase examined for NO inducing activity. The aqueous phase was dried, re-dissolved in 120μl culture medium, serially diluted 1 in 3 each time and added to IFNγ activated (6ng/ml) J774 cells. NO inducing activity was significant (* = $p < 0.005$, student's t-test), dose dependent and is expressed against the dilution of the aqueous phase where 1:1 represents the addition of 25μl of un-diluted solution to a final volume of 125μl per well. NO induction by the GPI-PLC treated sample was also significantly greater than the untreated sample at the point of maximal response ($p < 0.005$, student's t-test). NO inducing activity is represented as nitrite concentration in 100μl of tissue culture supernatant. The positive control was IFNγ activated cells with LPS (20ng/ml), which induces strong NO production. Negative controls were IFNγ activated cells only (IFN+MED) and cells with medium only (MED only). Values shown are the mean of triplicates +/- standard deviation.

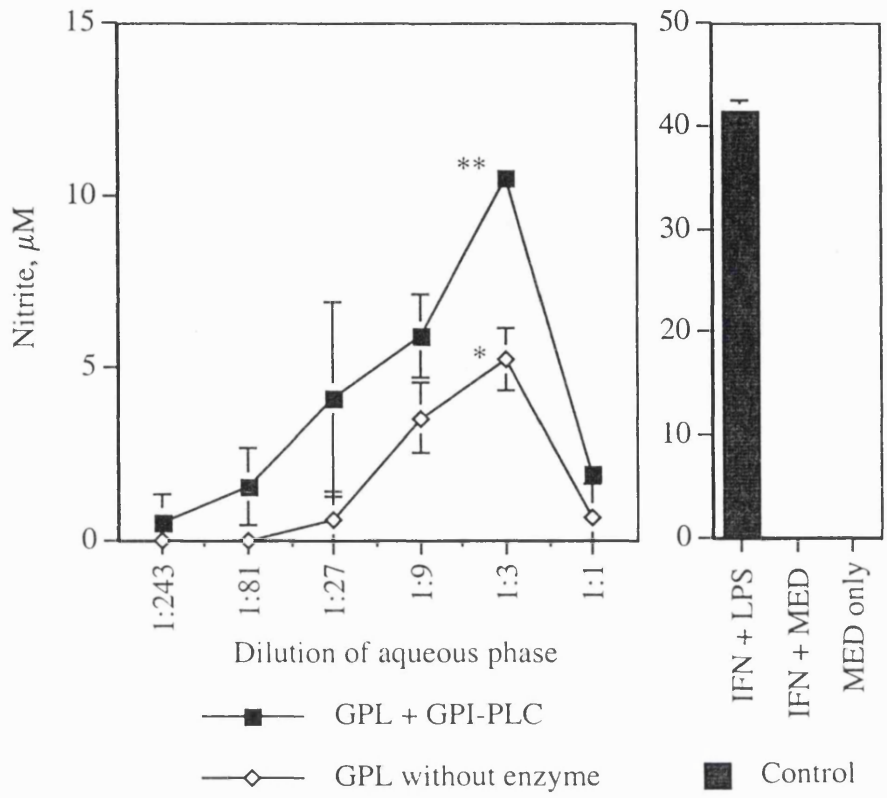


Figure 2.17. NO inducing activity of the aqueous fraction of GPI-PLC treated GPL from *M tuberculosis* H37Rv. After incubation of 450mg equivalent oganism starting mass of GPL with 0.025U of GPI-PLC or without enzyme for 90 minutes the reaction was stopped by phase separation and the aqueous phase examined for NO inducing activity. The aqueous phase was dried, re-dissolved in 120μl culture medium, serially diluted 1 in 3 each time and added to IFNγ activated (6ng/ml) J774 cells. NO inducing activity was dose dependent and is expressed against the dilution of the aqueous phase where 1:1 represents the addition of 25μl of the re-dissolved aqueous phase to a final volume of 125μl per well. Addition of the neat (1:1 dilution) aqueous phase was toxic to the cells in this experiment. The GPI-PLC treated sample induced significantly more NO then the untreated sample (** = $p < 0.005$, student's t-test). However, the untreated sample also gave significant NO production compared with the negative control, IFN + MED (* = $p < 0.005$, student's t-test). NO inducing activity is represented as nitrite concentration in 100μl of tissue culture supernatant. The positive control was IFNγ activated cells with LPS (20ng/ml), which induces strong NO production. Negative controls were IFNγ activated cells only (IFN+MED) and cells with medium only (MED only). Values shown are the mean of triplicates +/- standard deviation.

In addition to the demonstration of sensitivity to GPI-PLC, these data also show that native GPL from *M. tuberculosis* H37Rv is active in NO production from J774 cells. This is not surprising as many bacterially derived lipids and glycolipids cause macrophages to release inflammatory mediators and other substances involved in bacterial killing. This was confirmed by adding GPL completely untreated to J774 cultures (figure 2.18). GPL from both *M. vaccae* R877R and *M. tuberculosis* H37Rv were able to induce NO production from J774 cells and the dose response was bell shaped, with higher doses inducing less NO production. This was probably due to overstimulation of the cells resulting in toxicity, as seen in previous experiments. GPL from *M. tuberculosis* H37Rv was significantly more active than that from *M. vaccae* R877R, providing an explanation for why activity in the non-enzyme treated samples of *M. vaccae* R877R GPL was not observed earlier.

Having established that the GPL could induce NO production from murine J774 macrophages, the effect of GPL from *M. tuberculosis* H37Ra on a human macrophage cell line, THP-1, was also investigated. Human macrophages do not so readily produce NO *in vitro*, so to investigate the activity of GPL IL-12 production was measured by ELISA instead. Figure 2.19 shows dose dependent IL-12 production from THP-1 cells induced by GPL from *M. tuberculosis* H37Ra, showing that GPL can induce the production of inflammatory mediators other than NO, and that there is also an effect upon human cells. In this experimental system GPL was not toxic to the cells at high doses. Unfortunately insufficient material was available at the time to use the same dose range as was used to determine NO release, but at a dose of 100mg GPL *M. tuberculosis* H37Ra organism equivalent per well there was no diminution of the IL-12 response whereas this clearly was in the case of the NO release assay. The positive control was bacterial LPS, another bacterial glycolipid known to induce IL-12 production from THP-1 cells.

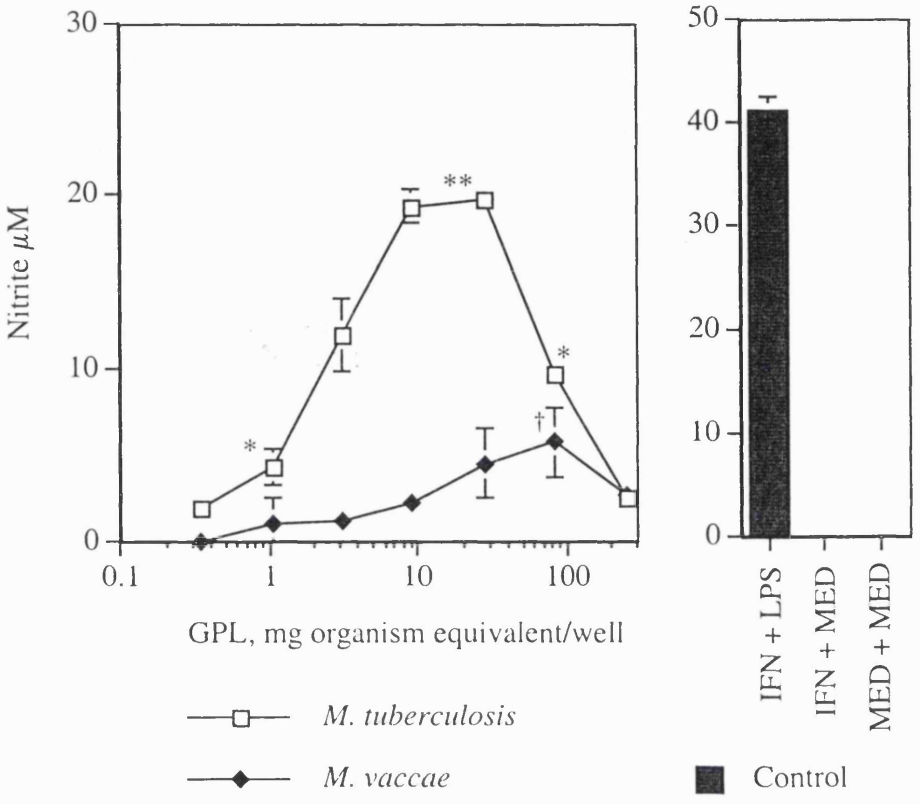


Figure 2.18. NO inducing activity of native GPL from *M. tuberculosis* H37Rv and *M. vaccae* R877R. IFNγ (6ng/ml) activated J774 monocyte/macrophages (10^5 per well) were cultured with mycobacterial GPL. GPL was dissolved in methanol and then diluted in tissue culture medium to a final methanol concentration of 0.5% v/v. GPL was derived from an equivalent amount of starting mass of both *M. tuberculosis* H37Rv and *M. vaccae* R877R cells. The amount of GPL is expressed in terms of the extraction starting mass equivalent of GPL present in each well after dilutions. NO production induced by *M. tuberculosis* H37Rv GPL was significantly greater than that induced by *M. vaccae* R877R GPL (* = $p < 0.05$, ** = $p < 0.005$, student's t-test). NO production induced by *M. vaccae* R877R GPL was also significant († = $p < 0.01$, student's t-test). NO inducing activity was measured by reaction of the Griess reagent with an equal volume of tissue culture supernatant and is represented as nitrite concentration in 100μl of tissue culture supernatant. Values shown are the mean of triplicates +/- standard deviation.

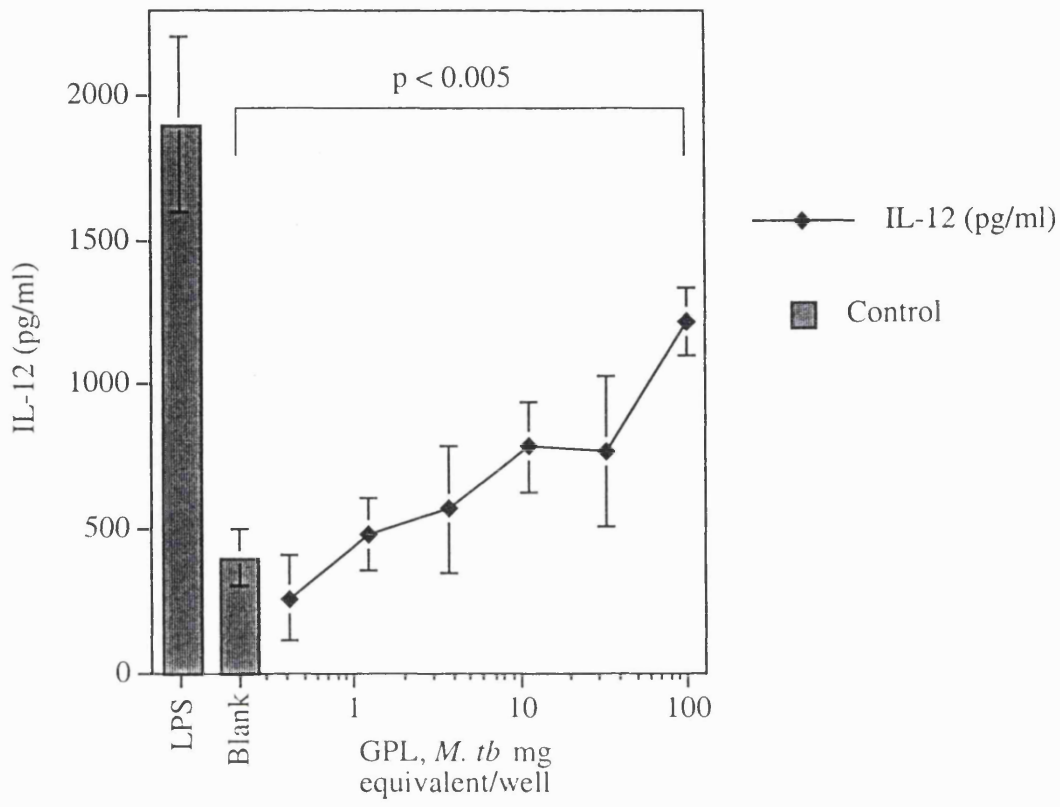


Figure 2.19. GPL from *M. tuberculosis* H37Ra induces IL-12 production from THP-1 cells. THP-1 cells (human monocyte/macrophages) differentiated by overnight culture with 1.2% DMSO were incubated with GPL from *M. tuberculosis* H37Ra at the concentrations indicated (expressed as mg equivalent of organisms per well). After 18 hours of culture with GPL supernatants were collected, centrifuged to remove residual cells and assayed for the presence of IL-12 by ELISA. Data plotted represent pg/ml IL-12 and are the mean of triplicate assays ± one standard deviation of the mean. GPL from *M, tuberculosis* H37Ra caused significant ($p < 0.005$ indicated, student's t-test), dose dependent IL-12 release from THP-1 cells. The positive control was 20ng/ml LPS and the negative control was culture medium only.

Although there is evidence that GPL from *M. vaccae* R877R and *M. tuberculosis* H37Rv were able to act as substrates for GPI specific phospholipases, TLC analysis of the organic phase of a GPI-PLC reaction did not reveal a decrease in native GPL, as would be expected. Figure 2.20 shows TLC analysis of GPI-PLC treated and untreated GPL from *M. tuberculosis* H37Rv and *M. vaccae* R877R with no discernable difference between the GPI-PLC treated and untreated samples. Clearly the GPI specific phospholipases were not working at maximal efficiency. This also provides an explanation for why the activities seen in enzyme treated and untreated samples were so similar on some occasions (for example figure 2.16).

The enzyme treatments described were performed in detergent solution, however GPI specific phospholipases do not exhibit maximal efficiency in detergent solution. Despite the fact that such enzymes are commonly used in detergent solution, they work most efficiently when the substrate is incorporated into unilamellar lipid bilayer vesicles, as described by Villar *et al* (Villar *et al.* 1998). The authors found that the efficiency with which the GPI specific phospholipases work varies with the lipidic composition of the vesicles. PI-PLC (used in the previous section, and having no glycan specificity) derived from *Bacillus thuringenesis*, however, does not require lipid vesicles to function effectively; detergent is sufficient (figure 2.11).

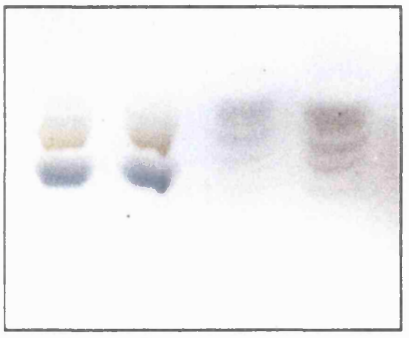


Figure 2.20. TLC of glycolipid from *M. tuberculosis* H37Rv and *M. vaccae* R877R after incubation in the presence and absence of GPI-PLC. After incubation of glycophospholipid with 0.025U of GPI-PLC or without enzyme for 90 minutes, the reaction was stopped by phase separation and the organic phase applied to an HPTLC plate. The plate was developed in solvent V, allowed to dry and visualised with anisaldehyde and heat. Samples are: lane 1, glycophospholipid from *M. tuberculosis* H37Rv without enzyme; lane 2, glycophospholipid from *M. tuberculosis* H37Rv with GPI-PLC; lane 3, glycophospholipid from *M. vaccae* R877R with GPI-PLC; lane 4, glycophospholipid from *M. vaccae* R877R without enzyme. In this solvent system, glycophospholipid from *M. vaccae* R877R separated into three bands, unlike the acidic solvent, solvent III. Glycophospholipid from *M. tuberculosis* H37Rv separated into two bands, and can be seen to be distinct from *M. vaccae* R877R glycophospholipid. This figure also shows the green colour after reaction with ansialdehyde, indicating the presence of a glycolipid. The image is shown blown up greater than actual size for clarity.

2.4.5 *Reaction of mycobacterial glycophospholipid fluorescamine*

The fluorescamine reaction was performed to establish the presence of free primary amino groups in GPL extracted from mycobacteria. The results of preliminary experiments on GPL from *M. vaccae* R877R and *M. tuberculosis* H37Rv are shown in table 2.3. Amino groups were derivatised and a fluorescent product was observed in five separate experiments (four in the case of *M. vaccae* R877R and one in *M. tuberculosis* H37Rv).

	Source of glycophospho-lipid	Equivalent mass of organisms assayed	Fluorescence Units	approximate nmol NH_2/g
Experiment 1	*M. vaccae*	1.4g	25.3	36.1
Experiment 2	*M. vaccae*	3g	31.1	20.7
Experiment 3	*M. vaccae*	1g	23.2	46.4
Experiment 4	*M. vaccae*	1.8g	22.7	25.2
Experiment 5	*M. tb* H37Rv	1.7g	36.2	43.4

Table 2.3. Fluorescence of mycobacterial GPL after reaction with fluorescamine. Varying mass equivalents of GPL from *M. vaccae* R877R and *M. tuberculosis* H37Rv were reacted with fluorescamine as described. Fluorescence was measured by emission at 480nm after excitation at 390nm. All the samples assayed gave a fluorescent product, but the amount of amino groups apparently measured gave a varying result with the amount of material assayed. Amino groups were quantified by reference to glucosamine standards.

The amount of amino groups detected per gram of starting cell mass, however, appeared to be variable. In particular it appeared that as the amount of GPL assayed was increased, the amount of amino groups measured per gram of organisms decreased This is shown schematically in figure 2.21. This has two possible explanations. Firstly this may mean that the extraction efficiency decreases with increasing starting cell mass. However some of the assays shown above were performed using varying amounts of the same extraction. Secondly these data suggest that the presence of GPL acted as an inhibitor of the fluorescamine reaction and diminished the sensitivity of the assay. This was tested directly by addition of equal quantities of GPL to the standards, whereupon flattening of slope of

the curve was observed in case of both *M. vaccae* R877R and *M. tuberculosis* H37Ra (figure 2.22).

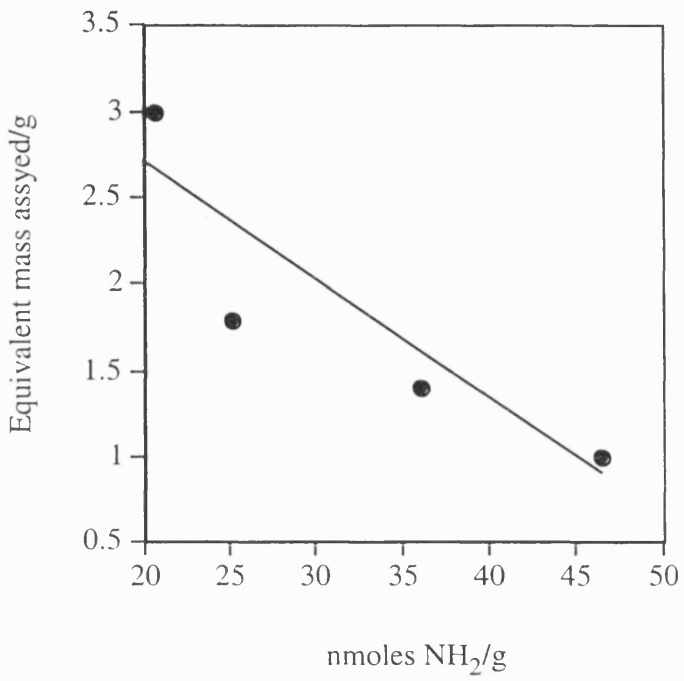


Figure 2.21. Variability of fluorescence following fluorescamine reaction with GPL purified from *M. vaccae* R877R on four separate occasions: influence of the amount of material assayed on nmol of amino groups detected per gram of starting material. The fluorescamine reaction was performed as described in materials and methods. After reaction of GPL with fluorescamine fluorescence was measured in a fluorimeter with an excitation wavelength of 390nm and a detection wavelength of 480nm. Calculation of nmols of amino groups per gram of cell mass at the start of the GPL extraction was performed by reference to glucosamine standards.

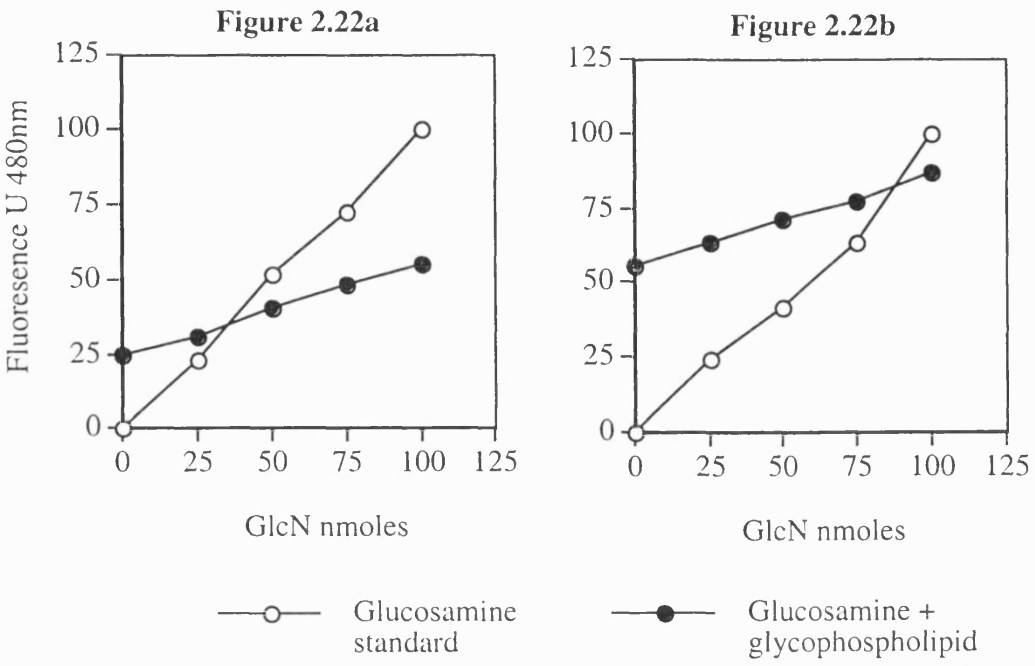


Figure 2.22. Method of standard additions on GPL from *M. vaccae* R877R and *M. tuberculosis* H37Ra. GPL from *M. vaccae* (figure 2.22a) and *M. tuberculosis* H37Ra (figure 2.22b) was added to each of the points on the standard curve in equal amounts (closed circles) and assayed in parallel to identical standards with no GPL (open circles). The fluorescamine reaction was performed as described in materials and methods. After derivatisation fluorescence was measured in a fluorimeter with an excitation wavelength of 390nm and a detection wavelength of 480nm. Calculation of the amino group content using the slopes thus generated gave values of 180nmol NH_2/g equivalent extraction starting mass for *M. vaccae* and 400nmol NH_2/g for *M. tuberculosis* H37Ra.

Using this method the amount of amino groups present in GPL per gram of starting mass for each organism was calculated to be 180nmol NH_2/g for *M. vaccae* R877R and 400nmol NH_2/g for *M. tuberculosis* H37Ra. Insufficient material from *M. tuberculosis* H37Rv was available for precise assay by the method of standard additions, nevertheless this material also gave a fluorescent product after reaction with fluorescamine (table 2.3). From the graphs, the presence of GPL caused an approximately three-fold decrease in sensitivity of this assay.

Detection of amino groups was also attempted using ninhydrin, but GPL from mycobacteria did not stain with ninhydrin on a TLC plate or when spotted onto chromatography paper (data not shown). This was not the expected result in light of the fluorescamine derivatisation experiments. Failure of material immobilised on a TLC plate to stain may be due to a steric problem, the amino group not being accessible on the surface of the TLC plate to react with ninhydrin. Peptide normally does stain with ninhydrin on TLC plates, so this result is perhaps best interpreted that there is no peptide present in the purified GPL. Failure to stain on filter paper strongly contradicts the results obtained using fluorescamine. A greater discussion of the significance of these data is given in section 2.5.

2.5 Discussion

In order to characterise unidentified material as being a GPI species, the following chemical properties should be demonstrated. The material should act as a substrate for PI or GPI specific phospholipases, it should have a free primary amino group, it should contain sugar residues and it should contain phosphate. These properties are, to some extent, overlapping, providing more than one line of evidence for the same chemical property. For example, sensitivity to PI-PLC and reaction with molybdate both independently indicate the presence of phosphate. The findings presented here show that a glycophospholipid with some properties compatible with those of a GPI structure may be isolated from the mycobacterial species *M. tuberculosis* H37Ra, H37Rv and *M. vaccae* R877R.

2.5.1. Properties compatible with those of a glycolipid

The material purified from *M. vaccae* R877R, *M. tuberculosis* H37Ra and H37Rv was clearly hydrophobic in nature, being isolated off a TLC plate and *M. vaccae* R877R incorporated radiolabelled glycerol and palmitate substrates. The purification process used also separates GPL from LAM, another mycobacterial phosphatidylinositol containing glycolipid. This is because LAM has a very large carbohydrate would be expected to partition into the aqueous phase of the aqueous/organic separation that occurs early in the isolation process. It should also be noted that the purification procedure in unlikely to be 100% efficient as some GPL does partition into the aqueous phase and must be lost at that stage, as shown by the experiment in figure 2.13. Anisaldehyde staining (figures 2.3 and 2.20) reveals green coloured staining indicating the presence of sugar residues in the preparation. The purified material from *M. vaccae* R877R did not co-run with phosphatidic acid, phosphatidylethanolamine, diacylglycerol or phosphatidylinositol, other phospholipids known to be present in mycobacteria (Jackson *et al.* 2000). Although the material from *M. tuberculosis* was not run next to some of these standards, it had exactly the same Rf as the material from *M. vaccae* in all the solvent systems used, making it very likely that it also did not co-run with these standards. On the basis of purification procedure, TLC analysis and glycerol and palmitate incorporation (*M. vaccae* R877R only), therefore, the purified material may be identified as a glycolipid.

Interestingly, figure 2.20 appears to show the GPL from *M. tuberculosis* H37Rv and *M. vaccae* R877R separating into three bands by HPTLC. This was not previously observed for the radiolabelled material or for the anisaldehyde stained preparations shown in figures 2.2 and 2.3. The chromatograms shown in figures 2.2 and 2.3 were run using solvents IV and III whereas that shown in figure 2.20 was run using solvent V. The use of a different solvent may explain this phenomenon, as two of the bands seen in figure 2.20 may have the same TLC mobility in an alternative solvent. However, this does not explain the discrepancy between the radiolabelled material (figures 2.7 and 2.11) and that shown in figure 2.20 as these were all developed using solvent V. It may be that one of the species seen in figure 2.20 does not incorporate glycerol, or that the different species incorporate glycerol at very different rates, thus meaning that one would label relative more than the others. This can be seen to occur in figure 2.11, where the sample not treated with PI-PLC shows another band that is only just visible. The autoradiograph shown in figure 2.7 was incubated with the TLC plate for a shorter time than that shown figure 2.11, so the film may not have been allowed sufficient exposure this second band for it to become visible. All studies relating to biological activity of GPL were performed using material not separated in these apparent sub-fractions, so any properties described are probably those of a few closely related species, perhaps varying only in the length of their lipid chains, for example.

2.5.2 Compositional analysis of the mycobacterial glycophospholipid

GPL from *M. vaccae* R877R has been the most extensively characterised, as this species is easier to work on than *M. tuberculosis*. Material is more abundantly available from *M. vaccae* R877R due to its more rapid growth rate and the safety precautions required are considerably less stringent. GPL from *M. vaccae* R877R was metabolically labelled by [^{3}H]-glycerol, [^{3}H]-palmitate, [^{3}H]-inositol, [^{3}H]-mannose and [^{3}H]-galactose. Notably GPL did not incorporate [^{3}H]-glucosamine. *M. vaccae* GPL was found to contain mannose and galactose, the same sugars as have been reported in the core structure of some other GPI molecules, principally those that are known to function as protein anchors (Homans *et al.* 1988, Ferguson *et al.* 1988). Interestingly all such GPI anchor molecules reported to date have been found to contain glucosamine. GPL, therefore, has properties that do not precisely fit with those of GPI molecules described to date. These compositional analyses also indicate that the purified material is distinct from PIM (another abundant

mycobacterial phosphatidylinositol-glycolipid), at least in the case of *M. vaccae* R877R because PIM does not contain galactose. The presence of galactose was not demonstrated in GPL from *M. tuberculosis*, but in every other respect the material from *M. tuberculosis* was very similar to that from *M. vaccae* R877R. This represents yet more indirect evidence that the material is not LAM, as PIMS is the biosynthetic precursor to LAM. Unfortunately constraints of time prevented more complete compositional analysis.

GPL from *M. vaccae* and both strains of *M. tuberculosis* was found to contain inorganic phosphate using a modified version of the protocol originally described by Bartlett (Bartlett 1958). Pilot experiments suggested that the amount of detectable phosphate was very low. However substitution of inorganic phosphate with phosphatidylinositol as a standard revealed a one hundred fold reduction in the sensitivity of the assay when the phosphate group was incorporated into a larger molecule as a phosphodiester. It may be that this assay is best suited to the determination of phosphomonoesters, perhaps because these are more easily released to take part in the reaction. The possibility exists, therefore, that GPL does not contain any phosphomonoesters on the glycan portion and that the only phosphate is present as the phosphodiester linkage between the lipid part and the glycan part of the molecule.

Reaction with fluorescamine and ninhydrin

The fluorescamine reaction was performed to determine the existence of free primary amino groups within GPL extracts. Upon calculation of the amount of amino groups present, however, it was found that the stoichiometric ratio of phosphate to amino groups was approximately one to 40 in every case. This ratio is not compatible with current understanding of the likely structure of a GPI molecule. Although it is possible that this result is real and a large glycan very rich in amino sugars is present, this is unlikely, perhaps in view of the fact that GPL from *M. vaccae* did not metabolically label with [^{3}H]-glucosamine. Moreover, material that is active in the PDH phosphatase assay is usually of low molecular weight (Prof. T.W. Rademacher, personnal communication and Dr. S Kunjara, unpublished observations). It is more likely that, despite the apparent robustness of this assay, this result is an artefact. This artefact may be due to the effect of divalent cations present bound to GPL (see chapter 1), for example zinc or manganese. The presence of divalent cations might cause opening of the fluorescamine ring to artificially

increase the measured fluorescence. Alternatively another contaminant within the preparation might cause opening of the fluorescamine ring and give artefactual fluorescence. The fluorescamine reagent has previously been applied to the study of IPG molecules that are also thought to contain divalent cations (Caro *et al.* 1997), but in this case fluorescence was detected using HPLC. This would certainly have separated any artefactual fluorescence from the carbohydrates of interest. In the present experiments purified GPL and reaction mix were not subject to any form of separation before fluorescence was measured. Therefore there was not the opportunity to separate fluorescence truly due to reacted amino groups from background arising for any other reason. However, the presence of divalent cations in GPL has not been determined so this explanation remains hypothetical.

Consistent with the above explanation is the observation that GPL did not stain with ninhydrin when immobilised upon a TLC plate or on paper, suggesting that measured fluorescence was an artefact. There was no evidence of the presence of protein (a potential source of amino groups) by coomassie protein assay, SDS polyacrylamide gel electrophoresis followed by transfer to nitrocellulose and staining with India ink and ninhydrin staining on paper or a TLC plate. The presence of phosphoethanolamine was not examined.

If the above hypotheses are correct, they predict that the glycan portion of GPL will not contain any phosphate or amino groups if derived by PLD cleavage, or will contain phosphate groups but no amino groups if derived by PLC cleavage. Although all mammalian GPI derived IPG structures described so far contain both phosphate and amino groups (Jones and Varela-Nieto 1998), this is not universally the case. IPG like structures have been described in plants that lack phosphate and amino groups within their inositol and hexose containing core structures (Smith and Fry 1999, Smith *et al.* 1999). As described in chapter 1, IPG may contain divalent cations within their structure. If this is indeed the case, and the cations are essential for biological activity, then one current hypothesis is that the determinant of activity may be the ability to bind these cations by whatever means, rather than the absolute presence of phosphate or amino groups.

The mycobacterial phospho-oligosaccharide molecules, described more fully in the next chapter, also gave very high fluorescence measurements after reaction with fluorescamine. So great was the fluorescence that it would be impossible to account for it even if the entire mass of material extracted was composed entirely of nitrogen and hydrogen in molar ratio one to two. These data clearly demonstrate that it is possible to generate a large amount of artefactual fluorescence (chapter 3). However, a principal difference between GPL and POS was that POS did stain with ninhydrin, whereas GPL did not.

2.5.3 Cleavage of mycobacterial glycophospholipid by phospholipases

GPL from *M. vaccae* radiolabelled with [^{3}H]-glycerol was able to act as a substrate for PI-PLC (figure 2.11). The evidence that GPL from either species acts as a substrate for the GPI specific phospholipases is not as firm. In one experiment, although treatment with GPI-PLC and GPI-PLD appeared to yield dose dependent aqueous soluble activity, the level of activity was low (figure 2.14). In a subsequent experiment the level of activity was greater but due to poor availability of material at the time the correct negative control was not included (figure 2.15). In the light of PI-PLC treatment, however, which clearly demonstrates the presence of PI in GPL from *M. vaccae* R877R, it seems reasonable to conclude that this material is indeed GPI phospholipase sensitive. The evidence that GPL from *M. tuberculosis* is PI-PLC sensitive is not as conclusive as that from *M. vaccae*. The solubility and activity of the native glycolipid also make interpretation of NO production after GPI-PLC treatment less clear. However, on at least one occasion NO induced by the GPI-PLC treated sample was obviously and significantly greater than that induced by the untreated sample ($p < 0.005$, figure 2.17). These data suggest that GPL from *M. tuberculosis* is also GPI-PLC sensitive.

Interpretation of the data derived from PI-PLC treated GPL in the PDH phosphatase activation assay is more complicated as there are several other factors to be taken into account in addition to whether or not GPL is a PI-PLC substrate. These considerations are discussed in more detail below, along with a discussion of the biological activity of GPL.

Sensitivity of all the GPL studied to (G)PI-PLC also provides evidence that GPL is a novel glycolipid of mycobacteria. The PI containing glycolipids of mycobacteria, PIM, LM and LAM, are all based on a PIM (phosphatidylinositol dimannoside) structure. Such a

structure would not be expected to be (G)PI-PLC sensitive. (G)PI-PLC generates *myo*-inositol-(1,2)-cyclic phosphate, and the mannose residues of PIM are attached to positions 2 and 6 of the inositol ring, making it is impossible for a PLC to form a cyclic phosphate of any kind. For the same reason, mammalian or trypanosome GPI molecules are not (G)PI-PLC sensitive if they are acylated at position 2 of the inositol ring (Deeg and Davitz 1995). Moreover, it has been reported in the literature that LM/LAM is resistant to PI-PLC from *Staphylococcus aureus* (Hunter and Brennan 1990, Chatterjee *et al.* 1992b). The glycan of GPL is probably attached at a position other than position 2 of the inositol ring.

2.5.4 Biological activity of the mycobacterial glycophospholipids

Two assay methods were used to show the appearance of aqueous soluble biological activity derived from phospholipase treated GPL. The J774 cell nitric oxide release assay does not show definitive IPG activity because murine macrophages will release NO in response to a wide variety of different bacterial carbohydrates. Nevertheless this was a useful assay for detecting the presence of a water soluble phospholipase reaction product. GPL, when added directly to J774 cells, was also highly active in this assay. Confirmation of the effect of GPL from *M. tuberculosis* H37Ra on macrophage like cells was obtained by showing that it caused release of IL-12 from the human monocyte/macrophage cell line, THP-1.

The aqueous phase of PI-PLC treatments were examined for activity more defining of an IPG structure, PDH phosphatase activation. The results obtained showed that only the presence of GPL in the reaction was required to induce PDH phosphatase activation (figure 2.12). This result seems puzzling if we are to assume that the phospholipase reaction product is the bioactive species. These data indicate, however, that it is not the phospholipase reaction product alone, but the substrate as well, that is active in this assay. For this to occur it is a pre-requisite that at least some GPL partitions into the aqueous portion of a phase separation. This was shown to be the case in figure 2.13.

Because the PDH assay was only performed as single data points, it was not possible to determine whether or not the difference the PI-PLC treated or untreated samples shown in figure 2.12 is significant. This result may suggest that there is no difference in activity between un-cleaved GPL and the cleaved glycan portion in this assay. Alternatively, these

data may suggest that treatment with PI-PLC reduced the activity of GPL in this assay. This is opposite to the accepted cell signalling dogma described in chapter one where the reaction product is active and the substrate is not, however this has been demonstrated to occur with synthetic compounds in our laboratory. Figure 2.23 shows the effect of the compound man-α4-GlcNH$_2$-α6-D-*myo*-inositol with and without phosphatidic acid covalently attached. The lipidic form, man-α4-GlcNH$_2$-α6-D-*myo*-inositol-(DAG)-1-mono-PO$_3$, was active whereas the non-lipidic form was not. Other work in our laboratory has shown that GPI derived from human placenta is also active both before and after cleavage with GPI-PLD (Sylvie Deborde, PhD thesis 2000). Under some conditions (perhaps due to failure to incorporate the appropriate divalent cations into the IPG structure) the reaction product was inactive (Sylvie Deborde, personal communication). It was postulated that the GPI is physically sequestered in the plasma membrane away from its site of action. Cleavage of the GPI structure by GPI-PLD represents activation of the molecule not by actually inducing bio-activity but by allowing the glycan portion to become biologically available.

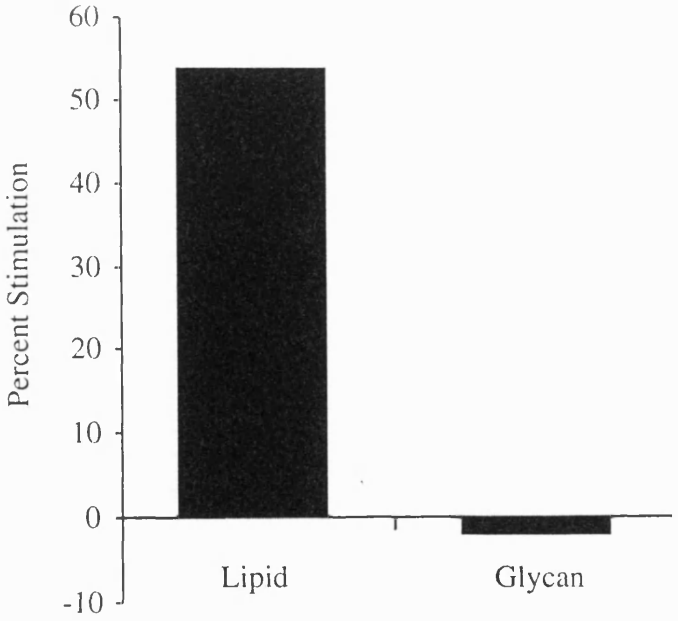


Figure 2.23. Activity of synthetic IPG and GPI species in the PDH phosphatase activation assay. The lipid man-α4-GlcNH$_2$-α6-D-*myo*-inositol-(DAG)-1-mono-PO$_3$ (labelled lipid) and the related glycan man-α4-GlcNH$_2$-α6-D-*myo*-inositol (labelled glycan) were assayed for PDH phosphatase activation as described previously. Data shown are percentage stimulation above baseline. In the case of these compounds the lipidic form was

active, whereas the non-lipidic form (in this case it would be the PLD, not the PLC reaction product) was not active. These data are courtesy of Dr. S Kunjara, Molecular Medicine Unit, UCL.

Whether the glycan reaction product of GPL phospholipase treatment was indeed inactive because the correct cations were not incorporated into the structure by PI-PLC from *B. thuringenesis* or because it does not have the correct structure remains unknown. The activity of mycobacterial GPL in the PDH phosphatase activation assay provides the most intriguing evidence yet, however, that GPL is related to the IPG-like material, phospho-oligosaccharide (POS) also purified from *M. vaccae* R877R, *M. tuberculosis* H37Ra and H37Rv. This is discussed in more detail in chapter 6.

2.5.5 Conclusion

In summary it has been shown by a number of different methods that *M. vaccae* R877R contains a glycophospholipid that has some, but not all, of the chemical properties expected of a GPI species. *M tuberculosis* H37Ra and H37Rv contain material highly similar in many of the key characteristics. If this material is indeed a GPI structure then it may represent a novel class of such structures if it does not contain a free primary amino group. As discussed above, mycobacterial GPL is not a PIM based structure so therefore is not related to LM/LAM, because of its sensitivity to GPI-PLC. GPL, therefore, most likely represents a novel class of mycobacterial glycolipid with interesting biological activity, such as the ability to stimulate inflammatory mediator production from macrophages and to activate PDH phosphatase.

Almost no information is available on the function of GPL purified from mycobacteria. Although no protein was detected by two different methods, and there is no report of protein linked GPI molecules being purified using the method that has been used here, the possibility remains that these molecules functioned as protein anchors in mycobacterial cells. The possibility that GPL has a physiological function was suggested by the observation that, when labelled using [^{3}H]-glycerol only, GPL appeared to turn over faster than the rest of the membrane in *M. vaccae* R877R. However, this result was not repeated using alternative radiolabelled substrates, so no conclusion may be drawn about its significance. A 24 hour incubation may give time for the radiolabelled glycerol to be used

as a carbon source, thus labelling parts of the lipid pellet other than glycerol containing lipids relatively more at this time point, although this was not observed with palmitate or inositol (figure 2.8). A more intriguing speculation is that GPL may act as Toll like receptor ligands, on the basis of their ability to induce NO and IL-12 production from macrophage like cell lines. This is discussed in more detail in chapter 6.

Chapter 3

Isolation and partial characterisation of mycobacterial phospho-oligosaccharide

3.1 Introduction

The presence of IPG or IPG like biological activity within the mycobacterial species *M. tuberculosis* and *M. vaccae* has already been observed in our laboratory. The purpose of the work described in this chapter was twofold: to confirm the observations already made and to attempt to further characterise the material responsible for this activity. For the purification of IPG like activity from mycobacteria the protocol originally devised by Nestler *et al.* was used (Nestler *et al.* 1991). This method is based upon anion exchange chromatography of the supernatant derived from disrupted mycobacterial cells. IPG-like material isolated from mycobacteria is referred to here as phospho-oligosaccharide (POS). The reason for this nomenclature in that there is evidence that sugar and phosphate are present within the preparations. The presence of inositol is yet to be formally demonstrated, although there has been some suggestion of its presence (see later). One POS fraction from *M. tuberculosis* H37Ra was found not to contain phosphate. Although technically incorrect, this fraction has also been named POS for reasons of consistency, as the various fractions are referred to collectively on some occasions.

3.2 Materials

Anion exchange resin AG 1-X8 29-50 mesh, hydroxide form, analytical grade 8% cross linked resins and cation exchange resin, AG50W X-12 was obtained from Bio-Rad Laboratories. Cell culture media, divalent cation free Hank's balanced salt solution (HBSS) and tissue culture additives were obtained from Gibco (Life Technologies, Paisley, UK); all tissue culture plastics were obtained from Nunc (Life Technologies, Paisley, UK). Recombinant human epidermal growth factor (rhEGF) was from Calbiochem. Recombinant murine interferon gamma (rmIFNγ) was from Pharmingen (San Diego, CA, USA). [^{3}H]-thymidine and [U-^{14}C]-glucose were obtained from Amersham Radiochemicals (Amersham, Buckinghamshire, UK). Volumetric solutions for acids, ammonia, organic solvents and salts for buffers were AnalaR grade and were obtained from BDH (Poole, Dorset, UK). β-mercaptoethanol, ethylenediaminetetraacetic acid (EDTA) and all other chemicals were from Sigma (Poole, Dorset, UK). Bond elute C8 columns were obtained from Phenomenex ltd. (Cheshire, UK). 3MM paper for paper

chromatography was obtained from Whatman (Maidstone, Kent, UK). Reagents for the PDH phosphatase assay are described in chapter 2. Antibodies for the detection of mycobacterial POS are described in detail in chapter 4.

3.3 Methods

3.3.1 Conversion of AG 1X-8 hydroxide to formate form

AG 1-X8 anion exchange resin was washed twice with deionised water. The resultant slurry was transferred into a liquid chromatography column and allowed to settle. The excess water was drained off and five bed volumes of 1M formic acid passed down the column. After conversion, the resin was washed with 4-5 bed volumes of deionised water until the eluent pH (measured using pH paper) was stable. The converted resins were stored as a slurry in water at 4°C.

3.3.2 Extraction and purification of phospho-oligosaccharide from mycobacteria

The isolation of mycobacterial POS was based on that described by Nestler *et al.* used for the isolation of mammalian IPG (Nestler *et al.* 1991). Mycobacteria were maintained as described in chapter 2. The confluent mycobacterial growth was harvested from Sauton's medium and transferred into a Duran bottle. The typical starting mass of an extraction was 10-20g wet weight of bacteria. The bacteria were boiled for 5 minutes in 50mM formic acid, 1mM β-mercaptoethanol and 1mM EDTA (3ml per gram of organisms) with about 50 glass beads. After boiling, the solution was allowed to cool on ice for 15 min. The suspension was sonicated for 30 minutes using a 100-Watt ultrasonicator, VibraCell™ model (Sonics and Materials, Inc., Danbury, Connecticut, USA) and the sonicate was centrifuged at 29 500g for 90 min at 4°C in a Beckman J2-21M/E Ultracentrifuge (Rotor JA-20, Beckman Instruments Inc, Palo Alto). The supernatant was mixed with activated charcoal (10 mg per ml of supernatant) for 10 minutes at 4°C. The charcoal was removed by centrifugation at 29 500g for 30 min at 4°C. The resulting solution was diluted with 10 volumes of deionised water and the pH adjusted to 6.0 with 10% ammonium hydroxide. 50 ml of AG 1-X8 (20-50 mesh) formate resin was added and gently shaken in a 5-litre conical flask overnight at room temperature. The resin was transferred into a liquid

chromatography column and eluted with 100 ml (2 bed volumes) of deionised water followed by 100ml of 1mM HCl, pH 3.0. The eluent was discarded; the column was eluted with 250ml (5 bed volumes) 10mM HCl, pH 2.0 (P-type fraction) and 250ml 50mM HCl, pH 1.3 (A-type fraction). Both fractions were adjusted to pH 4.0 with 10% ammonium hydroxide and dried *in vacuo*. The POS were redissolved in sterile deionised water, aliquoted into smaller volumes, lyophilised and stored at -20°C until use.

3.3.3 J774 Cells and nitrite assays (Griess reaction)

The J774 cell line was maintained and nitrite production, which is indicative of nitric oxide production (Marletta *et al.* 1988), was measured as previously described (chapter 2). POS was substituted for glycolipid in the method described in chapter 2, dissolved in DMEM supplemented with penicillin and streptomycin only. Final concentrations were typically IFNγ 5-6ng/ml, LPS 20ng/ml and POS as indicated in the text and figures.

3.3.4 3T3 EGFr cell proliferation assays

The 3T3 EGFr cell line is a murine fibroblast cell line stably transfected with cDNA for the human epidermal growth factor (EGF) receptor. This cell line has previously been shown in our collaborators' laboratory to be a sensitive and reliable biological assay for IPG and IPG-like activity. 3T3 EGFr cells were maintained in DMEM supplemented with 2mM L-glutamine, 50U/ml penicillin/50μg/ml streptomycin and 10% FBS at 37°C in a humidified atmosphere containing 5% CO_2. Cells were grown until confluent (every two to three days) and removed from the tissue culture flasks by washing once in divalent cation free Hank's balanced salt solution (HBSS) then incubating for five minutes in trypsin/EDTA solution. The cells were centrifuged and then split 1 in 4 to 6. For proliferation assays cells were plated at 10^4 cells per well in 96 well plates in complete culture medium and left overnight to adhere to the wells. The following day the wells were washed with FBS free medium to remove any FBS present then POS solutions were added to a final volume of 100μl. Recombinant human EGF was added as a positive control. POS and EGF concentrations were as indicated in the text. After 24 hours 0.1μCi 3H-thymidine (specific activity 115Ci/mmol) per well was added for 4-5 hours. Cells were trypsinised and harvested for liquid scintillation counting.

3.3.5 PDH phosphatase and lipogenesis assays

The pyruvate dehydrogenase phosphatase activation assay for P type IPG bioactivity was performed as described in chapter 2.

The effect of POS sub types on lipogenesis was tested in rat adipocytes isolated from the epididymal fat pads of young rats, following the proceedure described by Rodbell (Rodbell M 1964). Adipocytes from two young rats (weighing approximately 200g each) epididymal fat pads were suspended in 8ml of Krebs Ringer bicarbonate buffer. 250µl of the cell suspension were added to 1 ml Krebs Ringer bicarbonate buffer containing 1% w/v albumin and 5mM [U-^{14}C]-glucose (specific activity 3 mCi/mmol), 1µCi per sample, with POS samples (added in a 10µl volume from stock) and incubated at 37°C for two hours. The rate of incorporation of uniformly labelled glucose into fatty acids was used as a measure of lipogenesis. The reaction was stopped by addition of 0.1ml 2N H_2SO_4. 1ml of reaction mixture was withdrawn, 10ml of iospropanol/hexane/2N H_2SO_4 (10:6:6 v/v/v) was added and the mixture shaken well. A further 6ml hexane was added to form two phases. The upper, hexane phase was withdrawn taking care not to disturb the interface. The hexane phase was washed with an equal volume of 2N H_2SO_4, allowed to separate and an aliquot of the hexane was analysed by liquid scintillation counting. One unit of POS lipogenic activity was defined as the amount required to increase the basal rate of [U-^{14}C]-glucose incorporation by 50%.

3.3.6 Fluorescamine and ninhydrin reactions for free primary amino groups and inorganic phosphate determination

Reaction with fluorescamine and phosphate determinations were performed as described for the glycophospholipid extracted from mycobacteria (chapter 2) except that the fluorescamine reaction buffer did not include 0.05% nonidet P-40.

For ninhydrin reaction to detect free amino groups, mycobacterial POS fractions were spotted onto filter paper in water and allowed to dry in air. The ninhydrin reagent was made up 0.3g ninhydrin dissolved in 100ml butanol with 3% glacial acetic acid. The ninhydrin reagent was sprayed onto the filter paper which was then heated at 60°C for 15

minutes. Separation of POS from contaminating material prior to reaction with ninhydrin was achieved by descending paper chromatography using the method described by Takasaki *et al.* (Takasaki *et al.* 1982). POS samples were dissolved in water, spotted on to 3MM chromatography paper and developed for 4 hours using a butanol/ethanol/water solvent (4:1:1 v/v/v) (solvent VI). After chromatography the paper was allowed to dry and then treated as described above.

3.3.7 Passage of phospho-oligosaccharide over AG50W X-12 cation exchange resin

0.2ml AG50W X-12 resin in deionised water was placed in a disposable column and eluted with 1ml deionised water to remove water soluble material. POS samples were dissolved in 1ml deionised water and allowed to pass through the resin column. A further 1ml deionised water was passed over the column. All eluents were collected, pooled and lyophilised. Samples were stored at -20°C until assayed.

3.3.8 Detection of mycobacterial phospho-oligosaccharide by ELISA

Detection of mycobacterial POS by ELISA was performed using the method that was established as described in chapter 4. Briefly, POS were coated onto a polysorp ELISA plate by incubation overnight in 0.1M phosphate at pH 7. The plate was washed and blocked, after which the primary antibody, a rabbit serum raised against CFA and IPG-P from pre-eclamptic urine that also recognises POS from *M. tuberculosis*, was added. The secondary antibody was HRPO-goat anti rabbit IgG. 100µl 3,3',5,5'-tetramethylbenzidine (TMB) was used as a substrate and optical density was measured at 630nm.

3.3.9 Passage over C8 columns

C8 columns were washed prior to use with 60% propanol and equilibrated with 0.1M ammonium acetate in 5% propanol. POS samples were dissolved in 0.5ml 0.1M ammonium acetate 5% propanol and allowed to pass over the column. The column was eluted with a further 0.5ml 0.1M ammonium acetate 5% propanol and both ammonium acetate fractions were pooled to give the aqueous fraction. The column was eluted with 0.5ml 40% propanol and 0.5ml 60% propanol, which were collected and pooled to give the organic fraction. Both eluate fractions were dried *in vacuo* and stored at -20°C prior to use.

3.4 Results

3.4.1 Mycobacteria contain active phospho-oligosaccharide

POS were extracted using the method described and each batch was tested for several different bioactivities. Figure 3.1 shows the proliferogenic activity of POS from *M. vaccae* on EGF receptor transfected 3T3 fibroblasts. Both POS-A and P fractions were active in a dose dependent manner. POS from *M. tuberculosis* H37Rv were able to induce nitric oxide (NO) production in J774 murine monocyte/macrophages whereas POS from *M. vaccae* R877R exhibited only very weak NO inducing activity (data not shown, and see chapter 2, figure 2.14). In particular, POS fractions from *M. vaccae* R877R were consistently poor at inducing NO production from J774 cells, whereas the GPI-PLC treated GPL aqueous fraction varied considerably in the respect (chapter 2, figures 2.14 and 2.15).

Figure 3.2 shows a representative experiment demonstrating the NO inducing activity of POS from *M. tuberculosis* H37Rv, both fractions exhibit dose dependent activity. POS from *M. tuberculosis* H37Ra has previously also been shown to be active in this assay (Kiajoo Puan, PhD thesis, 1999, and figure 3.8). The pH 3 eluate from the anion exchange column was assayed and found to be inactive (data not shown). POS-A displays a bell shaped dose response with lower activity at the highest dose of POS. There are two likely explanations for this. Firstly, POS may be present in such a high amount at this dose that it may become directly toxic to the cells, as was seen with GPL and its phospholipase cleavage product(s) (chapter 2). Secondly, the POS preparations contain some salts consequent to pH adjustment during purification. Repeated lyophilisation removes these salts as they are mainly comprised of ammonium, formate and chloride so form volatile ammonia, formic acid and HCl in aqueous solution, but this process is not completely efficient. It is consistent with this explanation that the effect is seen in the case of POS-A and not POS-P, as POS-A was eluted off the anion exchange resin at a lower pH and therefore required more ammonia to adjust to pH 4, thus generating more salts. When put into cell culture these salts can render the culture medium hyperosmotic and therefore toxic to the cells.

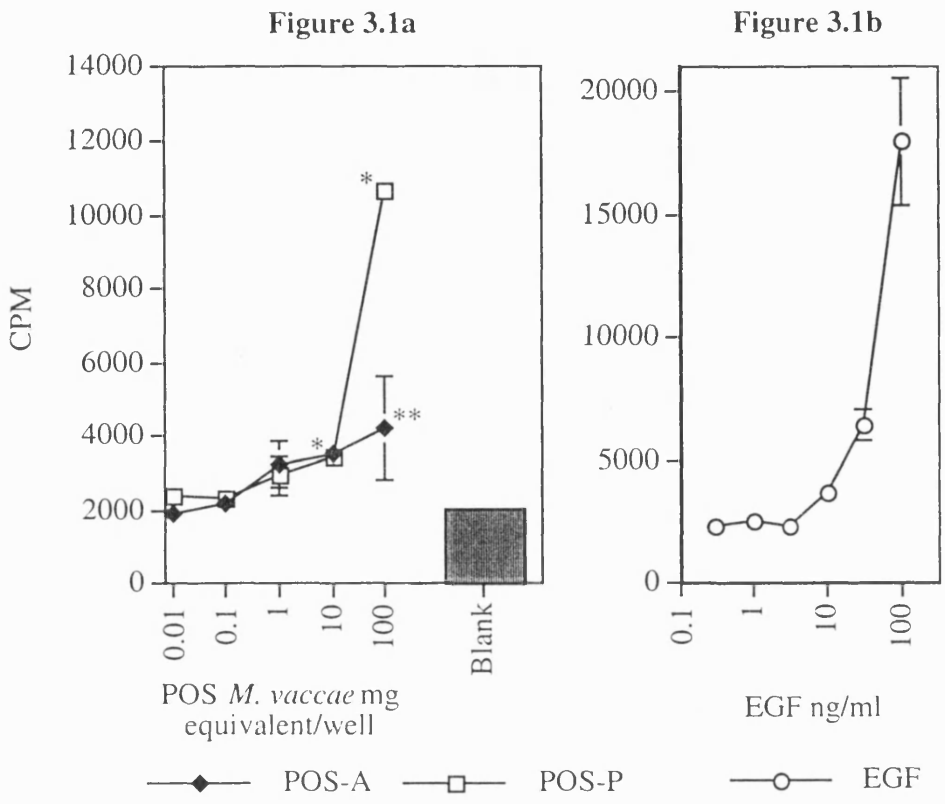


Figure 3.1. POS extracted from *M. vaccae* R877R have EGF receptor transfected (EGFr) 3T3 fibroblast proliferogenic biological activity. POS were extracted from *M. vaccae* R877R as described. EGFr 3T3 fibroblasts (10^4 cells per well) were cultured with POS-A and P at the concentrations indicated in serum free culture medium for 24 hours after which time 0.1μCi [^{3}H]-thymidine was added to each well. After incubation for a further five hours cells were trypsinised and harvested for liquid scintillation counting. Both POS-A (filled diamond) and POS-P (open squares) fractions were biologically active as shown by their ability to stimulate significant (* = $p < 0.005$, ** = $p < 0.05$, student's t-test) proliferation of EGFr 3T3 cells in a dose dependent manner (figure 3.1a). Proliferation induced by recombinant human EGF is shown as a positive control (figure 3.1b). Data plotted are counts per minute (CPM) and are the mean of triplicate assays +/- one standard deviation of the mean. POS fractions are expressed as the mass of organisms from which the amount of POS in each well is derived in mg.

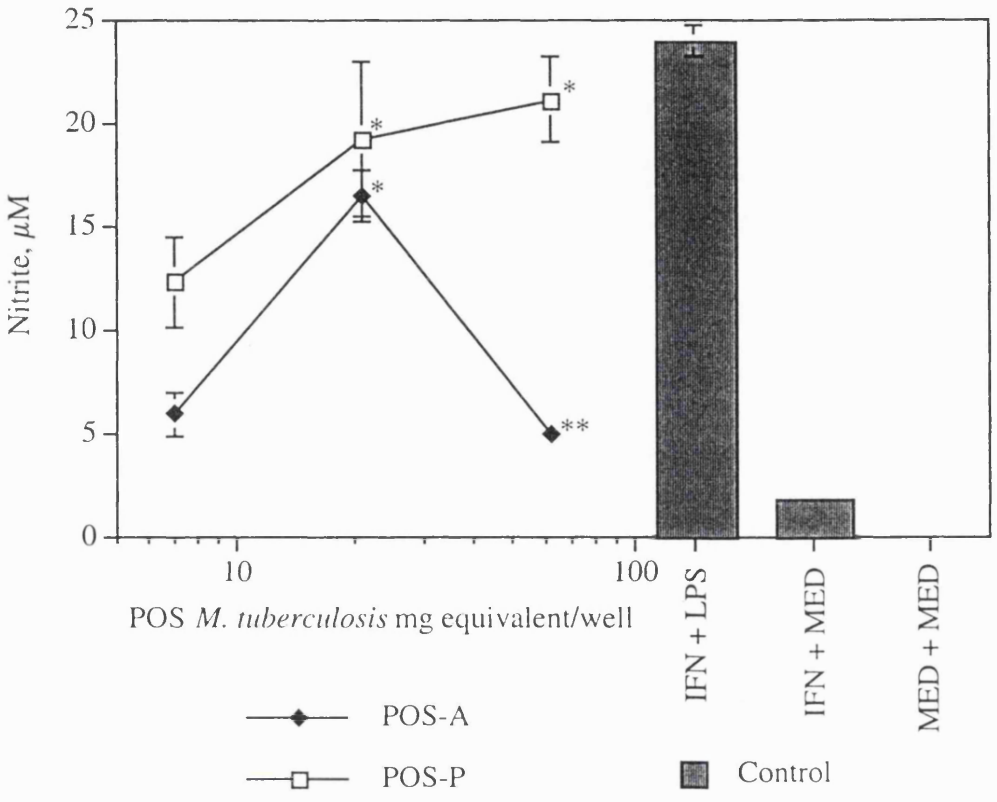


Figure 3.2. POS extracted from *M. tuberculosis* H37Rv induce nitric oxide (NO) production by J774 murine monocyte/macrophages. POS were extracted from *M. tuberculosis* H37Rv as described. J774 monocyte/macrophages (10^5 cells per well) were cultured with POS-A and P at the concentrations indicated in serum free medium in the presence of 6ng/ml IFNγ for 24 hours. The culture supernatants were withdrawn and assayed for the presence of nitrite by reaction with an equal volume of the griess reagent followed by optical density determination at 570nm with reference to sodium nitrite standards. Both POS-A and P were active as shown by their ability to induce significant NO production by J774 cells (* = $p < 0.005$, ** = $p < 0.025$, student's t-test). Data plotted represent nitrite concentrations and are the mean of triplicate assays +/- one standard deviation of the mean. POS fractions are expressed as the mass of organisms for which the amount of POS in each well is derived in mg.

Biochemical assays used to measure the activity of mammalian IPG include induction of activity of the enzyme pyruvate dehyrogenase (PDH) phosphatase and an adipocyte lipogenesis assay. POS from *M. vaccae* R877R, *M. tuberculosis* H37Ra and H37Rv were found to be active in these assays. Figure 3.3 shows P type activity of mycobacterial POS. POS-P from *M. vaccae* R877R and *M. tuberculosis* H37Rv both contained readily detectable activity that stimulated PDH phosphatase whereas *M. tuberculosis* H37Ra contained very little. POS-A from *M. vaccae* R877R weakly stimulated PDH phosphatase activity and, interestingly, POS-A from *M. tuberculosis* H37Rv and H37Ra actually inhibited PDH phosphatase activity. IPG from rat liver are included in figure 3.3 for comparison.

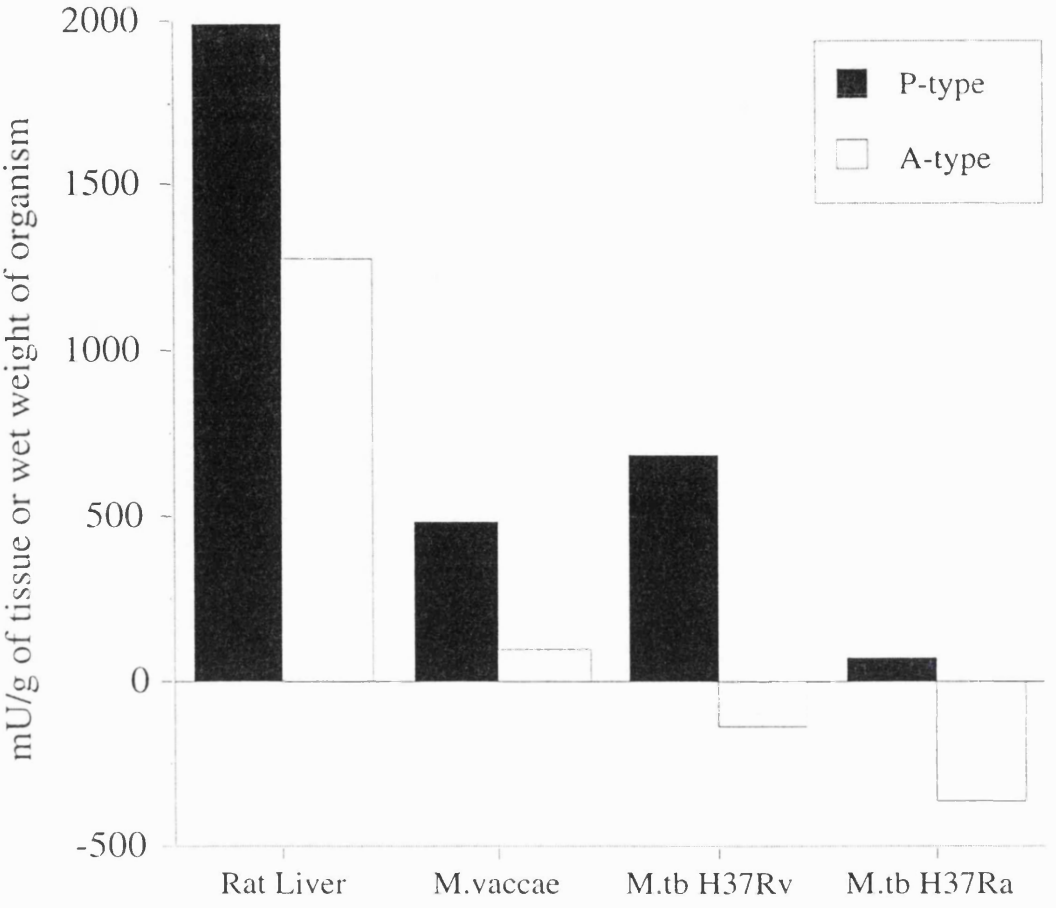


Figure 3.3. PDH phosphatase activation by different fractions of POS from mycobacteria. POS extracted from *M. tuberculosis* H 37Ra, H37Rv and *M. vaccae* R877R were incubated with PDH phosphatase and pyruvate dehydrogenase complex (PDC) for two minutes. The activation of PDH phosphatase was determined by measuring the rate of production of NADH catalysed by PDC. The values plotted are expressed as milliunits (mU) of activity per gram of wet weight of tissue or organisms. One unit of PDH phosphatase activity is defined as the activity required to increase the basal level of NADH production by 50%. Rat liver IPG-A and P are included as a positive control. This assay was performed by Dr. S Kunjara.

M. vaccae R877R and *M. tuberculosis* H37Ra POS-A both contained large amounts of lipogenic activity, comparable with that exhibited by IPG-A from rat liver. POS-A from *M. tuberculosis* H37Rv, however, inhibited lipogenesis. *M. tuberculosis* H37Ra POS-P also stimulated lipogenesis, *M. vaccae* R877R POS-P did so only very weakly and *M. tuberculosis* H37Rv POS-P had no lipogenic activity. These data are shown in figure 3.4.

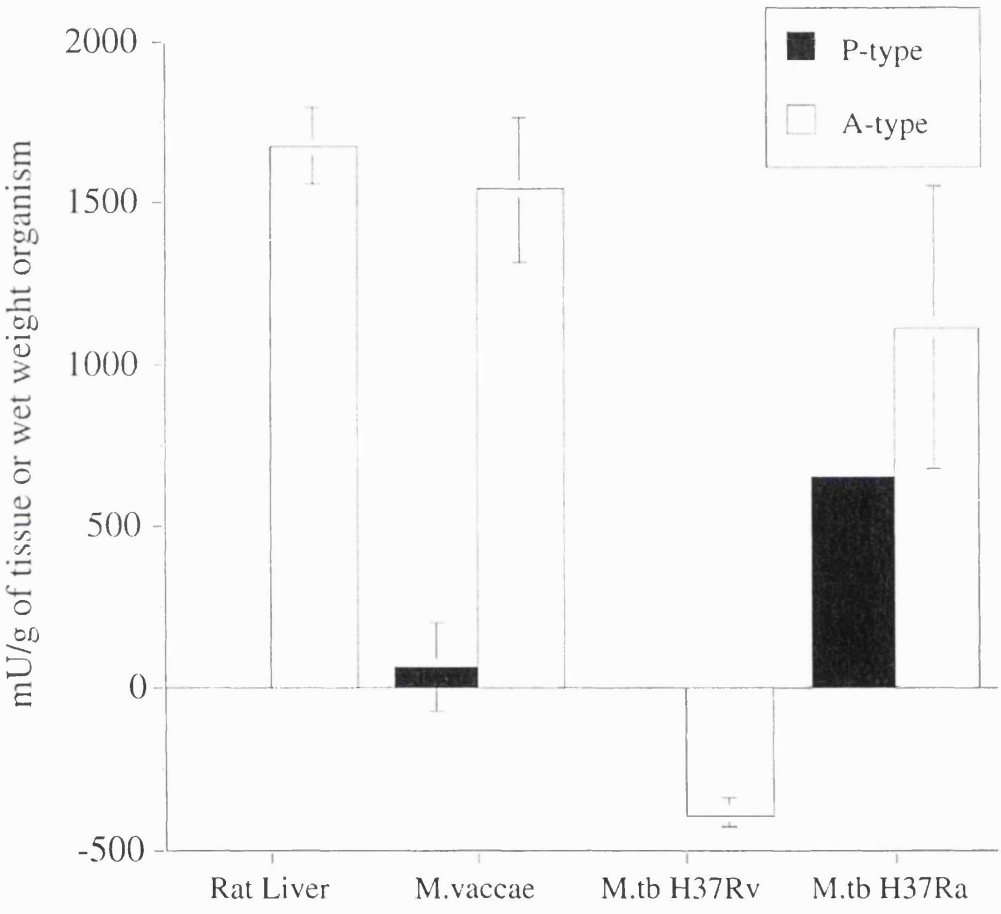


Figure 3.4. Lipogenic activity of different fractions of POS from mycobacteria in adipocytes. POS extracted from *M. tuberculosis* H 37Ra, H37Rv and *M. vaccae* R877R were incubated with adipocytes isolated from rat epididymal fat pads. Lipogenic activity was measured by the amount of incorporation of 5mM [U-^{14}C] glucose into fatty acids after two hours. The values plotted are expressed as milliunits (mU) per gram of wet weight of tissue or organisms and represent the mean of triplicate assays ± the standard error of the mean. One unit of lipogenic activity is defined as the activity required to raise the basal level of [U-^{14}C] glucose incorporation by 50%. Rat liver IPG-A was used as a positive control and rat liver IPG-P functioned as a negative control as this fraction is known to inactive in this assay. This assay was performed by Dr. S Kunjara.

3.4.2 *Phospho-oligosaccharide from mycobacteria contains phosphate*

POS from *M. vaccae* and *M. tuberculosis* were analysed for the presence of phosphate using a method based on that originally described by Bartlett (Bartlett 1958) involving 70% perchloric acid treatment and reaction with molybdate. Reaction of phosphorous with molybdate gives phosphomolybdate, a coloured reaction product with a peak absorbance at 830nm. POS from *M. tuberculosis* were subject to phosphate analysis by reference to potassium phosphate standards and all found to contain detectable phosphate except POS-P from *M. tuberculosis* H37Ra. The absence of detectable phosphate in this fraction showed that free phosphate did not come of the column, as phosphate should be in all fractions if it was a contaminant. Therefore, the presence of free phosphate was not examined directly, only the perchloric acid extractable phosphate was determined. Phosphate levels are presented in table 3.1 as nmols detected per gram of bacteria in the extraction.

Organism	**POS fraction**	**nmol phosphate/g**
M. tuberculosis H37Ra	POS-A	4nmol/g
	POS-P	not detected
M. tuberculosis H37Rv	POS-A	3.8nmol/g
	POS-P	1.1nmol/g
M. vaccae R877R	POS-A	4.3nmol/g
	POS-P	1.2nmol/g

Table 3.1. Phosphate content of mycobacterial POS. Mycobacterial POS were treated with 70% perchloric acid and reacted with molybdate as described to determine phosphate content. Phosphomolybdate was measured by absorbance at 830nm and phosphate determination performed by comparison with potassium phosphate standards.

If one of the mycobacterial POS fractions described here does not contain phosphate, there must be another explanation for why this fraction and possibly others can be purified using anion exchange. Mammalian IPG are believed to bind anion exchange resin through the metal ions bound in turn to EDTA present in the extraction buffer (Dr S. Kunjara, unpublished observations and Prof. T.W. Rademacher, personal communication). The fact that a mycobacterial POS fraction that did not contain phosphate was purified by this method provides some indirect evidence that it too may contain metal ions. Alternatively

these structures may contain other chemical groups that give them a negative charge, such as sulphate or carboxyl groups.

3.4.3 Detection of free amino groups in mycobacterial phospho-oligosaccharide

In the mammal IPG is derived from glycosylphosphatidylinositol and therefore contains a free primary amino group. Our hypothesis predicts that both GPL and POS should have a free amino group. However, as we have already shown there is some doubt over whether or not GPL does contain an amino group. Reaction of mycobacterial POS with fluorescamine always yielded a fluorescent product, apparently indicating the presence of a free primary amino group (table 3.1). However the amount of fluorescent product detected was so great that if all the material extracted consisted of amino sugars the predicted mass in this total extraction based on the fluorescence measured would be approximately 10mg in the case of H37Rv POS-P (where the greatest level of fluorescence was detected). This value is in fact approximately equal to the total mass of POS actually extracted from this sub fraction, which was measured at 10mg, including the residual salts from the extraction. It is not possible that the material purified consists solely of amino groups. The fluorescamine reagent has not been reported to react with ammonia or with ammonium ions. A mock extraction mixture of 50mM HCl, however, to which 10% ammonia was added until pH 4 (as in the extraction) or pH 9 (the pH at which the fluorescamine reaction is normally performed) gave a fluorescent product, even after lyophilisation.

Re-lyophilisation of the POS samples did not reduce the amount of fluorescence after derivatisation (data not shown). Moreover the amount of amino groups detected varied considerably between different reactions upon the same material. The level of fluorescence was generally so great that dilution of the reaction mixture after the reaction had taken place was necessary to bring the fluorescence into the range of the fluorimeter. This dilution process may have been non-linear and would account for the large variation seen in the amount of fluorescence detected. Greatly reducing the amount of material assayed also did not appear to yield any better result. Addition of standards to equal aliquots of test material was performed and it was established that the presence of the unknown, at least in one case, did profoundly affect the sensitivity of the assay. Calculation of the molar

amount of amino groups present, however, again gave an "impossible" result. The use of fluorescamine as a reagent to detect amino groups in this material was therefore abandoned.

Sample assayed	Fluorescence Units	Approximate nmol amino groups/g
M. vaccae R877R POS-A	23.4	748.8
M. vaccae R877R POS-P	26.9	860.8
M. tb H37Rv POS-A	47.2	1510.4
M. tb H37Rv POS-P	76.0	2432
M. tb H37Ra POS-A	70.1	2243.2
M. tb H37Ra POS-P	78.6	2515.2
	Fluorescence Units	nmol amino groups/ml
HCl pH 1.3 + NH_3 to pH 4, lyophilised	36.4	72.8
HCl pH 1.3 + NH_3 to pH 9, lyophilised	35.3	70.6
HCl pH 1.3 + NH_3 to pH 9, liquid	35.5	71

Table 3.2. Fluorescence of mycobacterial POS after reaction with fluorescamine. 625mg equivalent organism mass of POS from *M. vaccae* R877R, *M. tuberculosis* H37Rv and H37Ra were reacted with fluorescamine as described. Fluorescence was measured by emission at 480nm after excitation at 390nm. POS samples from *M. tuberculosis* all required a tenfold dilution to be brought into the range of the fluorimeter. Controls shown are glycolipid from *M. vaccae*, HCl and ammonia mixed to give pH 4 and pH 9, lyophilised, then assayed exactly as the POS samples, and HCl and ammonia mixed to give pH 9 then assayed directly as a liquid.

Previous work on mammalian IPG in our collaborators' laboratory had shown that amino sugars dissolved in water bind to strong cation exchange resins, probably by way of their amino groups, and that IPG bio-activity was unrecoverable from AG50W X-12 cation exchange resin. POS from *M. tuberculosis* H37Ra were passed over AG50W X-12 resin in water with a view to show that they were not retained on the column, and therefore did not contain a free amino group. POS from *M. tuberculosis* H37Ra were passed over AG50W X-12 resin, the column eluents were dried *in vacuo* and coated onto a polysorp ELISA plate for detection by ELISA. POS-P immunoreactivity (measured using a polyclonal rabbit serum raised against complete Freund's adjuvant and IPG-P from pre-eclamptic human urine described in chapter 4) was completely retained on the AG50W X-12 resin and approximately half of the POS-A immunoreactivity was retained on the

AG50W X-12 column (figure 3.5). Unfortunately this result is not interpretable because there may be other reasons why the material does not pass through a cation exchange column in water, for example the possibility that these structures also contain divalent cations. This would give two potential reasons for retention on AG50W X-12 resin, and would not distinguish between them.

To further examine for the presence of free amino groups POS was reacted with ninhydrin. POS fractions from *M. vaccae* R877R, *M. tuberculosis* H37 Ra and H37 Rv were spotted onto filter paper which was then sprayed with the ninhydrin reagent. After heating at 60°C for 15 minutes a blue colour developed in every POS fraction tested (figure 3.6), indicating that mycobacterial POS fractions do contain free primary amino groups. Because of the suspected presence of material that interfered with amino group assays (see above), however, POS were subject to paper chromatography to separate them from any potential contaminants. Carbohydrate has been reported to remain at the origin whilst salts, hydrophobic material and peptide run in a paper chromatogram developed using solvent VI (Takasaki *et al.* 1982). Monosaccharides have very low mobility in this solvent. Mycobacterial POS fractions were spotted onto 3MM paper strips and run for approximately 20cm. Development of the strips with ninhydrin showed that all ninhydrin reactivity remained at the origin (figure 3.7). (Only the origin and surrounding few centimetres, not the full length of the paper strips is shown in figure 3.7 for reasons of clarity.) Therefore mycobacterial POS fractions contain amino groups within a carbohydrate structure and do not contain any contaminating ninhydrin positive material, such as peptide.

The POS-A fraction from *M. tuberculosis* H37Rv separated into two ninhydrin positive spots, one remaining at the origin and one migrating approximately 3cm (Rf = 0.17) in solvent VI. This additional material may be a single amino sugar, but it migrated further than the glucosamine standard. This material has not been definitively identified.

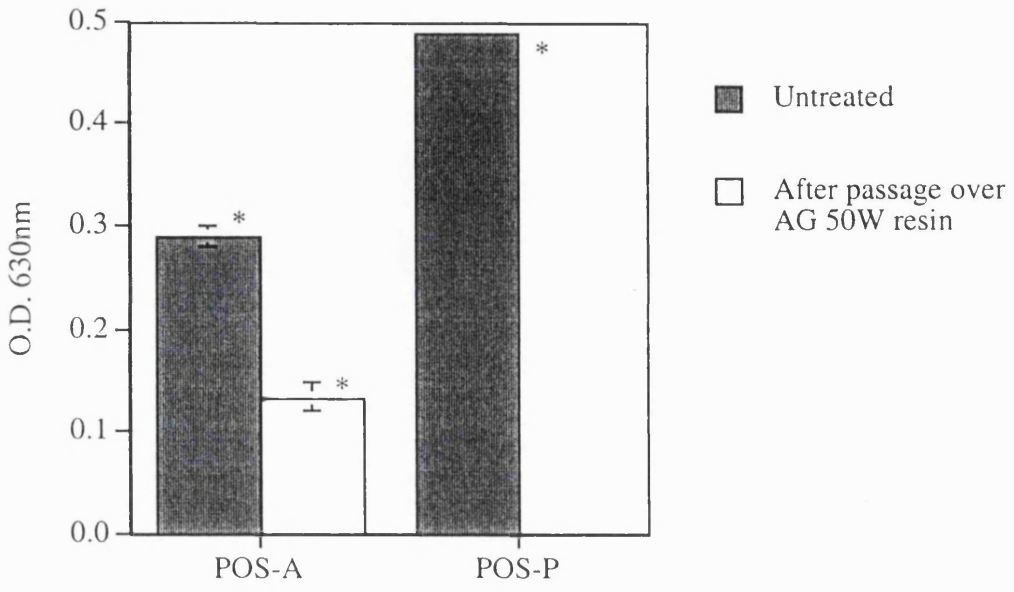


Figure 3.5. Immunoreactivity of POS from *M. tuberculosis* H37Ra after passage over AG50W X-12 strong cation exchange resin. POS-A and P from *M. tuberculosis* H37Ra were loaded onto an AG50W X-12 cation exchange column in 1ml of deionised water. The column was eluted with a further 1ml of water and all the eluents collected, pooled and lyophilised. POS equivalent to 62.5mg starting wet weight of organisms per well (assuming no loss on the cation exchange column) was coated onto polysorp ELISA plates in 0.1M phosphate at pH 7 overnight at 4°C. POS was detected using a polyclonal rabbit sera raised against CFA and IPG-P from pre-eclamptic urine that is reactive against mycobacterial POS. Passage over completely abolished all ELISA reactivity of POS-P and approximately half the ELISA reactivity of POS-A; both untreated samples gave significantly greater immunoreactivity than the samples passed over AG50W X-12 cation exchange resin. The immunoreactivity of POS-A after passage over AG50W X-12 was significantly greater than the negative control, that of POS-P was not (* = $p < 0.005$, Student's *t*-test). The values plotted represent optical density at 630nm and are the mean of quadruplicate assays ± one standard deviation of the mean.

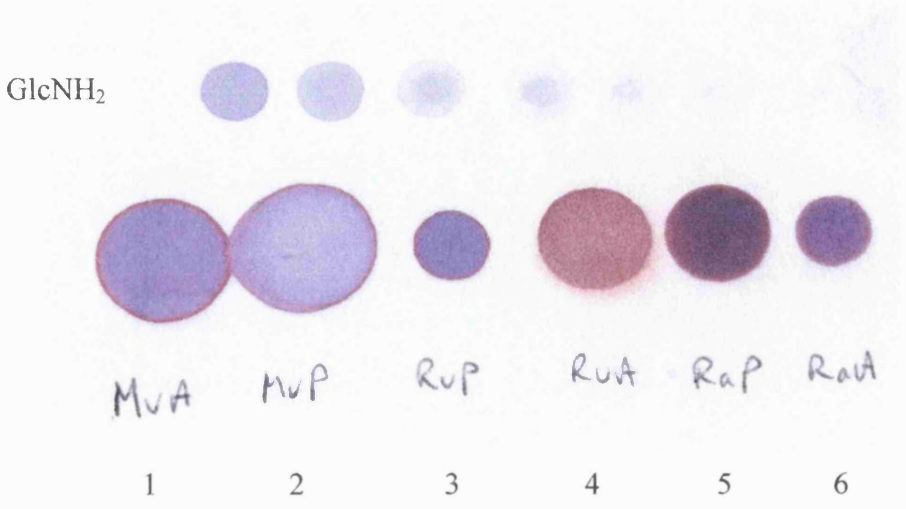


Figure 3.6. POS from mycobacteria stain with ninhydrin. POS isolated from *M. vaccae* R877R, *M. tuberculosis* H37Ra and H37Rv as described were spotted onto 3MM paper, allowed to dry and treated with ninhydrin in butanol with 3% glacial acetic acid, followed by heating at 60°C for 15 minutes. All POS fractions gave a reaction with ninhydrin, indicating the presence of free amino groups. 1 & 2 = *M. vaccae* R877R POS-P and POS-A, 3 & 4 = *M. tuberculosis* H37Ra POS-A and POS-P, 5 & 6 = *M. tuberculosis* H37Rv POS-A and POS-P. $GlcNH_2$ = glucosamine standard.

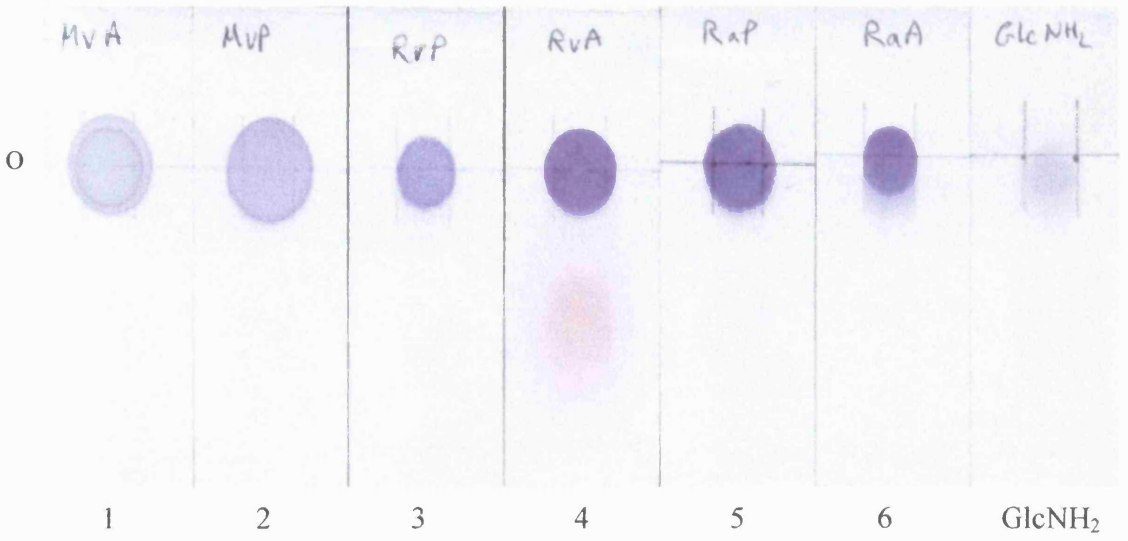


Figure 3.7. Ninhydrin staining of mycobacterial POS after butanol/ethanol/water paper chromatography. POS isolated from *M. vaccae* R877R, *M. tuberculosis* H37Ra and H37Rv as described were spotted onto 3MM paper strips and further purified by descending paper chromatography using solvent VI. After chromatography the paper was allowed to dry and treated with ninhydrin in butanol with 3% glacial acetic acid, followed by heating at 60°C for 15 minutes. All POS fractions gave a reaction with ninhydrin, indicating the presence of free amino groups. 1 & 2 = *M. vaccae* R877R POS-P and POS-A, 3 & 4 = *M. tuberculosis* H37Ra POS-A and POS-P, 5 & 6 = *M. tuberculosis* H37Rv POS-A and POS-P, $GlcNH_2$ = glucosamine standard, O = origin. The solvent front is not shown on this figure for reasons of space.

3.4.4 Phospho-oligosaccharide from M. tuberculosis *H37Ra are mainly not hydrophobic: passage over octyl sepharose.*

Some GPI structures in mammalian cells are acylated at position 2 of the inositol ring. Mycobacteria also contain structures that are acylated on the inositol ring, or on a mannose residue (chapter 1). GPI-PLD derived IPG from acylated GPI still bear the single lipid chain attached to position 2 giving them amphiphilic properties. Such structures are water soluble, but also would be expected to display lipidic characteristics such as retention on octyl sepharose. POS from *M. tuberculosis* H37Ra were loaded onto a C8 column in 0.1M ammonium acetate in 5% propanol. The column was eluted with the same solvent to give the aqueous phase and then eluted with 40% and 60% propanol, which were pooled to give the organic phase. Both phases were assayed for bioactivity by NO production from J774 cells (figure 3.8). In terms of fold increase over the respective column blank, 65% of the POS-A activity and 75% of the POS-P activity coming off the column was in the aqueous fraction. These data suggest that the majority of the NO inducing active material coming off the column is not amphiphillic and is therefore not acylated, but a significant proportion of the material may be acylated. These data also show that acylation is not essential for some biological activity in this assay system, unlike the findings of Barnes *et al.* who observed that deacylated LAM was inactive in a human PBMC TNFα release assay (Barnes *et al.* 1992).

Unfortunately, insufficient material was available to allow comparison with the total activity present in un-fractionated POS preparations or to perform mild alkali treatment. Mild alkali treatment could confirm the presence of acylation by showing that the activity present in the organic fraction moves into the aqueous fraction after deacylation.

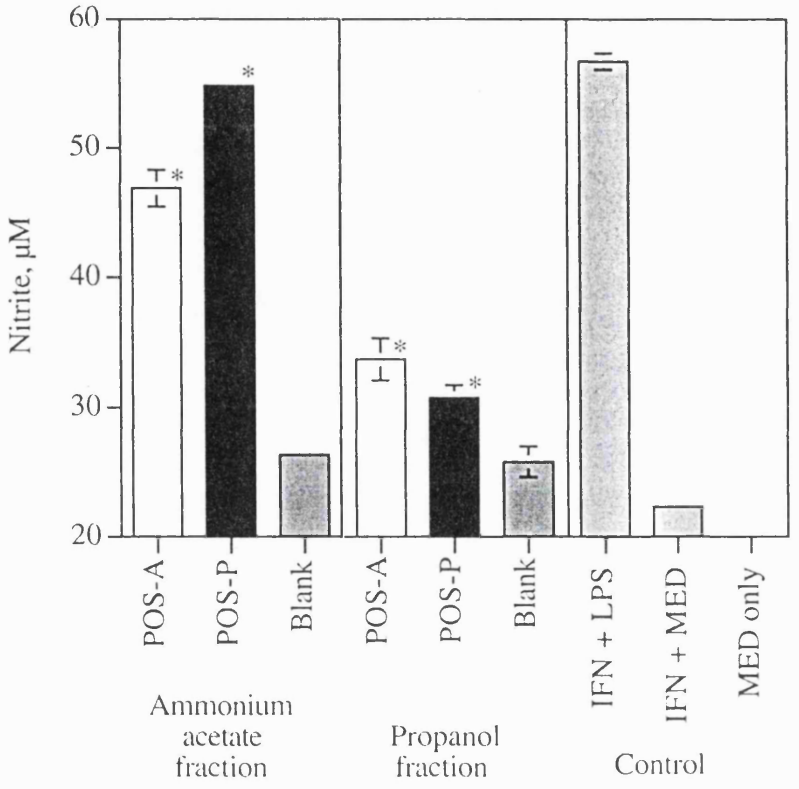


Figure 3.8. Nitric oxide (NO) inducing activity of POS from *M. tuberculosis* H37Ra in the aqueous and organic fractions eluted from a C8 (octyl sepharose) column. POS-A and P from *M. tuberculosis* H37Ra were loaded onto a C8 column in 0.5ml 0.1M ammonium acetate 5% propanol and the column was eluted with a further 0.5ml 0.1M ammonium acetate 5% propanol. The column was then eluted with 0.5ml 40% propanol followed by 0.5ml 60% propanol. The ammonium acetate fractions were pooled and lyophilised. The propanol fractions were pooled and dried *in vacuo*. The ammonium acetate fraction and the propanol fraction were tested for NO inducing activity. J774 cells (10^5 cells per well) activated with 6ng/ml rmIFNγ were cultured with POS equivalent to 40mg starting mass of organisms in serum free medium for 24 hours. NO production was measured by determining the nitrite concentration after reaction with an equal volume of the Griess reagent followed by optical density determination at 570nm with reference to sodium nitrite standards. 75% of the POS-P NO inducing activity and 65% of the POS-A NO inducing activity was found in the aqueous fraction. All experimental fractions were significantly greater than the mock experiment for the respective column fraction (* = $p < 0.005$, Student's *t*-test). Data plotted represent nitrite concentrations and are the mean of triplicate assays ± one standard deviation of the mean.

3.5 Discussion

In mammalian tissues second messengers derived from free membrane GPI (functionally and chemically distinct A and P-type subfamilies) are released outside the cell following growth factor or hormone stimulation, possibly in a tissue-specific manner. The second messengers are then taken up by the cell where they act by inhibiting or stimulating a variety of intracellular enzymes.

3.5.1 Biological activity

In this study the presence of bioactive POS that may be isolated from mycobacteria has been observed, confirming the observations originally made by Rook and co-workers in our laboratory and that of our collaborators. Since this material co-purifies with mammalian IPG, eluting from AG1 X-8 anion exchange resin at exactly the same molarity of HCl, it is possible that the material extracted from mycobacteria is structurally similar to IPG from mammalian tissues. However, co-elution in a chromatographic system constitutes only indirect evidence that two or more materials are the same or closely related.

The principal bioactivities examined; EGFr 3T3 cell proliferation, J774 cell NO production, PDH phosphatase activation and lipogenesis may all be properties of different molecular species within the same column fraction. The PDH phosphatase assay, for example, is divalent cation sensitive, so contaminating divalent cations concentrated during the purification procedure giving what appears to be PDH phosphatase activation in the final product. However, this is unlikely for a number of reasons. Some of the mycobacterial POS fractions were actually inhibitory in the PDH phosphatase activation assay, despite representing the same column fraction as stimulatory material from a different species, whereas contaminating cations would be expected to always give stimulatory activity. POS were purified by anion exchange chromatography, so any cations present should not have been co-purified. Cations may have been co-purified by binding to EDTA, but then they should be effectively chelated, whereas IPG-like biological activity is not (Rademacher and Caro 1997). It remains to be determined if mycobacterial POS may contain cations within their structure, but this would not represent contamination.

Other potential artefacts explaining observations described in this chapter include the effects of hexose concentration on the behaviour of cells in culture. Simply adding more hexose to a culture medium may increase the metabolic rate of cells and result in an apparent increase in an experimental end point measured; for example, in this case, lipogenesis or proliferation. As above, the observation that column fractions purified in the same way from different species have differing, sometimes absent and sometimes completely opposite activities provides good evidence against such artefacts.

NO production by macrophages is triggered by a wide variety of microbial sugars and other structures. Many different bacterial structures that contain sugars such as mannose and glucosamine may induce NO production from murine macrophages under conditions very similar to those described here. Previous observations in our laboratory have established that NO production in response to mycobacterial POS (purified in exactly the same way as the material used here) is not inhibited in the presence of polymixin B (Dr Kiajoo Puan, PhD thesis, 1999), eliminating contamination with LPS, also a powerful activator of macrophages.

POS from all three mycobacterial species, including two strains of *M. tuberculosis*, were distinct in their biological activities (figures 3.3 and 3.4). This cannot be due to varying amounts of POS in each strain, as some equivalent had opposite effects between different organisms. Whether or not this has any bearing on pathogenicity would require *in vivo* experiments to be performed, ideally with some way of removing or neutralising POS, such as by specific antibodies. Experiments such as this cannot be performed at the present time because such reagents are, as yet, unavailable (chapter 4).

3.5.2 Chemical characterisation

The chemical characterisation presented here is preliminary as there were many methodological problems encountered and the work was restricted by the small amounts of material available, particularly in the case of *M. tuberculosis*. The work presented here focuses on *M. tuberculosis* as the pathogenic and therefore more interesting of the two species studied. Although this preliminary characterisation is incomplete, the data presented are compatible with the material purified from mycobacteria being an IPG related structure.

Reaction with fluorescamine was inconclusive, as was found to be the case for mycobacterial GPL (chapter 2). Ninhydrin staining clearly showed that all mycobacterial POS fractions contain free amino groups and that ninhydrin reactivity was not due to a contaminant, but was present within material that has the characteristics of carbohydrate in butanol/ethanol/water paper chromatography (figures 3.6 and 3.7). The fact that at least some *M. tuberculosis* H37Ra POS-A appeared not to be retained on AG50W X-12 resin suggests that not all of this fraction contains amino groups. All mammalian IPGs described to date have free amino groups (Jones and Varela-Nieto 1998) so the *M. tuberculosis* H37Ra POS-A fraction at least appears to display some difference from the mammalian material. Nevertheless, the presence of amino groups in all the mycobacterial POS fractions indicates a significant similarity with mammalian material.

The presence of phosphate was established in POS extracted from mycobacteria by treatment with 70% perchloric acid and reaction with molybdate. Phosphate was detected in all the preparations except the P fraction from *M. tuberculosis* H37Ra. It is possible that phosphate was present but in very small amounts such that it was undetectable in an amount of material in which phosphate was readily detectable in the case of all the other mycobacterial POS fractions.

3.5.3 Further characterisation

Subsequent to the experiments described here some HPLC profiles for POS from *M. tuberculosis* have been obtained in our collaborators' laboratory. These profiles were obtained using pulsed amperometric detection, a modality that detects carbohydrates separated on an HPLC column. These profiles show peaks between 3 and 6 glucose units in size, around the size that would be expected of an IPG structure, and also a peak at 1.7 glucose units that may be a contaminant (Dr. Alan Jaques, unpublished observations). These results therefore confrim that POS preparations from *M. tuberculosis* contain material that is carbohydrate in nature and is probably a small oligosaccharide, as suggested by the retention of ninhydrin reactivity at the origin of butanol/water/ethanol chromatography. This represents an advance from previous work in our laboratory in which hexose determination was attempted by chemical means but failed due to the small amounts of POS available (Dr. Kiajoo Puan, unpublished observations).

Some preliminary mass spectra by electrospray and matrix assisted laser desorption ionisation ion cyclotron resonance mass spectrometry (MS) have also been obtained in our collaborators' laboratory. They reveal ions that have a mass consistent with di-hexose phosphate and tri-hexose phosphate. The 1.7 glucose unit peak seen by HPLC is suspected to be contaminating material (Dr. A Jaques, personnal communication) so interpretation of these spectra is so far speculative. It is of note that none of the ions analysed so far has given a mass compatible with a hexosamine containing structure, contradicting the data from reaction with ninhydrin, and casting further doubt on the validity of these spectra. Interestingly, application of MS/MS to one of the ions followed by fragmentation gave a change in mass compatible with the loss of inositol, the first direct chemical evidence that inositol is present in the mycobacterial POS preparations. However these mass spectra remain only partially interpreted and drawing any firm conclusions is therefore premature. One of the major concerns with the application of mass spectrometry to biological samples is that the ions seen do not necessarily represent all the molecular species that were in the original sample. Much more work is required to confirm and extend these observations; experiments involving HPLC-MS could be particularly revealing.

3.5.4 Conclusion

In summary several lines of evidence suggest that the mycobacterial POS preparations are carbohydrate in nature, contain phosphate (except *M. tuberculosis* H37Ra POS-P), contain amino groups and exhibit biological activity. Taken together these observations point towards the existence of material with some similarity to IPG in the mycobacterial species *M. tuberculosis* H37Ra, H37Rv and *M. vaccae* R877R, although little is known about this material at present. One conclusion which may be drawn is POS from *M. tuberculosis* H37Ra and *M. tuberulosis* H37Rv are different, itself an interesting observation. The presence of POS or of IPG-like activity is not, however, a universal property of mycobacteria. One other species, *M. paratuberculosis*, was examined in our laboratory and found not to contain any detectable IPG activity (Dr. K.J. Puan and Dr. S. Kunjara, unpublished observations).

Chapter 4

Serological studies of mycobacterial phospho-oligosaccharide

4.1 Introduction

Previous work by our collaborators had resulted in the generation of two polyclonal rabbit sera that were reactive against some mammalian IPG fractions. The aim of this chapter was to examine for cross-reactivity between this antibody and mycobacterial POS, and to investigate the generation of antibodies, artificially or naturally against mycobacterial POS. The reasons for attempting to carry out these experiments were:

- To investigate the structural similarities or differences between POS derived from the different mycobacterial species described and mammalian sources.
- To investigate the immunogenicity of POS derived from mycobacteria in animal models and in humans.
- Possibly to generate reagents for the further investigation of the role of POS in the immune response to *M. tuberculosis*. This could involve neutralisation experiments of POS fractions or sub fractions of more crude mycobacterial preparations *in vitro* and eventually *in vivo*.

4.2 Materials

ELISAs were performed using 96 well flat-bottomed polysorp ELISA plates obtained from Nunc (Life Technologies, Paisley, UK). The blocking reagents used were Boehringer Mannheim blocking reagent (BMBR) and 5% skimmed milk powder. BMBR (1% gelatine hydrolysate in tris buffered saline) was obtained from Boeringher Mannheim, (Lewes, East Sussex, UK). 5% skimmed milk powder was obtained from marvel. Sterile, pyrogen free water was obtained from Baxter Healthcare Ltd. (Thetford, Norfolk, UK). Complete Freund's adjuvant and incomplete Freund's adjuvant were obtained from Difco. Salts for buffers (AnalaR grade) were obtained from BDH (Poole, Dorset, UK). All other chemicals were obtained from Sigma (Poole, Dorset, UK).

Antibodies

Primary antibodies were one of the following:

1) The IgG (protein A) fraction of a polyclonal rabbit serum raised against rat liver PI-PLC treated GPI in complete Freund's adjuvant (CFA) at a dilution of 1 in 200, corresponding to a protein concentration of 100μg/ml, or at the dilutions indicated in the text. Rabbit serum IgG (Sigma, Poole, Dorset, UK) was used as a negative control. This antibody was used in all experiments up to and including that represented in figure 4.5.

2) An IgM monoclonal anti-mammalian IPG antibody, 2D1, cloned from a mouse immunised with PI-PLC treated rat liver GPI at an antibody concentration of 2.5μg protein/ml. Mouse IgM was used as a negative control at the same protein concentration.

3) A polyclonal rabbit serum pooled from two sera raised against P type IPG extracted from a pre-eclamptic urine sample and CFA at a dilution of 1 in 10 000 of the original serum. In addition, pre-sacrifice bleeds from these animals were available, as were sera from two rabbits immunised with rat liver IPG-P and CFA. Pre-immunisation bleeds were used as negative controls. Experiments performed using this antibody are shown in figures 4.6 and 4.7.

4) Rabbit sera raised against mycobacterial POS in CFA or against CFA alone, as indicated in the text, at the dilutions indicated in the text. Pre-immunisation bleeds were used as negative controls.

5) Mouse sera raised against mycobacterial POS in CFA, in Ribi's adjuvant or against CFA alone. These experiments were unsuccessful and are not represented in any figures, however they are discussed briefly in section 4.4.6.

6) Sera from 16 tuberculosis patients from East London and sera from 7 healthy volunteers, 6 PPD positive and one PPD negative at a dilution of 1 in 100 of each original serum.

1), 2) and 3) were gifts from Dr. Phil Williams, Molecular Medicine Unit, UCL.

The following secondary antibodies were used in these experiments:

HRPO-goat anti-mouse Ig, HRPO-goat anti-rabbit IgG, HRPO-goat anti-rabbit Ig, HRPO-goat anti-human Ig, HRPO-goat anti-human IgM and HRPO-goat anti-human IgG were obtained from Harlan Seralab, Loughborough, Leicestershire, UK. HRPO-goat anti-human IgA was obtained from Dako, Glostrup, Denmark.

4.3 Methods

4.3.1 Mycobacterial phospho-oligosaccharide ELISA method

ELISA plates were coated with POS from *M. tuberculosis* or *M. vaccae* in 50μl volumes of 0.1M Na_2PO_4 and 0.1M KH_2PO_4 mixed to give pH 7 overnight at 4°C. Other coating times were used as indicated in the text. For some coating times of greater than 18 hours 0.1% sodium azide was included in the buffer to prevent false positives due to bacterial growth. Coating dilutions are indicated relative to the amount of POS extractable per unit mass of mycobacterial cells. A typical coating concentration, therefore, corresponds to the amount of material extractable from 2.5mg of bacterial cells dissolved in 50μl of coating buffer per well.

Plates were washed with 100μl per well of phosphate buffered saline (PBS, 137mM sodium chloride, 1.47mM anhydrous potassium dihydrogen phosphate, 8mM anhydrous disodium hydrogen orthophosphate, 2.7mM potassium chloride) three times and blocked for one hour at 37°C with 100μl per well of BMBR for rabbit and mouse ELISAs 5% marvel for human ELISAs. Plates were washed once with PBS/0.05% tween 20 prior to incubation with the primary antibody.

Incubation with the primary antibody was performed in appropriate blocking reagent (see text) at 37°C for one and a half hours, following which the plates were washed three times with PBS/0.05% tween 20. Secondary antibodies were HRPO-goat anti-mouse Ig and HRPO-goat anti-rabbit IgG or Ig at a dilution of 1 in 5000 in BMBR for mouse and rabbit ELISAs, respectively. Secondary antibodies for human ELISAs were HRPO-goat anti-human Ig, IgM, IgG or IgA at a dilution of 1 in 5000 in 5% marvel. The plates were incubated with the secondary antibody for one hour at 37°C after which they were washed five times with PBS/0.05% tween 20 and once with PBS. Colour was developed with TMB

complete liquid substrate system. Optical density was read before acidification at 630nm and after acidification at 450nm with a reference filter at 570nm.

4.3.2 Animal immunisations

Rabbit

Female rabbits weighing 2.2Kg were immunised with either *M. tuberculosis* H37Rv POS-A in CFA, *M. tuberculosis* H37Rv POS-P in CFA or CFA only. 0.625g equivalent starting mass of bacterial cells of each POS fraction were re-dissolved in 350μl of pyrogen free water and emulsified with 1050μl of CFA. A total of 1ml was administered to each rabbit in 5 subcutaneous sites. Prior to immunisation 5ml bleeds were taken to provide control sera. After 4 weeks 1ml bleeds were taken to assay for anti-POS immunoreactivity. The next day the rabbits were boosted with 75% of the first immunising dose in incomplete Freund's adjuvant (IFA), or IFA and water for the CFA only rabbit. After another 12 days and 25 days further 1ml bleeds were taken to assay for anti-POS immunoreactivity.

Mouse

Two Balb/c mice were immunised with *M. tuberculosis* H37Rv POS-A or P in Ribi's adjuvant, *M. tuberculosis* H37Rv POS-A or P in CFA or CFA only. 0.625g equivalent starting masses of bacterial cells of each POS fraction were re-dissolved in 200μl pyrogen free water and emulsified with 600μl CFA. Each animal received 200μl of the emulsion subcutaneously. Two weeks later the animals were bled to assess their immune response. Normal (non-immune) Balb/c mouse serum was used as a negative control.

4.4 Results

4.4.1 IgG from a polyclonal rabbit serum raised against PI-PLC treated rat liver GPI in complete Freund's adjuvant binds mycobacterial phospho-oligosaccharide: the effect of time and pH on binding of mycobacterial phospho-oligosaccharide to polysorp ELISA wells.

Effect of pH and coating dilution

POS from *M. tuberculosis* H37Ra was coated for three days at 4°C in a pH range of 5 to 9. Buffers used to attain a pH range of 5 to 9 were acetate (pH 5 and 6), phosphate (pH 7) tris (pH 8) and HCO_3^-/CO_3^{2-} ions (pH 9). All buffers were made at 0.1M and adjusted to the appropriate pH with the corresponding anion except tris which was adjusted with 0.1M HCl. POS were coated in single wells only at the dilutions indicated in figure 4.1. Figure 4.1 shows that this antibody, raised against PI-PLC treated rat liver GPI in CFA, could recognise mycobacterial POS coated onto an ELISA well. Many of these data did not give very convincing curves, but they did suggest that a dilution of 2.5mg organism mass equivalent per well would be sufficient for future ELISAs, allowing the conservation of valuable material. The data from the points coated at pH 6 both before and after acidification are shown in figure 4.1 as a representation. A 3 day coating step was chosen by analogy to the protocol developed for use in detection of mammalian IPG by ELISA (Dr. Phil Williams, personal communication). Another group had given this antibody to our collaborators' laboratory and unfortunately the pre-immunisation bleed was not available. However, normal rabbit serum IgG at the same protein concentration displayed very little binding to POS from *M. tuberculosis* H37Ra. The degree of binding seen in figure 4.1 was also exceptionally high for this negative control, in general rabbit IgG did not bind mycobacterial POS at all (see, for example, figures 4.2, 4.3 and 4.4). Blank wells comprised coating buffer only. These wells were used as the reference blank for the optical density reading.

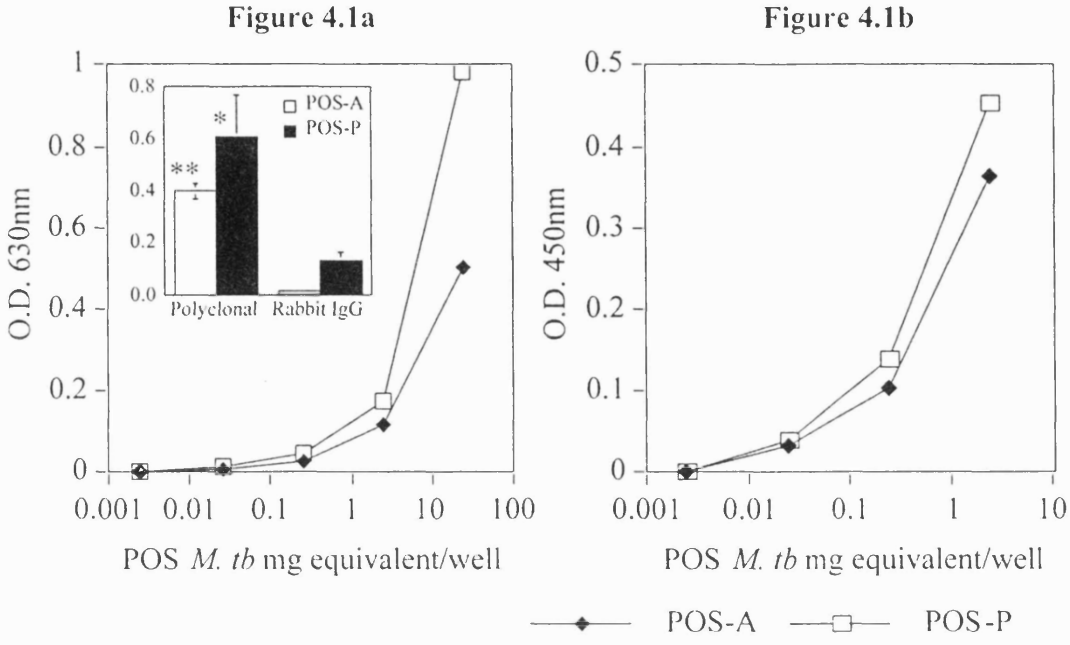


Figure 4.1. Immunoreactivity of different coating amounts of POS from *M. tuberculosis* to a rabbit polyclonal anti-POS antibody. POS-A and P from *M. tuberculosis* H37Ra were coated on ELISA plates at pH6 in 0.1M acetate for three days at 4°C. POS on the wells was recognised by a rabbit polyclonal serum IgG fraction immunised with PI-PLC treated GPI from rat liver in CFA at a protein concentration of 100μg/ml at POS coating dilutions of 2.5mg organism mass equivalent per well and above. Data shown are before acidification at 630nm (figure 4.1a) and after acidification at 450nm (figure 4.1b). Inset shows a separate experiment coating at 2.5mg organism mass equivalent per well and reading at 630nm, the polyclonal anti IPG serum bound to *M. tuberculosis* H37Ra POS significantly more than did non-immune rabbit IgG (* = $p < 0.01$, ** = $p < 0.005$, student's t-test). Representative data for pH 6 from a range of pH 5 to 9 are shown in this figure. Normal (non-immune) rabbit IgG served as a negative control (see inset).

Effect of pH using various different buffers

Data from the experiment represented in figure 4.1 showed a marked difference between the binding observed with some of the buffers compared with others that did not bear any apparent relationship with pH. In order to establish further the inter-relationship between pH, buffer composition and coating efficacy, POS from *M. tuberculosis* H37Ra were coated at 25mg organism mass equivalent per well in buffers of pH 5 to 9 using several different anions. The buffers used contained formate, acetate, tris and phosphate. A solution of 0.1M of each respective anion was mixed with 1M HCl or 1M NaOH (or with the appropriate anion of another valency in the case of phosphate, as above) to achieve the desired pH and used to coat the POS for three days at 4°C. The data for each pH are shown in figure 4.2. This assay was performed in triplicate, giving more convincing results. Optimal binding was observed in 0.1M phosphate at pH 7, statistical analysis was not performed because the conditions in each set of wells were different.

Effect of coating time: overnight compared with three days

The data shown in figure 4.3 again demonstrated that a coating concentration of 25mg organism mass equivalent per well gives an adequate optical density reading. In this experiment coating for three days and overnight was compared. True comparison of a three day coating step and an overnight coating step is not possible because these experiments were done on two different ELISA plates. There was a difference in colour development time between these two data series, the plate used in the overnight coating experiment was allowed to develop colour for longer than the plate used to coat for 3 days (25 minutes as opposed to 5 minutes). However the overnight coating gave adequate optical density values with this colour development time and the negative controls remained negative, so for reasons of convenience this method was used from then on. Inclusion of sodium azide at 0.1% in the coating buffer for the three day coating step in order to prevent to growth of bacterial cultures giving false results did not make any difference to the results obtained (data not shown).

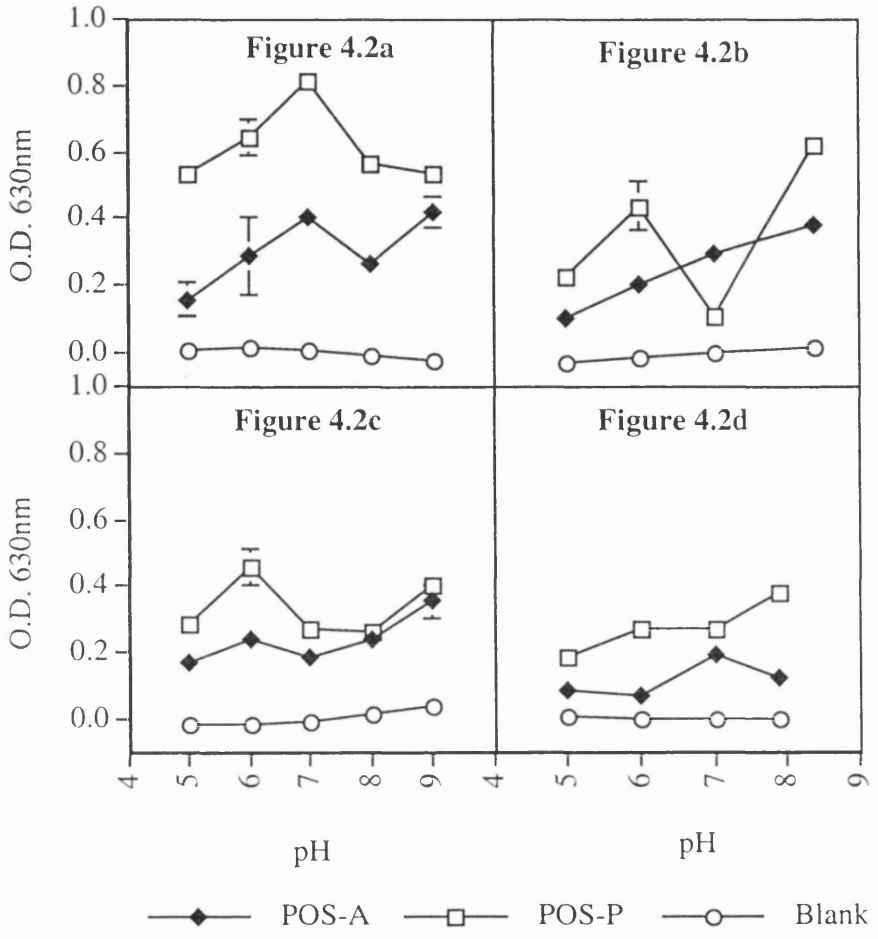


Figure 4.2. Effect of pH and of different buffers on coating of POS from *M. tuberculosis* H37Ra on ELISA wells. POS-A and P from *M. tuberculosis* H37Ra were coated on ELISA plates at 2.5mg organism mass equivalent per well for three days at 4°C. POS were coated in 0.1M phosphate at pH 5 to 9 (figure 4.2a), 0.1M formate at pH 5 to 8.4 (figure 4.2b), 0.1M tris HCl at pH 5 to 9 (figure 4.2c) and 0.1M acetate at pH 5 to 8 (figure 4.2d). The primary antibody was a rabbit polyclonal serum IgG fraction immunised with PI-PLC treated GPI from rat liver in CFA. Blank values represent the binding of normal rabbit serum IgG. Data are shown as optical density values at a wavelength of 630nm (without acidification). Each point represents the mean of triplicates and error bars (where visible) represent the standard deviation of the mean.

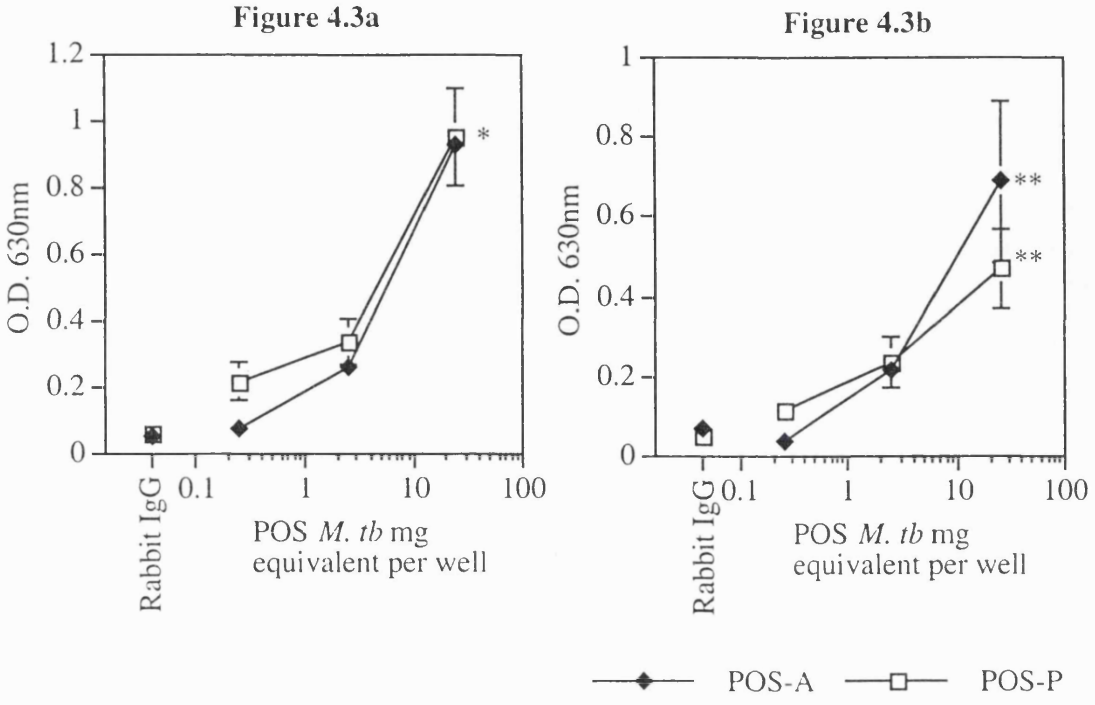


Figure 4.3. Comparison of three day and overnight coating steps for POS from *M. tuberculosis*. POS-A and P from *M. tuberculosis* H37Ra were coated on ELISA plates at a range of 25 to 0.25μg organism mass equivalent per well for 17 hours (figure 4.3a) or three days (figure 4.3b) at 4°C. The primary antibody was a rabbit polyclonal serum IgG fraction immunised with PI-PLC treated GPI from rat liver in CFA. This assay worked just as effectively using overnight coating as it did using coating for three days. Statistical comparison between each time-point was not performed because each experiment was done on a different ELISA plate, although the binding to POS was, as shown previously, significant (* = $p < 0.01$, ** = $p < 0.005$, student's t-test) compared with binding of normal rabbit IgG. Data are shown as optical density values at a wavelength of 630nm (without acidification), each point represents the mean of triplicates and error bars (where visible) represent the standard deviation of the mean.

4.4.2 *A monoclonal anti mammalian IPG antibody does not bind mycobacterial phospho-oligosaccharide*

A monoclonal IgM anti-mammalian IPG antibody, 2D1, was available in the laboratory. Figure 4.4 shows, however, that this antibody had no reactivity against mycobacterial POS. Normal mouse IgM served as a negative control. The murine antibodies were used at a protein concentration of 2.5μg/ml, a concentration at which 2D1 binds mammalian IPG fractions (Dr. Phil Williams, unpublished observations).

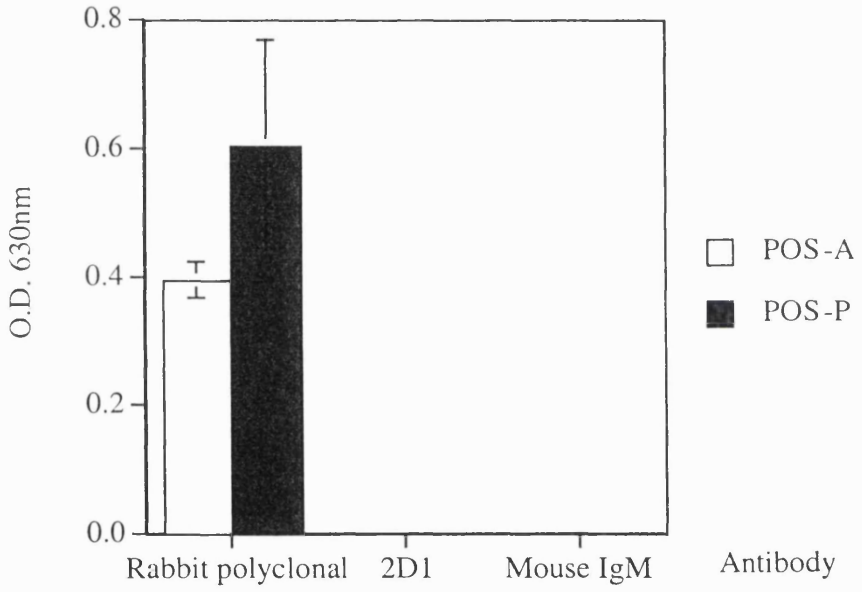


Figure 4.4. A murine monoclonal antibody, 2D1, does not recognise POS from *M. tuberculosis* H37Ra. POS-A and P from *M. tuberculosis* H37Ra were coated on ELISA plates at 2.5μg organism mass equivalent per well overnight at 4°C. Primary antibodies were a rabbit polyclonal serum IgG fraction immunised with PI-PLC treated GPI from rat liver in CFA (100μg/ml), a monoclonal antibody cloned from a mouse also immunised with PI-PLC treated rat liver GPI in CFA (2.5μg/ml) and normal mouse IgM (2.5μg/ml). Neither the monoclonal antibody 2D1 nor the normal mouse IgM isotype control were able to bind mycobacterial POS. Data are shown as optical density values at a wavelength of 630nm (without acidification). Each point represents the mean of triplicates and error bars represent the standard deviation of the mean.

4.4.3 IgG from rabbit polyclonal anti PI-PLC treated rat liver GPI also binds phospho-oligosaccharide from M. vaccae *R877R*

POS from *M. vaccae* were coated onto polysorp ELISA plates under the same conditions as POS from *M. tuberculosis*, in 0.1M phosphate at pH 7 overnight at 4°C. When coated alongside POS from *M. tuberculosis* H37Ra at 2.5mg organism mass equivalent in 50µl per well, the rabbit polyclonal serum IgG fraction did not appear to bind POS from *M. vaccae* (figure 4.5). However when coated at higher amounts, POS from *M. vaccae* become detectable by this polyclonal antibody (figure 4.5). By phosphate analysis the amount of POS-A fraction extractable from *M. tuberculosis* H37Ra and *M. vaccae* R877R is approximately the same (chapter 3). The same comparison cannot be performed for POS-P as POS-P from *M. tuberculosis* H37Ra does not contain phosphate. Possible explanations for this result are: 1) the polyclonal serum is only weakly cross-reactive for POS from *M. vaccae* R877R (either in terms of frequency of immunoglobulin molecules that recognise POS from *M. vaccae* or in their affinity), 2) there are more phosphate groups per molecule in the material from *M. vaccae* R877R or 3) the material from *M. vaccae* R877R does not bind the ELISA plates as well as the material from *M. tuberculosis* H37Ra. The monoclonal antibody, 2D1, did not bind POS from *M. vaccae* R877R at any of the coating dilutions used (data not shown).

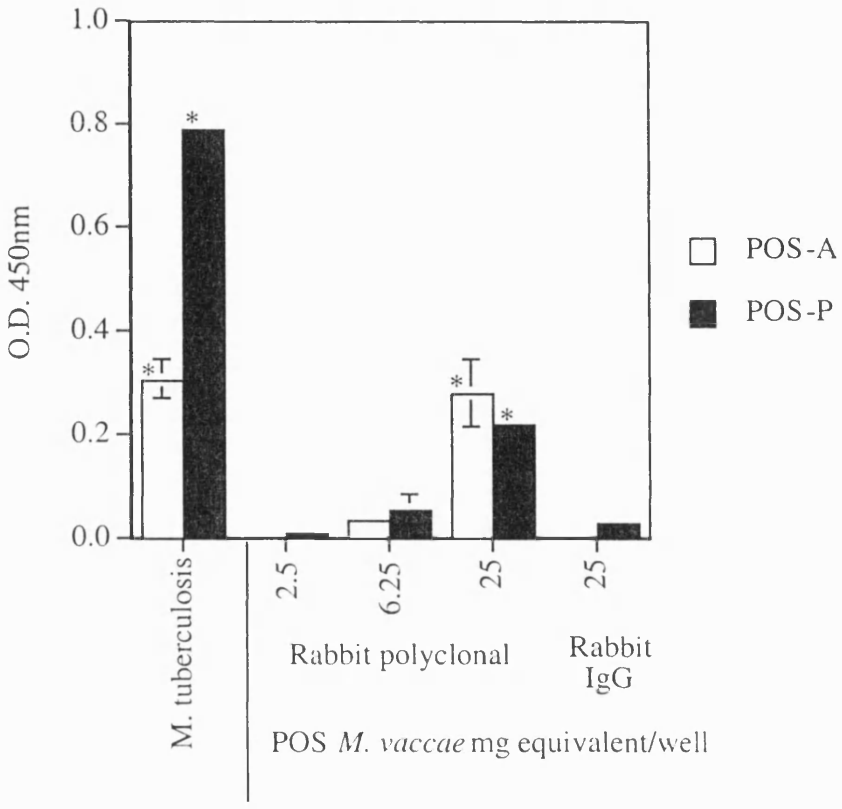


Figure 4.5. Rabbit polyclonal anti-IPG also binds POS from *M. vaccae* R877R. POS-A and P from *M. tuberculosis* H37Ra were coated on ELISA plates at 2.5μg organism mass equivalent, POS-A and P from *M. vaccae* R877R were coated at 25, 6.25 and 2.5μg organism mass equivalent (as indicated) overnight at 4°C. Primary antibodies were a rabbit polyclonal serum IgG fraction immunised with PI-PLC treated GPI from rat liver in CFA and normal rabbit IgG, both at 100μg/ml. Rabbit polyclonal serum bound POS from *M. vaccae* R877R, but a higher coating concentration was required. Binding to POS from both *M. vaccae* R877R and *M. tuberculosis* H37Ra was highly significant compared with respective negative controls (* = $p < 0.005$, student's t-test). Data are shown as optical density values at a wavelength of 450nm (after acidification). Each point represents the mean of triplicates and error bars (where visible) represent the standard deviation of the mean.

4.4.4 Polyclonal rabbit serum raised against pre-eclamptic urine P type IPG in complete Freund's adjuvant also binds mycobacterial phospho-oligosaccharide

P type IPG isolated from pre-eclamptic human urine was mixed with CFA and used to immunise two rabbits, giving a serum that binds mammalian IPG well (Dr. Phil Williams, personal communication). These sera were pooled to produce a single polyclonal serum that was also able to bind POS from *M. tuberculosis* H37Ra (figure 4.6a) and H37Rv (figure 4.6b). Interestingly, the same serum did not bind POS from *M. vaccae* R877R (data not shown). Figure 4.6 shows data from two different experiments, one for *M. tuberculosis* H37Ra POS and another for *M. tuberculosis* H37Rv POS, so comparison of the degree of binding to POS from the two strains is not possible. A pre-immunisation non-immune serum was available for comparison with this antibody, it did not bind POS from *M. tuberculosis* H37Ra or H37Rv (figure 4.6).

Experiments using these pooled sera and the previous bleeds were performed in duplicate, not in triplicate. Therefore statistical analysis has not been performed, however the interpretation of these data has generally been qualitative, to assess whether there is a response or not, rather than to quantify and compare responses. The variation between the duplicate assays was usually between 5 and 10%.

Previous, unpooled bleeds raised against pre-eclamptic urine IPG-P in CFA and two raised against rat liver IPG-P in CFA were also available. The sera raised against pre-eclamptic urine IPG-P were reactive to normal human placental IPG-P, pre-eclamptic placental IPG-P and were weakly reactive to placental GPI. The sera raised against rat liver IPG-P did not bind any mammalian IPG including the immunogen. As all the sera had been raised using CFA, they were tested against POS from *M. tuberculosis* H37Rv. Figure 4.7 shows that all of these sera were reactive against POS from *M. tuberculosis* H37Rv to at least some degree.

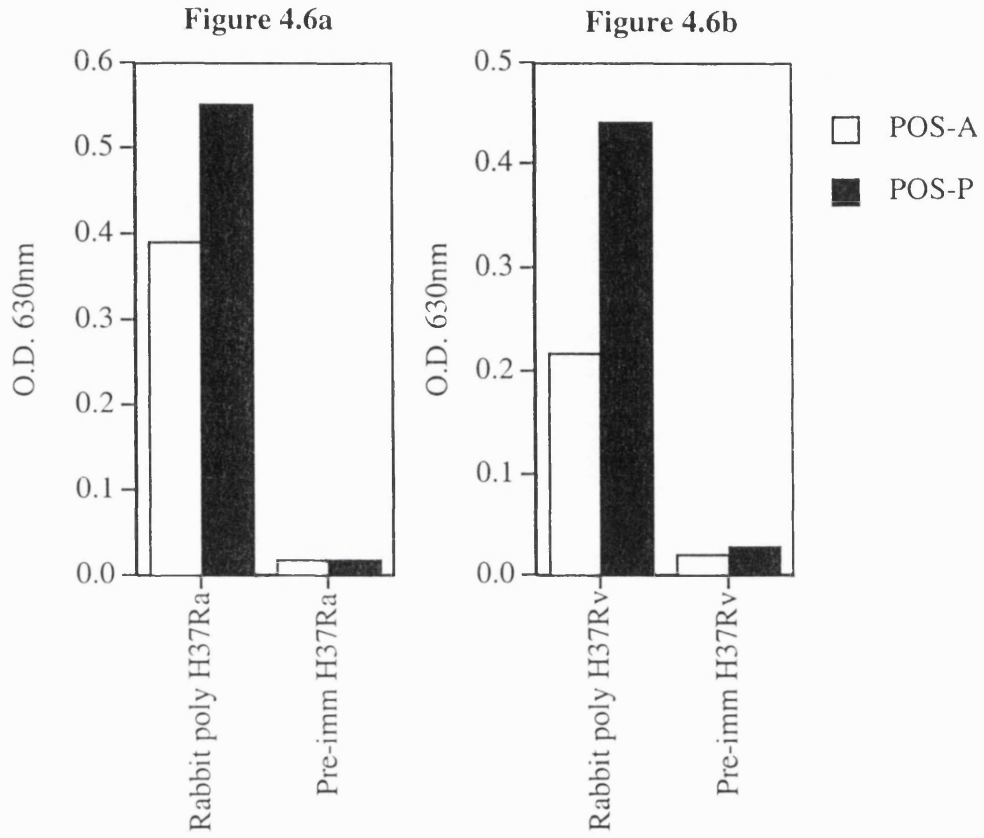


Figure 4.6. A rabbit serum raised against IPG-P from pre-eclamptic urine in CFA binds POS from *M. tuberculosis*. POS-A and P from *M. tuberculosis* H37Ra (figure 4.6a) and H37Rv (figure 4.6b) were coated on ELISA plates at 2.5mg organism mass equivalent per well overnight at 4°C. Primary antibodies were a serum pooled from two rabbits both immunised with POS-P from pre-eclamptic urine at 1 in 10 000 of the original serum and a pre-immunisation bleed from the same animals at 1 in 10 000. Rabbit poly = pooled, immune sera. Pre-imm = pree-immunisation sera. These data are derived from two separate experiments. Data are shown as optical density values at a wavelength of 630nm (without acidification), each point represents the mean of duplicates.

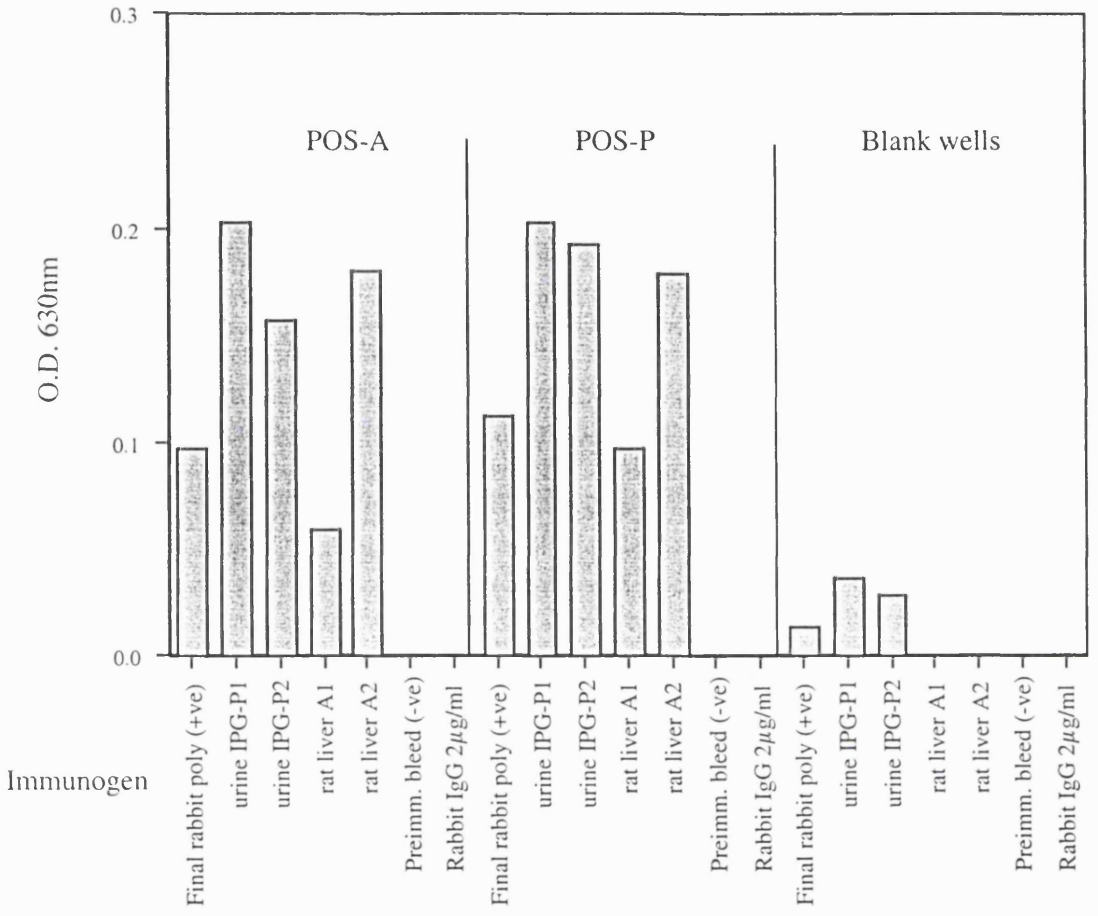


Figure 4.7. Rabbit sera that have been immunised with various mammalian IPGs all including CFA in the immunogen are reactive against POS from *M. tuberculosis*. POS-A and P from *M. tuberculosis* H37Rv were coated on ELISA plates at 2.5mg organism mass equivalent per well overnight at 4°C. Primary antibodies were two sera raised against pre-eclamptic urine IPG-P in CFA, the same sera pooled after sacrifice of the animals, pre-immunisation sera (pooled) from the same animals, two sera raised against rat liver IPG-P in CFA and normal rabbit IgG. All sera were used at 1 in 10 000 of the original. Data are shown as optical density values at a wavelength of 630nm (without acidification) and each point represents the mean of duplicates.

4.4.5 The only requirement for the presence of an anti-mycobacterial phospho-oligosaccharide antibody response in rabbits is the presence of complete Freund's adjuvant in the immunogen

The existence of polyclonal sera raised using CFA that recognise mycobacterial POS but do not recognise mammalian IPG (despite having mammalian IPG present in the original immunogen) suggests that CFA itself induced the anti-mycobacterial POS response, not cross-reactivity with mammalian IPG. This strongly implies the presence of POS or a POS-like epitope within CFA. This hypothesis was examined directly by immunising one rabbit with each of the following:

1) CFA only
2) *M. tuberculosis* H37Rv POS-A in CFA
3) *M. tuberculosis* H37Rv POS-P in CFA

Prior to immunisation, bleeds were taken to act as a negative control. When assayed for anti-POS IgG immunoreactivity these sera were all negative. After 4 weeks the animals were bled to assess their immune response and immunised again with 75% of the original POS dose in IFA. Figure 4.8 shows that only the animal that was immunised with CFA alone had responded at this point. The IgG response was detectable down to a 1 in 1000 dilution of the serum. This experiment proved that CFA alone is indeed sufficient to induce an anti-POS IgG response in the rabbit. These data, like those in section 4.4.4, were obtained in duplicate making statistical comparison impossible. Again, however, the data are used to qualitatively examine for the presence of an immune response, rather than to quantify and compare responses.

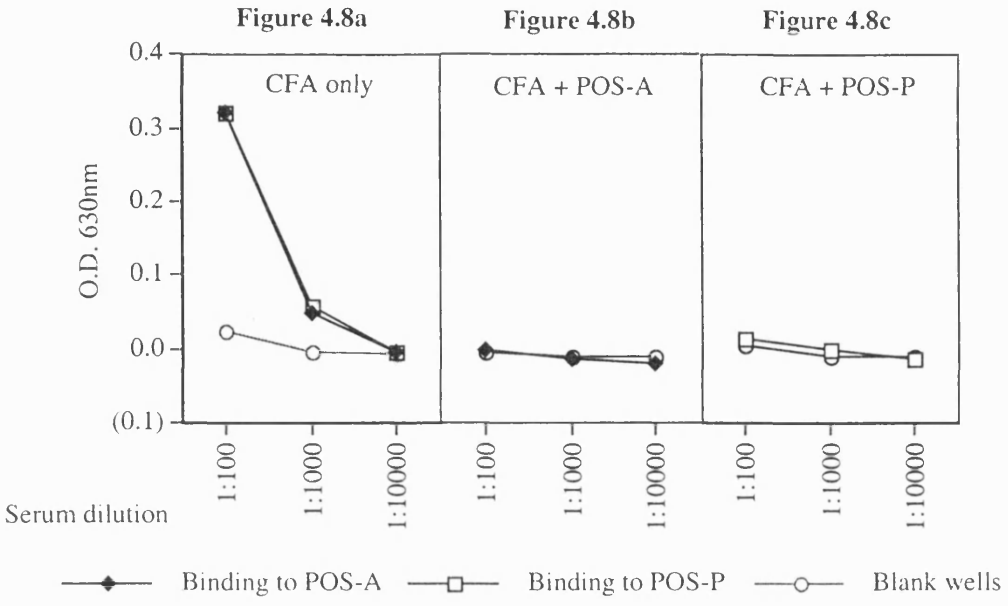


Figure 4.8. Immunoreactivity of rabbit sera against POS from *M. tuberculosis* H37Rv four weeks after immunisation with CFA alone or CFA and POS. POS-A and P from *M. tuberculosis* H37Rv were coated on ELISA plates at 2.5mg organism mass equivalent per well overnight at 4°C. Primary antibodies were sera from rabbits immunised with CFA only (figure 4.8a), CFA and POS-A (figure 4.8b) and CFA and POS-P (figure 4.8c) collected four weeks after immunisation. Rabbit sera immunoreactivities were assessed only against the relevant immunogen, or against both POS-A and P fractions in the case of the CFA only animal. Sera were used at the dilutions indicated. Data are shown as optical density values at a wavelength of 630nm (without acidification) and each point represents the mean of duplicates.

A second bleed was taken 2 weeks after the second immunisation and a third 2 weeks later still. The IgG response of the CFA only animal continued to be strong. Figure 4.9 shows the IgG responses of all animals throughout the course of the experiment examined after the final bleed. By this time, the animal immunised with *M. tuberculosis* H37Rv POS-A in CFA showed a markedly lower response than the CFA only animal. The animal immunised *M. tuberculosis* H37Rv POS-P in CFA showed no readily detectable IgG response against either POS fraction.

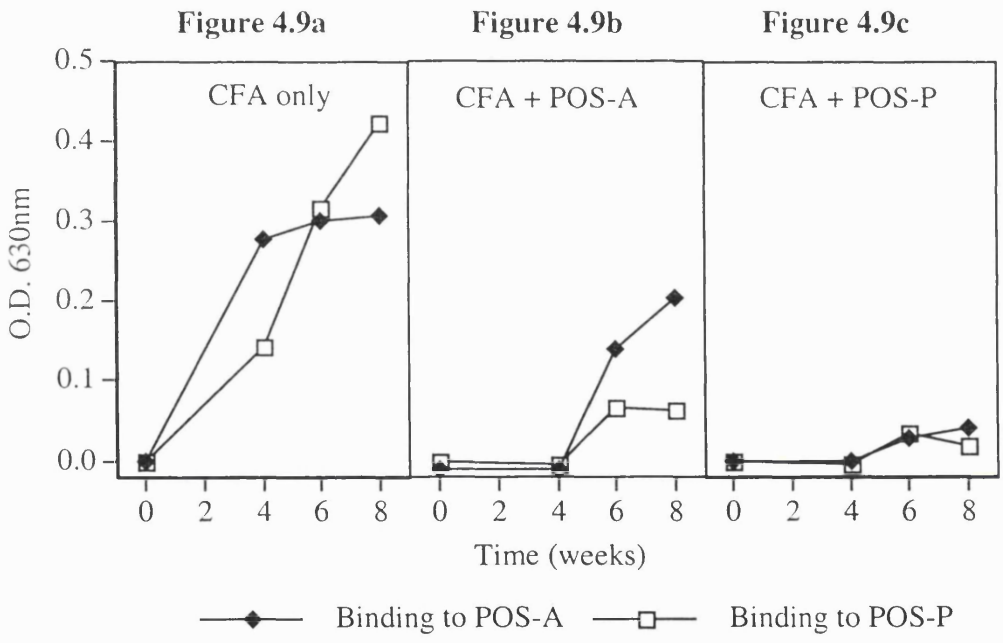


Figure 4.9. Immunoreactivity of rabbit sera raised against CFA and POS from *M. tuberculosis* H37Rv over the course of eight weeks. POS-A and P from *M. tuberculosis* H37Rv were coated on ELISA plates at 2.5mg organism mass equivalent per well overnight at 4°C. Primary antibodies were sera from rabbits immunised with CFA only (figure 4.9a), CFA and POS-A (figure 4.9b) and CFA and POS-P (figure 4.9c) at dilutions of 1 in 100 of the original sera. Anti POS reactivity of the rabbit sera were assessed against both POS-A and P, regardless of immunogen. Data are shown as optical density values at a wavelength of 630nm (without acidification) and each point represents the mean of duplicates.

Despite the absence of an IgG response against mycobacterial POS in the animal that received POS-P in CFA, this animal was not tolerised as a response was still detectable in the total immunoglobulin fraction (Figure 4.10). All animals exhibited easily detectable total Ig responses.

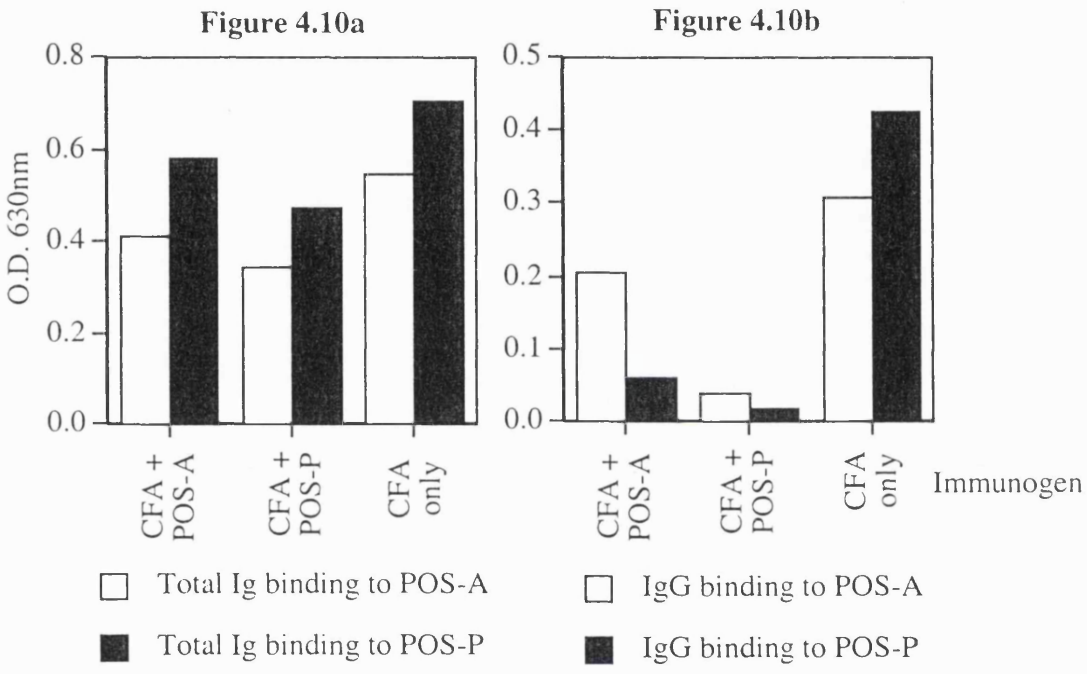


Figure 4.10. Co-immunisation of POS from *M. tuberculosis* H37Rv with CFA may prevent immunoglobulin class switching to IgG. POS-A and P from *M. tuberculosis* H37Rv were coated on ELISA plates at 2.5mg organism mass equivalent per well overnight at 4°C. Primary antibodies were sera from rabbits immunised with CFA only, CFA and POS-A and CFA and POS-P at dilutions of 1 in 100 of the original sera. Figure 4.10a shows total immunoglobulin responses and figure 4.10b shows IgG responses of the animals eight weeks after immunisation. Anti-POS reactivity of each rabbit sera was assessed against both POS-A and P, irrespective of immunogen. Data are shown as optical density values at a wavelength of 630nm (without acidification) and each point represents the mean of duplicates.

From this result we tentatively hypothesised that co-immunisation of a rabbit with CFA and POS prevented or reduced isotype switching to IgG, with POS-P perhaps being more effective. The assays had not been performed in triplicate, making statistical comparison between different animals impossible. However, as only one animal had been immunised in each group anyway true statistical comparison between the different immunisations remained invalid. Therefore, rather than repeat the ELISAs on existing sera, the immunisations were repeated using more animals in an attempt to confirm this observation.

This experiment was repeated with another extraction of POS from *M. tuberculosis* H37Rv that had been validated for biological activity by J774 NO release assay (data not shown). Nine animals were immunised, three in each group with the same immunogens as previously used:

1) CFA only
2) *M. tuberculosis* H37Rv POS-A in CFA
3) *M. tuberculosis* H37Rv POS-P in CFA

On this repeat occasion, however, there was no difference observed between the animals that were immunised with CFA only and those that had POS co-administered. Representative data from the third and final bleed are shown in figure 4.11, compared with the data from the pre-immunisation bleed as a negative control.

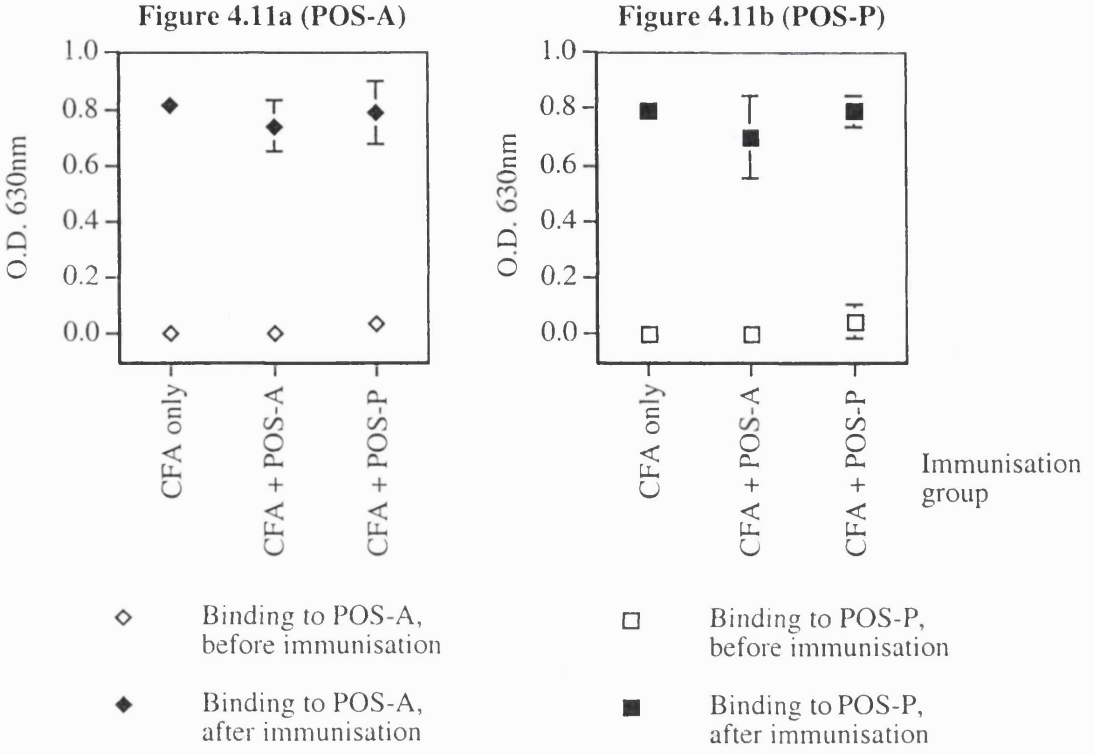


Figure 4.11. IgG immunoreactivity against POS-A and P from *M. tuberculosis* H37Rv of rabbit sera immunised with CFA and mycobacterial POS. Three rabbits were immunised with each of the following: CFA only, POS-A in CFA and POS-P in CFA. After 4, 6 and 8 weeks the rabbits were bled and anti POS immunoreactivity was assessed. The day after the 4 week bleed the rabbits received a booster immunisation. The data shown here represent relative IgG levels against both POS fractions of all the animals prior to immunisation and 8 weeks post immunisation. POS-A (figure 4.11a) and POS-P (figure 4.11b) from *M. tuberculosis* H37Rv (2.5mg organism mass equivalent per well) were coated on ELISA wells in 0.1M phosphate at pH 7 overnight. After blocking, the sera were incubated on the plate at a dilution of 1 in 100 in BMBR. The secondary antibody was HRPO-goat anti-rabbit IgG. Each animal was assayed in duplicate and the mean optical density calculated. There was no statistically significant difference between any of the groups for degree of binding to POS-A or to POS-P. The data shown are mean optical density values at 630nm for each group ± the standard deviation of the group.

4.4.6 *Phospho-oligosaccharide from* M. tuberculosis *H37Rv are non-immunogenic in mice*

Two female Balb/c mice were immunised with approximately 150mg wet weight equivalent starting mass of POS from *M tuberculosis* H37Rv in CFA as described in methods. The combinations were administered as follows:

1) CFA only
2) *M. tuberculosis* H37Rv POS-A in CFA
3) *M. tuberculosis* H37Rv POS-P in CFA

Two weeks after the booster dose given in IFA, the mice were bled and the immunoglobulin response assessed by ELISA. None of the animals responded to mycobacterial POS (data not shown). Sera were incubated on blocked ELISA plates coated with POS from *M. tuberculosis* H37Rv 2.5mg organism mass equivalent per well from a dilution of 1 in 10 000 up to 1 in 10 as the primary antibody, at which point non-specific binding was observed. Although the batch of POS used had been tested for bioactivity and shown to be active, the aliquots used for the immunisations were re-tested for activity by J774 cell NO release assay and again found to be active (data not shown). The experiment was repeated with the same result, and it was concluded that mycobacterial POS are not immunogenic in mice.

4.4.7 *Antibody responses to mycobacterial phospho-oligosaccharide in humans.*

Sera from 16 tuberculosis patients (8 male, 8 female, mean age 44 years, age range 21 to 85 years) were examined for the presence of anti-POS total immunoglobulins by ELISA. Seven normal volunteer sera were also assayed (5 male, 2 female, mean age 34 years, age range 23 to 52 years); six individuals were PPD positive and one was PPD negative. Milk protein was used as the blocking reagent for the human ELISAs because, unlike the rabbit ELISA method, BMBR was found to be an ineffective blocking reagent for human sera (figure 4.12).

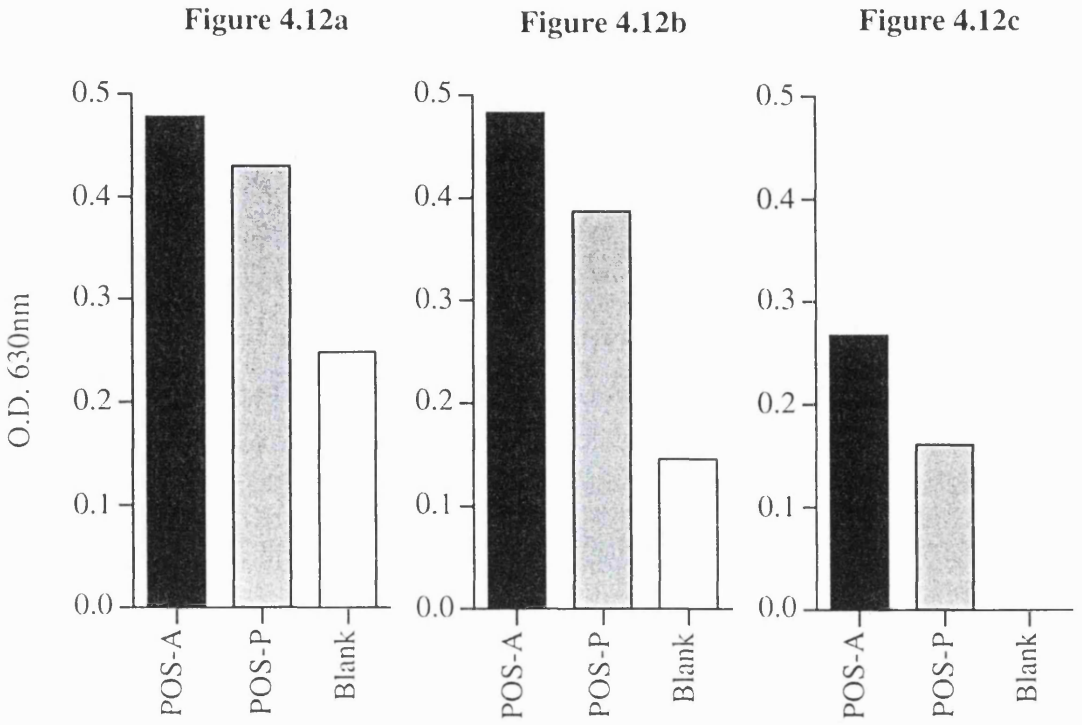


Figure 4.12. Suitability of various blocking reagents for detection of anti POS antibodies in human sera. POS A and P from *M. tuberculosis* H37Rv (2.5mg organism mass equivalent per well) were coated on to a polysorp ELISA plate in 0.1M phosphate at pH7 overnight at 4°C. Blank wells consisted of 0.1M phosphate only. Serum from a known positive TB patient at 1 in 100 dilution was used as the primary antibody. The secondary antibody was HRPO-goat anti-human total immunoglobulins. Blocking reagents were; 2% BSA in PBS (figure 4.12a), BMBR (figure 4.12b) and 5% skimmed milk protein (Marvel) in PBS (figure 4.12c). Each blocking reagent was used for every stage of the experiment. Only 5% Marvel in PBS achieved suitably low optical density values for the blank wells. Data plotted are the mean of duplicate optical density values at 630nm.

Significantly higher binding to both POS fractions of *M. tuberculosis* H37Ra was observed by the sera of tuberculosis patients compared with sera from healthy controls, $p < 0.05$ for POS-A and $p < 0.001$ for POS-P, Wilcoxon rank sum test. Control sera contained in many cases undetectable levels of anti POS-A and P immunoglobulins (figure 4.13).

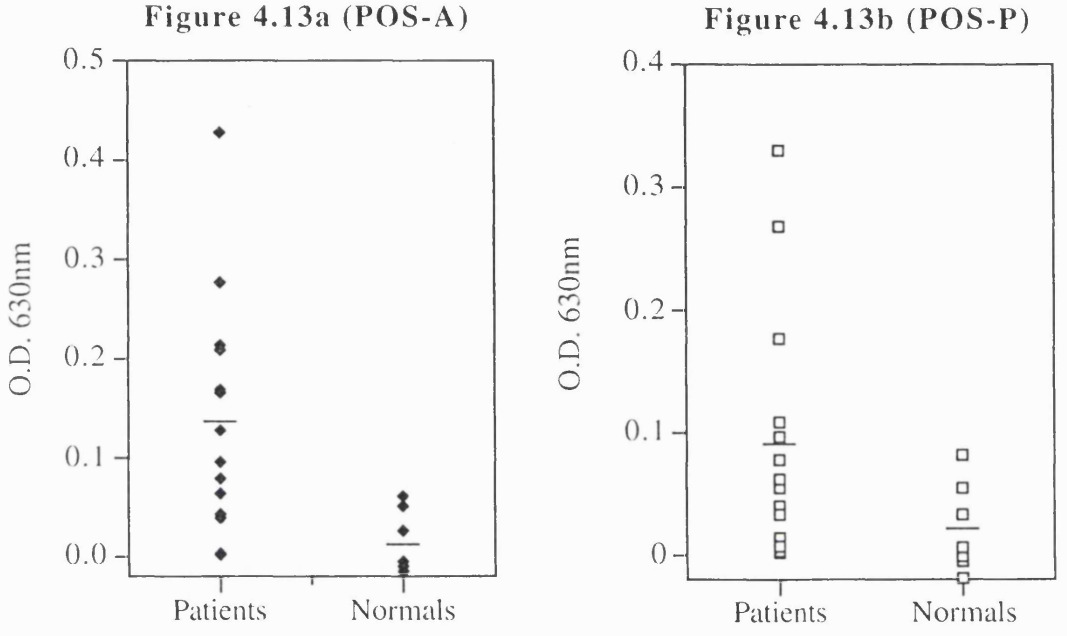


Figure 4.13. Sera from tuberculosis patients contain higher levels of anti-POS antibodies than healthy controls. POS-A and P from *M. tuberculosis* H37Ra (2.5mg organism mass equivalent per well) were coated on to a polysorp ELISA plate in 0.1M phosphate at pH7 overnight at 4°C. Sera from 16 tuberculosis patients and 7 healthy controls were used at 1 in 100 dilution as the primary antibodies. The secondary antibody was HRPO-goat anti-human total immunoglobulins. The patient group had significantly higher anti-POS antibody levels than the healthy control group, $p < 0.05$ for anti-POS-A Ig and $p < 0.001$ for anti-POS-P, Wilcoxon rank sum test. The mean optical density for the tuberculosis group was 0.091 for POS-A (filled diamonds, figure 4.13a) and 0.137 for POS-P (hollow squares, figure 4.13b), compared with 0.022 for POS-A and 0.012 for POS-P in the control group. Assays performed on separate ELISA plates always included a reference serum to allow comparison between plates. Data plotted are the mean of duplicate optical density values at 630nm.

Sera from tuberculosis patients were able to recognise POS from *M. tuberculosis* H37Ra and H37Rv (figure 4.14). The *M. tuberculosis* strains H37Ra and H37Rv are avirulent and virulent respectively in mice, but both strains are thought to be virulent in humans. The degree of binding to the POS fractions measured for the two different mycobacterial strains correlated together very significantly (figure 4.15) in the case of POS-P (correlation coefficient 0.867, $p < 0.001$, Spearman's rank correlation). The degree of binding to POS-A from each mycobacterial strain did not quite achieve statistical significance (correlation coefficient 0.566, $p = 0.055$). The number of serum samples investigated in this experiment was small, and it may be that this correlation would achieve significance if the sample size were greater. The degree of binding of patients' sera to one POS fraction correlated very highly with binding to the other fraction from the same organism (figure 4.16), $p < 0.0001$ for both organisms (Spearman's rank correlation), so the degree of response to one POS fraction was proportional to the degree of response to other for both bacterial strains in this population. However, the degree of binding to POS-P from either strain was generally higher than the degree of binding to POS-A (see, for example, figure 4.14). Whether this is due to the levels of antibody against POS or the respective amounts of POS-A and POS-P present in the extracts remains unknown, although the same pattern was observed with many of the rabbit sera. The Spearman rank correlation test was selected for these analyses because, based upon figure 4.13, these data do not appear to be normally distributed.

These results imply some similarity between the material derived from the two strains of *M. tuberculosis*. This similarity is clearly greater between the POS-P fractions than the POS-A fractions, but given the p value of 0.055, there may be still some similarity between the POS-A fractions from each strain of *M. tuberculosis*. The level of anti-POS antibodies in human sera was low, being not readily detectable in many samples at any dilution further than 1 in 100 of the original serum.

Immunoglobulin isotype testing of the sera from tuberculosis patients was performed against POS from *M. tuberculosis* H37Ra (figure 4.17). IgG levels were significantly greater than IgA and IgM levels ($p < 0.001$, Wilcoxon rank sum test), showing that class switching of the antibodies involved in the anti-POS immune response had taken place.

IgM and IgA binding to POS were also detectable in some of the sera; IgM anti-POS levels were higher than IgA anti-POS levels ($p<0.01$, Wilcoxon rank sum test).

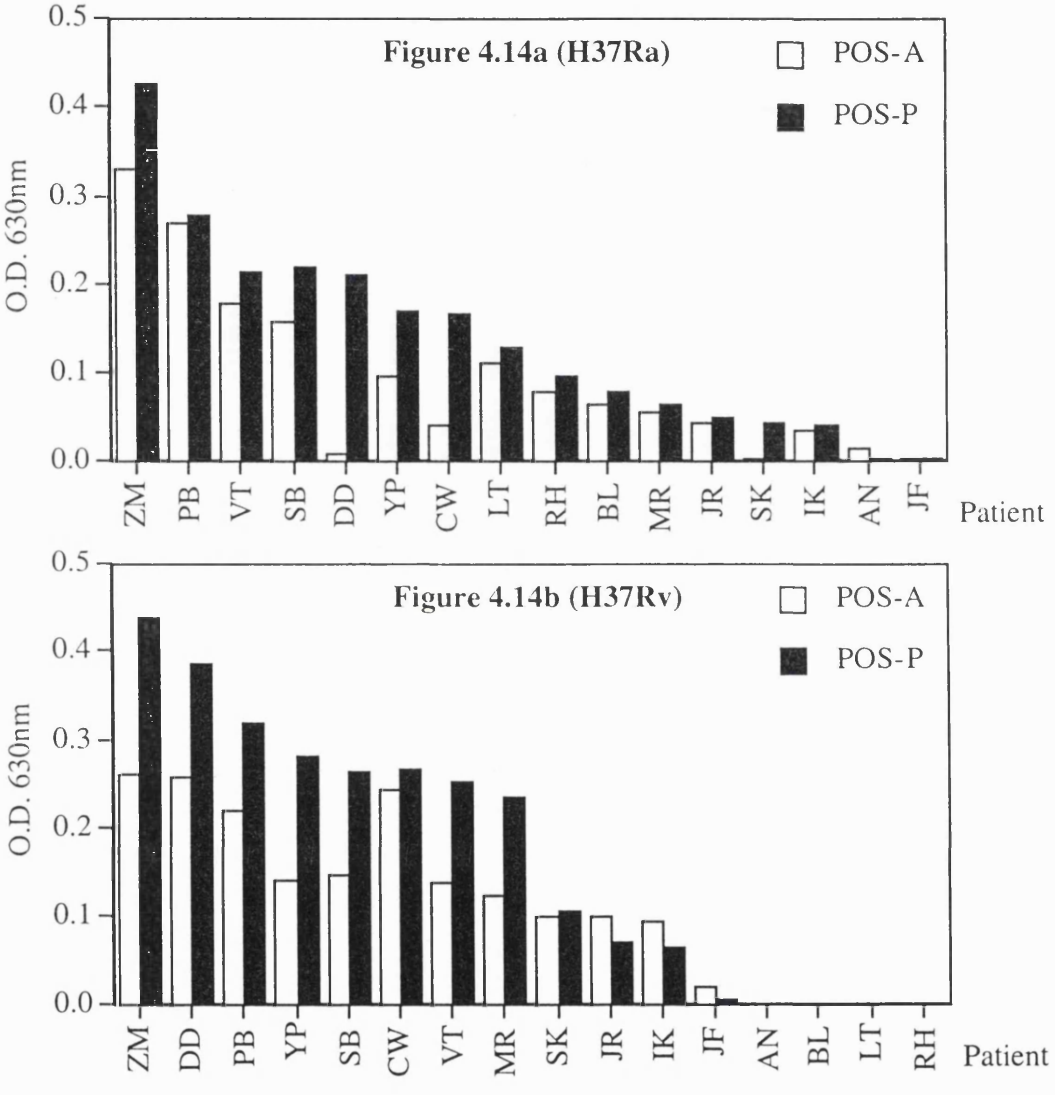


Figure 4.14. The sera of tuberculosis patients contain immunoreactivity against POS from *M. tuberculosis* H37Ra and H37Rv. POS-A and P from *M. tuberculosis* H37Ra (figure 4.14a) and H37Rv (figure 4.14b) were coated on to a polysorp ELISA plate at 2.5mg organism mass equivalent per well in 0.1M phosphate, pH 7, overnight at 4°C. Sera from tuberculosis patients were used at a dilution of 1 in 100 as the primary antibodies. The secondary antibody was HRPO-goat anti-human total immunoglobulins. Most of the patients responded to POS from both strains of *M. tuberculosis* although not in the same manner to each. Data plotted are the mean of duplicate optical density values at 630nm. (nd = not determined.)

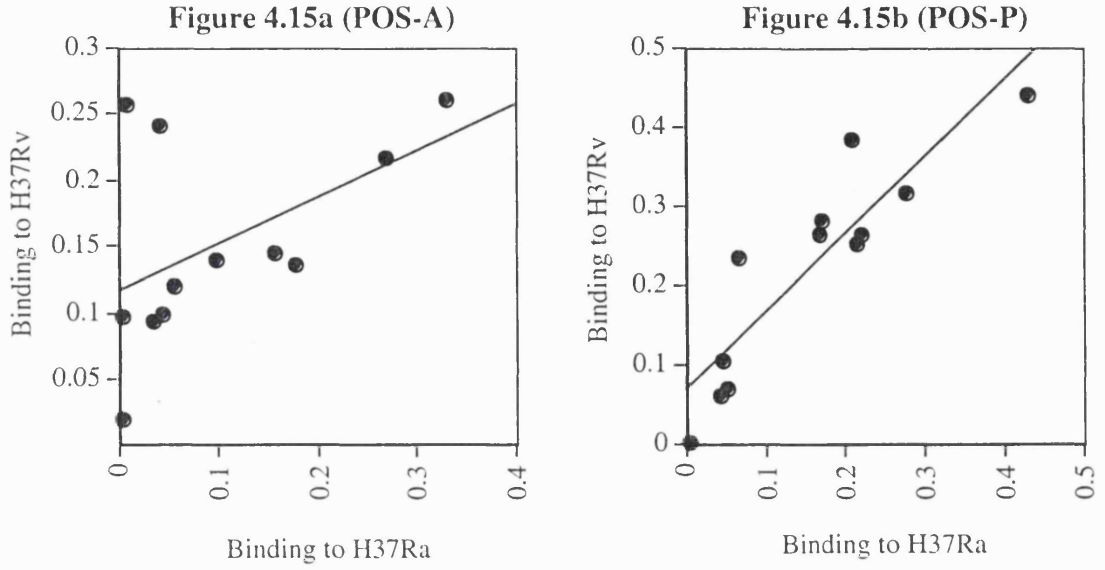


Figure 4.15. Correlation of binding to POS from *M. tuberculosis* H37Ra with binding to POS from *M. tuberculosis* H37Rv by sera from 12 tuberculosis patients. Data from figure 4.14 were re-plotted to show the correlation between binding to POS derived from *M. tuberculosis* H37Ra (y axes) and *M. tuberculosis* H37Rv (x axes). The axes represent optical density at 630nm. Figure 4.15a shows binding to POS-A and figure 4.15b shows binding to POS-P. Correlation between the optical density values observed for POS-A did not quite achieve statistical significance: correlation coefficient = 0.556, $p = 0.055$, by Spearman's rank correlation. Correlation between the optical density values observed for POS-P was highly significant: correlation coefficient = 0.867, $p < 0.001$, by Spearman's rank correlation.

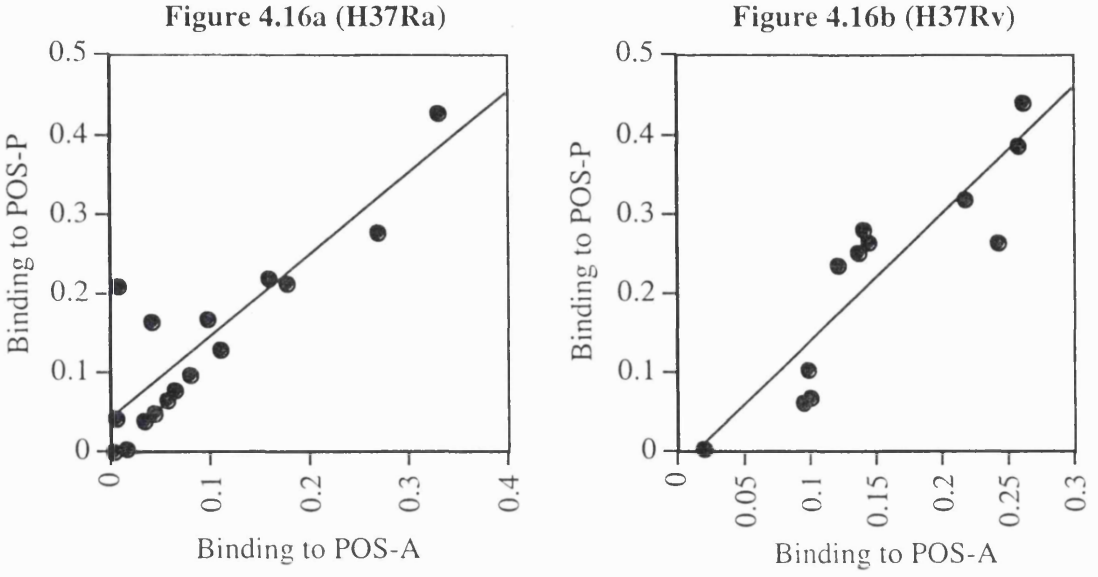


Figure 4.16. Correlation of binding to POS-A with POS-P from *M. tuberculosis* H37Ra and H37Rv by sera from tuberculosis patients. Data from figure 4.14 were re-plotted to show the correlation between binding to POS-A (x axes) and POS-P (y axes) from the two strains *M. tuberculosis* H37Ra and H37Rv. The axes represent optical density at 630nm. Figure 4.16a shows binding to *M. tuberculosis* H37Ra POS (16 patients) and figure 4.16b shows binding to *M. tuberculosis* H37Rv POS (12 patients). The degree of binding of patients' sera to one POS fraction was highly correlated with the degree of binding to the other fraction from the same organism. *M. tuberculosis* H37Ra correlation coefficient = 0.8, $p < 0.0001$; *M. tuberculosis* H37Rv correlation coefficient = 0.958, $p < 0.0001$ (Spearman's rank correlation).

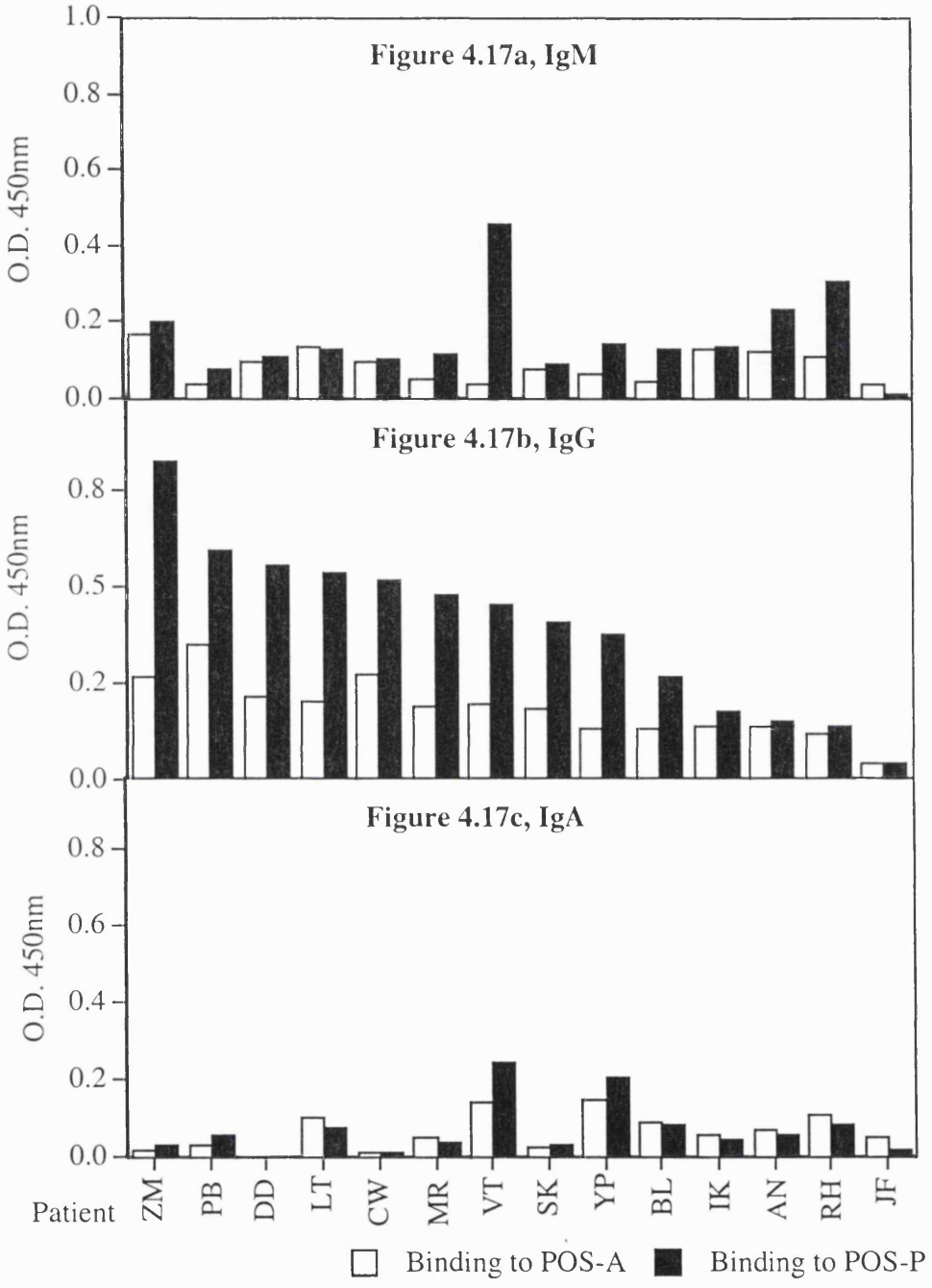


Figure 4.17. Isotype distribution of antibodies against POS from *M. tuberculosis* H37Ra in the sera of tuberculosis patients. POS-A and P from *M. tuberculosis* H37Ra (2.5mg organism mass equivalent per well) were coated on to a polysorp ELISA plate in 0.1M phosphate at pH7 overnight at 4°C. Sera from 15 tuberculosis patients were used at a dilution of 1 in 100 as the primary antibodies. The secondary antibodies were HRPO-goat anti-human IgM (figure 4.17a), IgG (figure 4.17b) and IgA (figure 4.17c). The most abundant isotype that bound POS was IgG, which was present in greater amount than IgM or IgA ($p < 0.001$,

Wilcoxon rank sum test). The IgM level was also significantly greater then the IgA level ($p < 0.01$, Wilcoxon rank sum test). Assays performed on separate ELISA plates always included a reference serum to allow comparison between plates. Data plotted are the mean of duplicate optical density values at 630nm.

4.5 Discussion

There were several reasons behind testing existing antibodies and sera for the property of binding to mycobacterial POS and for attempting to produce a variety of antibodies against mycobacterial POS, outlined at the beginning of the chapter. As can be seen from the data presented in this chapter, some of these aims were achieved and some were not.

4.5.1 Immunogenicity in rabbits

Because of the homologous activities between mycobacterial POS and mammalian IPG, we hypothesised that available antibodies that bind to mammalian IPG might also be able to bind mycobacterial POS, perhaps due to some structural similarity. This was indeed shown to be the case, and the coating conditions for POS from *M. tuberculosis* and *M. vaccae* have been described. However, no true negative control was available for the antibody (1), so it was not proven that this immunological cross reactivity was due to true structural cross reactivity between mammalian IPG and mycobacterial POS.

Although several polyclonal antibodies bound mycobacterial POS (antibody 3, rabbit sera raised against various mammalian IPGs with CFA), it seemed that it was the presence of CFA, rather than mammalian IPG, that was the essential factor in raising serological reactivity to POS from *M. tuberculosis*. This was suggested first by the observation that sera from rabbits immunised with mammalian IPG that did not bind their mammalian derived immunogen were still able to recognise mycobacterial POS. This observation was subsequently confirmed by a "blank" immunisation of CFA alone that also resulted in a mycobacterial POS reactive serum during an attempt to raise polyclonal sera that bind individual mycobacterial POS fractions. Moreover, many of the sera from rabbits immunised with CFA with or without mycobacterial POS fractions also responded well against both *M. tuberculosis* H37Rv POS fractions. The serum of every rabbit that was

immunised with CFA bound both *M. tuberculosis* POS fractions irrespective of whatever IPG or POS fraction (mammalian or mycobacterial) was also present in the immunogen. The only exceptions to this were the two sera that did not respond as well in the IgG fraction to mycobacterial POS when co-immunised with CFA, and these sera were still reactive in their total immunoglobulin fraction.

Effect of co-immunisation of CFA with phospho-oligosaccharide

As briefly mentioned above, two animals that had been co-immunised with *M. tuberculosis* H37Rv POS fractions and CFA did not mount IgG responses as strong as those observed with CFA alone (figure 4.9), although statistical verification was not performed. In particular we tentatively hypothesised that one animal which was immunised with POS-P in CFA did not isotype switch to IgG at all (figures 4.9 and 4.10). The repeat immunisation using more rabbits failed to show the same effect (figure 4.11). However, there remains a possibility that these results are explained by the fact that different batches of POS material were used on the two separate immunisation occasions. Although the POS fractions used in this immunisation were tested for activity by J774 cell NO release assay, there was insufficient material for more detailed assays, in particular quantification by phosphate analysis. There may be a rigid dose dependence of the effect observed in figures 4.9 and 4.10, and perhaps insufficient material was included in the second immunisation (figure 4.11). In favour of this is the observation that the *same* pattern appeared to occur for *both* animals co-immunised with mycobacterial POS fractions, at least at 4 weeks after immunisation (figure 4.9), although these data remain statistically unverified. It is of interest to note that the sera that did not bind mycobacterial POS strongly also showed reduced binding to hsp 65 from *M. bovis* (serologically indistinct to *M. tuberculosis*) compared with their binding to other antigens from *M. tuberculosis* (sonicate antigen and PPD, data not shown). It remains to be confirmed whether mycobacterial POS influence immunoglobulin isotype switching in rabbits.

4.5.2 Immunogenicity in mice

Notably, the effect described above did not take place in the mouse. Murine sera failed to recognise mycobacterial POS after immunisation with mycobacterial POS in Ribi's adjuvant, mycobacterial POS in CFA and CFA alone. An attempt at hybridisation to

generate monoclonal B cell lines from some of these immunised animals was successful in producing many clones but none of them made an antibody that bound mycobacterial POS. In addition, serum from a mouse immunised with CFA and human pre-eclamptic urine IPG-P that did bind the immunising IPG did not bind mycobacterial POS, nor did a murine monoclonal antibody (2D1) that recognises several different mammalian IPG fractions. This is of interest because mice do not naturally develop tuberculosis, this is discussed further below.

4.5.3 Immunogenicity in humans

Despite the small numbers of serum samples available for this study, it is reasonable to conclude that POS are immunogenic in humans. Although not all TB patients showed antibody binding to mycobacterial POS, the level of binding was found to be significantly higher than that of healthy controls (figure 4.13), and was mainly, though not exclusively, of IgG isotype (figure 4.17). Ideally it would be desirable to express these data as actual Ig concentrations, not as O.D. values, but the difference between the groups was sufficiently discernable that this transformation would be unlikely to render these data invalid, particularly for POS-P. All but one of the healthy controls were PPD positive and had BCG, indicating that the presence of an anti-POS antibody response is not dependent upon prior mycobacterial exposure but might be confined to individuals with active disease. In addition, rabbits immunised with CFA did develop an antibody response in the absence of infection, so it would seem that exposure to *M. tuberculosis*, whether alive or dead, is the crucial determinant in mounting an anti-POS antibody response. Expansion of this study to include more patients and healthy controls is needed to confirm this observation. It would also be very interesting to look at cured tuberculosis patients to see if such individuals retain anti-POS antibody after remission of active disease.

4.5.4 Implications of antibody work for structural characterisation

Our original hypothesis to explain the apparent cross-recognition of mammalian IPG and mycobacterial POS by the described antibodies construed structural similarity between the respective IPG and POS extracts. The observation that any serum from a rabbit that had been immunised with CFA, whether or not it recognised a mammalian IPG, could recognise mycobacterial POS failed to provide support for this hypothesis. Moreover, a

monoclonal antibody that binds mammalian IPG, 2D1, did not bind mycobacterial POS and a polyclonal murine serum that binds mammalian IPG also did not bind mycobacterial POS. However, one of the polyclonal sera raised against mammalian IPG (antibody 3) was able to bind POS from *M. tuberculosis* H37Rv but not POS from *M. vaccae* R877R. It is therefore possible that there is more structural similarity between *M. tuberculosis* H37Rv POS and mammalian IPG than there is between *M. vaccae* R877R POS and mammalian IPG. Importantly, however, there is still clearly significant difference between all mycobacterial POS fractions and mammalian IPG as shown by the discrepancy in monoclonal antibody binding.

Although the postulated structural *similarity* between mammalian IPG and mycobacterial POS was not supported by the data presented here, these data do show that there must therefore be some significant structural *difference* between mammalian IPG and mycobacterial POS, itself an important observation. It may also be concluded that POS from *M. tuberculosis* H37Rv and *M. vaccae* R877R are structurally different, as suggested by the difference in biological activity (chapters 1 and 3). Nevertheless, a degree of structural similarity between mammalian IPG and mycobacterial POS is not ruled out particularly as both mammalian and mycobacterial compounds share some properties in terms of activity. The apparent necessity of CFA, which also contains immunogenic POS, as an adjuvant and the cost of performing such immunisations in rabbits prevented further detailed investigation.

The work involving sera from tuberculosis patients shows that, on the basis of immunoglobulin binding, the POS-P fractions of the two strains of *M. tuberculosis* examined were very similar. The POS-A fractions did not display the same degree of similarity, although they show a lesser degree of similarity rather than being completely different.

4.5.5 Implications for the possible role of phospho-oligosaccharide in mycobacterial disease

Unfortunately, no reagents suitable for neutralisation of mycobacterial POS within the context of mixed antigen mycobacterial immunology experiments were generated. This was due to the failure of mice to respond POS preventing the generation of monoclonal

anti-POS antibodies. A further problem was the non-specific anti-mycobacterial nature of sera raised against CFA. In order to be useful in this context an antibody (polyclonal or monoclonal) must be selective for POS in order to block only the POS effects and not effects due to other mycobacterial antigens. Such a reagent could prove to be a useful tool for investigating the contribution of POS to the disease state in tuberculosis (especially in an *in vivo* model).

The observation that rabbits and humans are both able to mount an anti-POS antibody response whereas mice apparently are not was interesting because mice do not naturally suffer from tuberculosis, although the disease may be induced experimentally. Humans and rabbits are much more susceptible to the disease. Although it is premature to draw any conclusions, it would be interesting to seek a relationship between susceptibility to tuberculosis and the ability to mount an anti-POS antibody response. It is also of interest to note that, although POS from *M. tuberculosis* H37Ra and H37Rv are distinct in their biological activity (chapter 3), they appear to be very similar in their immunogenicity from the experiments shown here. The existence of anti-POS antibodies is clearly not a requirement for pathology based on the data presented here because some tuberculosis patients did not exhibit anti-POS antibodies although in some experimental models anti-mycobacterial antibodies have been associated with disease (see below). Immunisation with BCG was not associated with the presence of anti-POS antibodies in the subjects studied here (it is not known if *M. bovis* or *M. bovis* BCG contain POS material).

The question of the relevance of antibody to mycobacterial immunity is still an open one and has recently been comprehensively reviewed (Glatman-Freedman and Casadevall 1998). Over the last few decades the role of antibody in immunity to mycobacteria has become unfashionable, taking a back seat compared with the undeniable significance of cell-mediated immunity. However, as Glatman-Freedman and Casadevall have pointed out, there is much evidence that antibody may play an important role in mycobacterial immunity, as well as evidence that antibody plays no role, and evidence that antibody is harmful. Most interesting with regard to the present work is the frequency with which the presence of anti-carbohydrate antibody was found to be associated with protection. In two sets of experiments, antibody against protein and carbohydrate was associated with more protection than antibody against protein alone in rabbits (Seibert *et al.* 1956, Seibert and

Seibert M.V. 1957). In another study antibody against PPD in humans was found to be associated with disseminated, advanced disease but the presence of antibody against carbohydrate was not specifically examined (Lenzini *et al.* 1977). Tuberculosis patients displaying high antibody titres mounted weaker cellular responses than those who displayed low antibody titres, but both groups suffered the clinical disease. In yet a further report high levels of antibody against LAM protected children from disseminated disease, again showing a protective role for anti-carbohydrate antibody (Costello *et al.* 1992). This, however, is in contrast to the observation that infants who have received neonatal BCG do not mount an antibody response during the first year of life but are nevertheless protected against tuberculosis during this period (Peterson *et al.* 1952). What implications these data have for the role of POS and anti-POS antibodies are unclear, as anti-POS antibodies were associated with a state of disease in the small sample studied here.

More recent work has focused on animal models, in particular involving the use of animals that lack functional B cells by disruption of the μ heavy chain gene (Kitamura *et al.* 1991). However, these experiments too have given contradictory results, with one group reporting no difference in bacterial burden, pathology or survival using a low dose aerosol *M. tuberculosis* H37Rv infection model (Johnson *et al.* 1997), whereas another group reported increased bacterial burden in the organs of μ heavy chain gene KO mice after high dose intravenous administration of *M. tuberculosis* H37Rv, although there was no difference in survival between KO and wild type mice (Vordermeier *et al.* 1996). Furthermore, pre-incubation of *M. tuberculosis* Erdman with an IgG antibody specific for surface carbohydrate did improve survival after intra-tracheal infection even in IFNγ deficient mice (Teitelbaum *et al.* 1998). In these experiments the bacterial burden between the antibody treated and untreated infections was the same, but there was a marked difference in the nature of the granulomata formed, those formed after infection of antibody treated *M. tuberculosis* being better organised with greater containment of organisms. It is of interest to note that in a μ heavy chain gene KO mouse model of primary and secondary immunity to the intracellular bacterial pathogen *Francisella tularenis* live vaccine strain, transfer of B cells mediated secondary immunity whereas specific antibody did not (Elkins *et al.* 1999). These data demonstrate that B cells may have a role distinct to antibody production in immunity to intracellular pathogens, which may go some way to explaining the confusing results described above. What role B cells play in this model is unknown, but they may

have a role in defining the T cell response by biasing the response towards certain particular antigens that have been taken up specifically by the high affinity surface B cell receptors. Clearly the function of B cells and antibody in immunity to *M. tuberculosis* and other intracellular pathogens is yet to be completely defined, and POS from *M. tuberculosis* may yet play a significant role.

4.5.6 Serodiagnostic potential of anti phospho-oligosaccharide antibody responses

Clinical information concerning the tuberculosis patients whose sera were analysed was, unfortunately, not available in sufficient detail to investigate any potential relationship between the presence or level of anti-POS antibodies and the condition of the patient. Whilst most of the work in the first half of the 20th Century focused on serology with the aim of treating tuberculosis, during the second half of the century the emphasis switched to establishing a serological diagnosis of tuberculosis. A serological test for tuberculosis is of value because the current method of making a diagnosis is a clinical one, upon which the decision to treat is based. Formal bacteriological diagnosis takes several weeks at best, and it is desirable to start treatment as soon as possible after the presentation of the patient with tuberculosis.

Tuberculosis serology is an area of intensive research fraught with inadequacy in both sensitivity and specificity of the many tests that have been evaluated (Bothamley 1995, Pottumarthy *et al.* 2000). The usefulness of these tests can also be influenced by prior BCG vaccination (Turneer *et al.* 1988). The ELISA described here may be a suitable candidate for such a diagnostic test as on the sample here it appeared to be specific (although more work would be required to confirm this). For maximum usefulness the test would ideally need be made more sensitive as well. More modern serological tests using recombinant antigens have eliminated some, but by no means all, of the problems of false positives (Bothamley 1995, Julian *et al.* 2000). If an anti-POS ELISA could be confrimed to be specific (i.e. if a positive result made a diagnosis of clinical tuberculosis likely) further examination would be justified. The present results are at least consistent with a test of this nature, although the numbers involved are too small for any conclusions to be drawn. The potential usefulness of such a test gives sufficient reason for further investigation.

4.5.7 Conclusion

POS from *M. tuberculosis* H73Ra appear to have a greater affect upon B cells than T cells in culture, at least in some respects (chapter 5). The fact that this is so, coupled with the immunogenicity of POS to the humoral immune system in humans and rabbits, suggests that POS may represent a novel antigen of *M. tuberculosis* against which antibody responses are directed. Whether or not these antibody responses are protective, detrimental, or of diagnostic value remains to be determined, but the present results suggest this to be a potentially interesting topic within the still unresolved field of antibody immunity to tuberculosis.

Chapter 5

Immunological effects of mycobacterial phospho-oligosaccharide in human peripheral blood mononuclear cells

5.1 Introduction

The work described in the previous chapters suggests that mycobacterial POS has some properties and biological activity in common with mammalian IPG. Several mammalian cytokines and growth factors have been shown to signal using IPG as second messengers, and two immunologically important growth factors, insulin and IL-2, have been shown to cause GPI hydrolysis in T lymphocytes. For this reason the effects of mycobacterial POS on human immunocytes, and particularly T cells, is of interest, as the mycobacterial POS may have effects on such cells at the signalling level. In addition, as shown in chapter 4, mycobacterial POS are immunogenic. The aim of this chapter was to investigate the effects, if any, of mycobacterial POS on human peripheral blood mononuclear cells using measurements of proliferation and cell surface marker staining.

5.2 Materials

Tissue culture media and additives (penicillin/streptomycin and L-glutamine) were obtained from Gibco (Life Technologies, Paisley, UK). Tissue culture plasticware and ELISA plates were from Nunc (Life Technologies, Paisley, UK). Anti CD25-FITC, anti CD25-PE, anti CD3-PerCP and anti γδTCR-FITC monoclonal antibodies were obtained from Becton Dickinson (Cowley, Oxford, UK); anti CD30-FITC, anti CD19-FITC, anti CD19-PE, anti CD3-PE/Cy5 and HRPO-goat anti IgA were from Dako (Glostrup, Denmark). HRPO goat anti human Ig, IgM and IgG were obtained from Harlan Seralab (Loughborough, Leicestershire, UK). [^{3}H]-thymidine was obtained from Amersham Radiochemicals (Amersham, Buckinghamshire, UK). Ficoll hypaque (Lymphoprep) was from Nycomed. Sterile, preservative free sodium heparin 1000U/ml (Pump-Hep) was obtained from Leo Laboratories (Princes Risborough, Buckinghamshire, UK). PPD was obtained from Evans Medical Ltd. (Leatherhead, UK). Salts for PBS (AnalaR grade) were obtained from BDH (Poole, Dorset, UK) and all other chemicals were from Sigma (Poole, Dorset, UK).

Mycobacterial POS material used in the experiments presented in this chapter was purified according to the method described in chapter 3. Only one batch of POS from each species studied was used for all the *in vitro* immunological experiments.

5.3 Methods

5.3.1 PBMC separation

Human peripheral blood mononuclear cells (PBMCs) were separated from the blood of healthy male donors. 20ml of venous blood was drawn into a container with approximately 5U/ml sterile, preservative free sodium heparin. Blood was centrifuged at 2200rpm for eight minutes and the plasma was withdrawn. If the plasma was to be used in the experiment, it was further centrifuged at 4000rpm for eight minutes to remove any residual platelets. The cellular fraction was diluted with culture medium (RPMI 1640 containing 20mM HEPES, 2mM L-glutamine, 50U/ml penicillin, 50μg/ml streptomycin and 50μM 2-mercaptoethanol) to twice the original blood volume (typically 40ml) and layered over ficoll hypaque. Blood/ficoll was centrifuged at 1700rpm for 28 minutes after which the PBMC layer at the ficoll/RPMI interface was carefully removed using a pastette. Separated PBMCs were washed twice in complete culture medium containing at least 5% donor plasma, centrifuged at 1000rpm for ten minutes and counted using an improved Neubauer haemocytometer. For subsequent cell culture, cells were plated at $2.5x10^5$ cells per well in round bottomed 96 well plates in complete culture medium (see above) containing 10% donor plasma or in AIM V serum free lymphocyte medium, as indicated in the text. Cells were cultured with mycobacterial POS, phytohaemaglutinin-P (PHA-P) or purified protein derivative (PPD) at the concentrations indicated in the text.

5.3.2 [^{3}H]-thymidine uptake assays

PBMCs were cultured for the measurement of proliferation by [^{3}H]-thymidine uptake in 96 well plates for five days after which [^{3}H]-thymidine was added at a concentration of 0.5μCi per well. 18 hours later the cells were harvested onto glass wool paper, dried and transferred to vials for liquid scintillation counting using a 1211 Rackbeta liquid scintillation counter, LKB Pharmacia (Wolverton, UK). Results from [^{3}H]-thymidine uptake assays are expressed either as counts per minute or as stimulation index, where the stimulation index represents the fold increase in [^{3}H]-thymidine uptake over the negative control.

5.3.3 Flow cytometry

For the assessment of cell surface marker expression non adherent PBMCs were withdrawn from culture wells by pipetting vigorously up and down and washed once in staining buffer (PBS, 1% BSA, 0.1% azide). Cells were resuspended in 50µl aliquots and stained with 10µl of anti CD25-FITC, anti CD25-PE, or anti γδTCR-FITC and 8µl of anti CD30-FITC, anti CD19-FITC anti CD19-PE or anti CD3-PE/Cy5. Staining combinations were one of the following:

Anti CD25-FITC, anti CD19-PE, anti CD3-PE/Cy5.

Anti CD30-FITC, anti CD25-PE, anti CD3-PE/Cy5.

Anti γδTCR-FITC.

Compensation tubes with only one stain in each were included in each experiment. Cells were incubated with the stains indicated in the text for 30 minutes at 4°C in the dark, washed twice in staining buffer and centrifuged at 1500 rpm for five minutes. Stained cells were fixed by resuspending in 300µl PBS 1% paraformaldehyde and incubating for ten minutes at 4°C in the dark. Analysis was performed on a FACScalibur™ flow cytometer (Becton Dickinson, Erembodegem-Aalst, Denmark). For analysis the lymphocytes were gated on the basis of forward and side scatter (size and granularity). The cells within this gate were then subject to compensated quadrant analysis according to the respective stains that were included in each experiment.

5.4 Results

5.4.1 Effect of mycobacterial phospho-oligosaccharide on proliferation of human PBMCs in culture

Phospho-oligosaccharide from *M. vaccae* R877R

Proliferation of human PBMCs in culture was measured by [^{3}H]-thymidine uptake in the presence of POS from *M. vaccae* R877R. Pilot experiments Showed that POS from *M. vaccae* R877R were able to induce proliferation in human PBMCs but that the level of proliferation was low (data not shown). Culture of human PBMCs using AIM V serum free lymphocyte medium and round bottomed tissue culture wells has previously been reported to be suitable for detecting immune responses where the level of antigen induced

proliferation is low (Bjorksten *et al.* 1996). This method was applied to the present experiments for the detection of small proliferative responses to mycobacterial POS. The use of AIM V medium confirmed results obtained in the pilot experiments (performed using RPMI), *M. vaccae* R877R POS did induce proliferation of PBMCs. Representative results are shown in figure 5.1. Significant proliferation was observed, maximal proliferation being a stimulation index of 3.9 for POS-A and 3.4 for POS-P. The general proliferative effect of POS from *M. vaccae* R877R was reproducible: figure 5.2a shows a replica experiment. However, the pattern of the response was variable, as comparison of figure 5.1 with figure 5.2a shows. This is likely to represent variation in the responses of fresh human PBMCs harvested on different days. Figure 5.1 and figure 5.2a show the same bell shaped dose response curve, except that on the repeat occasion, figure 5.2a, this curve has shifted to the left and the level of proliferation induced is lower (maximal proliferation was stimulation index 2.3 for POS-A and 2.9 for POS-P). Reduced [^{3}H]-thymidine uptake byPBMCs at higher POS concentrations may represent increased stimulation resulting in exhaustion of the medium and death of the cells, or it may be due to direct toxicity of POS at high doses. A similar effect has previously been observed in other systems (see chapters 2 and 3). It appears that the optimal concentration for PBMC proliferation was variable between cells harvested on different occasions.

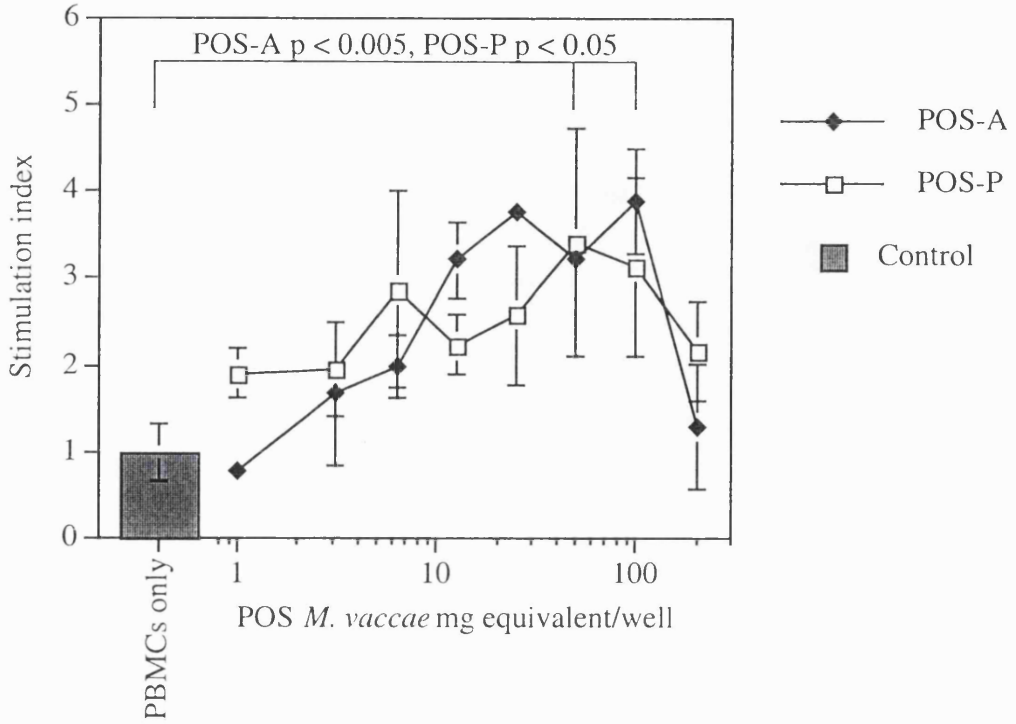


Figure 5.1. Effect of POS from *M. vaccae* R877R on proliferation of human peripheral blood mononuclear cells in culture. Human PBMCs (2 x 10^5 cells per well) were cultured for six days in AIM V medium with the indicated amounts of POS. The cultures were pulsed for the final 18 hours with 0.5μCi per well [^{3}H]-thymidine after which cells were harvested for liquid scintillation counting. POS-A (filled diamonds) and POS-P (unfilled squares) both induced a low but significant level of proliferation at this time point compared with the blank data value, cells in AIM V tissue culture medium only (values where $p < 0.005$ for POS-A and $p < 0.05$ by student's t-test for POS-P are indicated). Values are shown as stimulation index where the PBMC only CPM are assigned a value of 1 and are the mean of triplicate assays +/- one standard deviation of the mean. *M. vaccae* R877R POS fractions were active in a dose dependent manner, although the stimulation index goes down with high doses of POS, possibly representing overstimulation. POS fractions are expressed as the mass of organisms from which the amount of POS in each well is derived in mg.

Effect of adherent PBMC depletion

Interestingly, the proliferative effect of POS from *M. vaccae* R877R seen in figures 5.1 and 5.2a was completely inhibited by prior depletion of adherent PBMCs by pre-culture overnight in a tissue culture flask in the presence of donor plasma (figure 5.2b). This result indicates that POS may not be acting upon lymphocytes directly (the component of PBMCs that are able to proliferate) but rather are acting through, or at least are dependent upon the presence of adherent cells. Although the exact identity of the adherent cells required remains unknown, these cells are likely to be either macrophages, dendritic cells or both. In each case, these cells may be involved in antigen presentation.

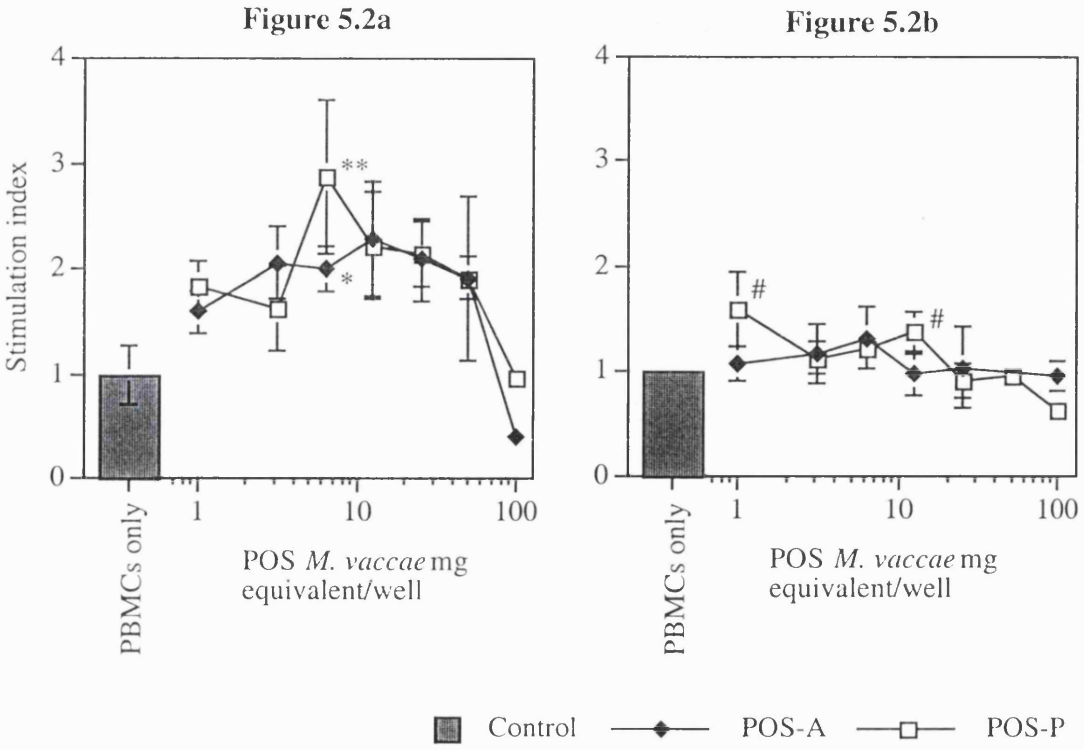


Figure 5.2. Effect of POS from *M. vaccae* R877R on proliferation of whole and non-adherent human peripheral blood mononuclear cells in culture. Whole, fresh human PBMCs (2 x 10^5 cells per well) were cultured at in an experiment identical to that represented in figure 5.1 (figure 5.2a). In parallel, PBMCs (2 x 10^5 cells per well) that had previously been incubated overnight in the presence of donor plasma to allow depletion of the adherent cells were cultured with identical POS doses (figure 5.2b). Both sets of cells were cultured for six days, and for the final 18 hours 0.5μCi per well [^{3}H]-thymidine was added. Values shown are stimulation index (fold increase in proliferation over the negative control) calculated from counts per minute after liquid scintillation counting where the negative control (PBMCs only) is assigned a value of 1. Data are the mean stimulation index of triplicate assays +/- one standard deviation of the mean. POS-A (filled diamonds) and POS-P (unfilled squares) both induced a low level of proliferation of whole PBMCS compared with blank (AIM V medium only), some significant values are indicated (* = $p < 0.01$, ** = $p < 0.025$, student's t-test). Depletion of adherent cells abolished this effect (figure 5.2b), although some of the points gave values significantly greater than the negative control (# = $p < 0.05$, student's t-test). *M. vaccae* R877R POS fractions are expressed as the mass of organisms from which the amount of POS in each well is derived in mg.

Effect of phospho-oligosaccharide from *M. vaccae* R877R on the PBMC response to PHA-P

The effect of co-culture of POS from *M. vaccae* R877R with the lymphocyte mitogen PHA-P on proliferation of human PBMCs was investigated. PBMC proliferation in response to a fixed dose of PHA-P was greater in the presence of POS. This assay was only carried out in the presence of two concentrations of POS, but from only these data points the effect was dose dependent at least for POS-P (figure 5.3), although this effect appeared to be additive, rather than synergistic. The presence of POS from *M. vaccae* R877R enhanced the proliferation of PBMCs in response to PHA-P at high doses, shown in figure 5.4. In this experiment the effect of POS also appears to be additive, rather than synergistic.

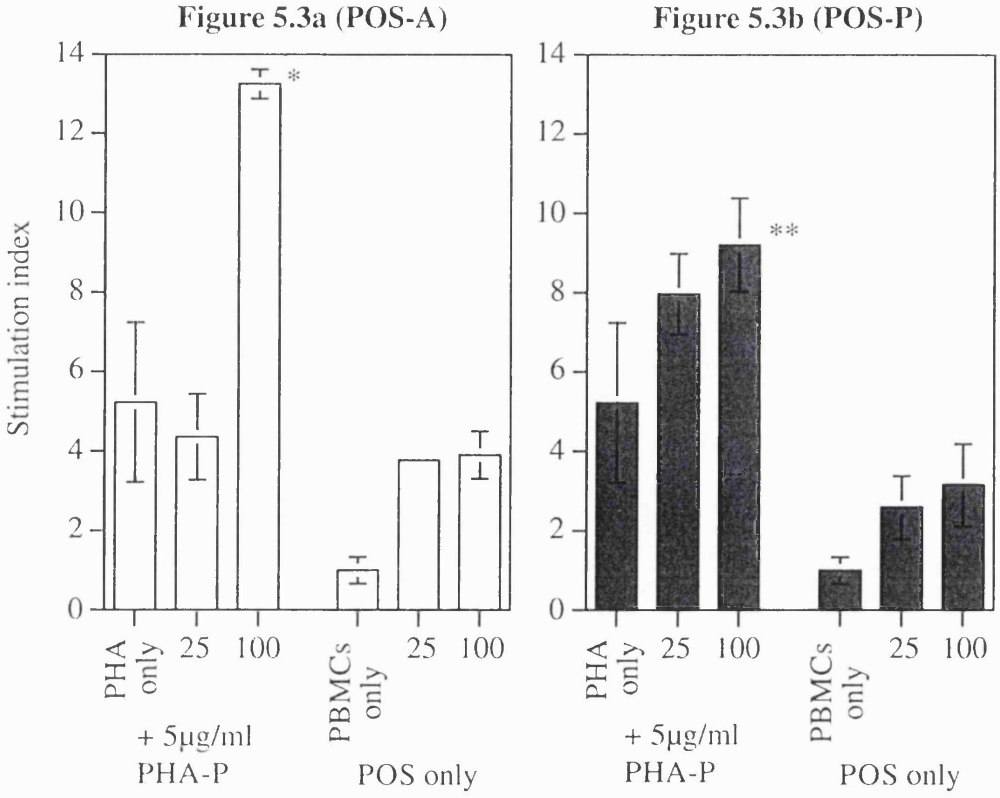


Figure 5.3. Effect of POS from *M. vaccae* R877R on proliferation of human peripheral blood mononuclear cells in the presence of PHA-P. Human PBMCs (2 x10^5 cells per well) were cultured for six days in AIM V medium in the presence and absence of PHA-P (5µg/ml) with POS from *M. vaccae* R877R at the concentrations indicated. PBMCs were pulsed with 0.5µCi per well [^{3}H]-thymidine for the final 18 hours of culture and harvested for liquid scintillation counting. Values shown are stimulation index (fold increase in proliferation over the negative control) calculated from counts per minute after liquid scintillation counting where the negative control (PBMCs only) is assigned a value of 1. Data are the mean of triplicate assays +/- one standard deviation of the mean. POS-A (figure 5.3a) and POS-P (figure 5.3b) both significantly enhanced the proliferative effect of 5µg/ml PHA-P at 100mg equivalent/well (* = $p < 0.005$, ** = $p < 0.05$, compared with respective negative controls, student's t-test). *M. vaccae* R877R POS fractions are expressed as the mass of organisms from which the amount of POS in each well is derived in mg.

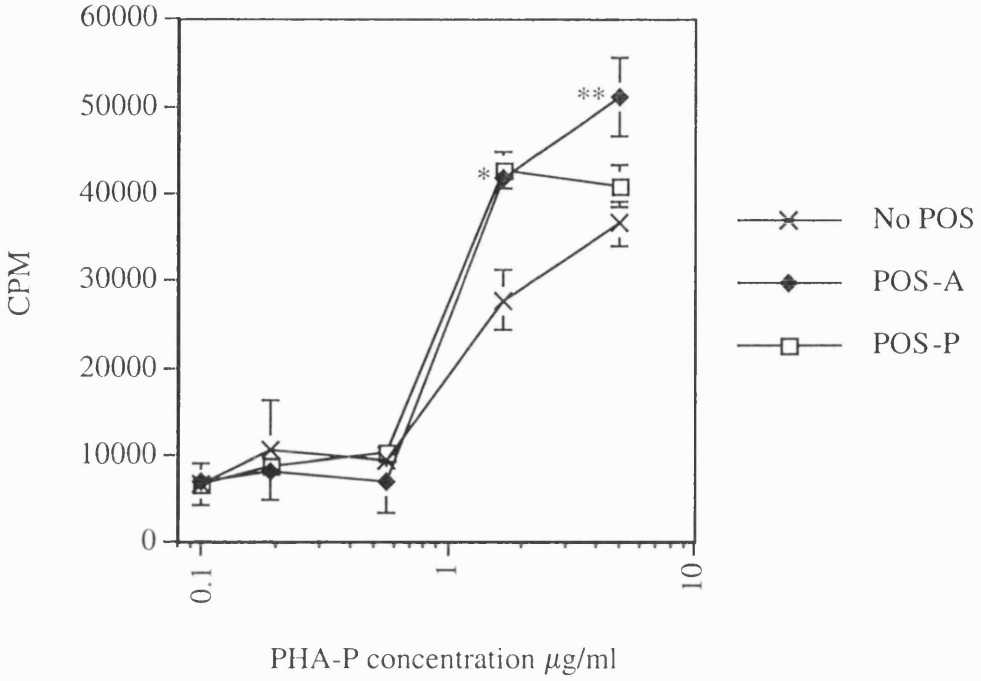


Figure 5.4. Effect of POS from *M. vaccae* R877R on the dose response curve of the proliferation of human peripheral blood mononuclear cells to PHA-P. Human PBMCs (2 x10^5 cells per well) were cultured for six days in the presence of PHA-P alone (crosses), with 100mg/well organism mass equivalent of POS-P (open squares) or POS-A (filled diamonds). PBMCs were pulsed with 0.5μCi per well [^{3}H]-thymidine for the final 18 hours of culture and harvested for liquid scintillation counting. Both POS fractions significantly enhanced the proliferation of PBMCs at 1.67μg/ml PHA-P (* = $p < 0.005$, student's t-test) and POS-A enhanced proliferation at 5μg/ml PHA-P (** = $p < 0.01$, student's t-test). POS-P no longer had a significant effect at 5μg/ml PHA-P. Values are shown as counts per minute (CPM) and are the mean of triplicate assays +/- one standard deviation of the mean.

Phospho-oligosaccharide from *M. tuberculosis* H37Ra

POS from *M. tuberculosis* H37Ra induced an increase in proliferation of 4.7 fold for POS-A and 5.6 fold for POS-P in human PBMCs over the negative control (figure 5.5). High doses of POS gave lower levels of [^{3}H]-thymidine uptake, as was observed in the case of POS from *M vaccae* R877R. The experiment represented in figure 5.5 was carried out in RPMI and donor plasma, not AIM V, showing no great advantage of the AIM V system over RPMI/donor plasma using POS from *M. tuberculosis* H37Ra in this assay. Overall, the effect of POS from *M. tuberculosis* H37Ra was slightly greater than that of POS from *M. vaccae.*

In order to mimic a more physiological system as would be in the course of an infection, 50μg/ml sonicate antigen from *M. tuberculosis* H37Ra was included in an identical set of culture wells to assess the effect of POS on PBMCs that were also responding to other mycobacterial antigens. The overall level of proliferation exhibited in this experiment when expressed as counts per minute (CPM), was greater than the proliferation of PBMCs in the absence of sonicate antigen, as would be expected, but in the presence of sonicate antigen POS no longer had any effect upon the proliferation of PBMCs. The reason for the abrogation of the POS response in the presence of other mycobacterial antigen is not clear. Expressed in CPM the highest level of proliferation in the cells incubated with POS alone was actually greater than the highest level of proliferation displayed by cells co-cultured with sonicate antigen. This suggests that some component of *M. tuberculosis* H37Ra sonicate antigen may to some degree prevent the full proliferative response to purified mycobacterial POS.

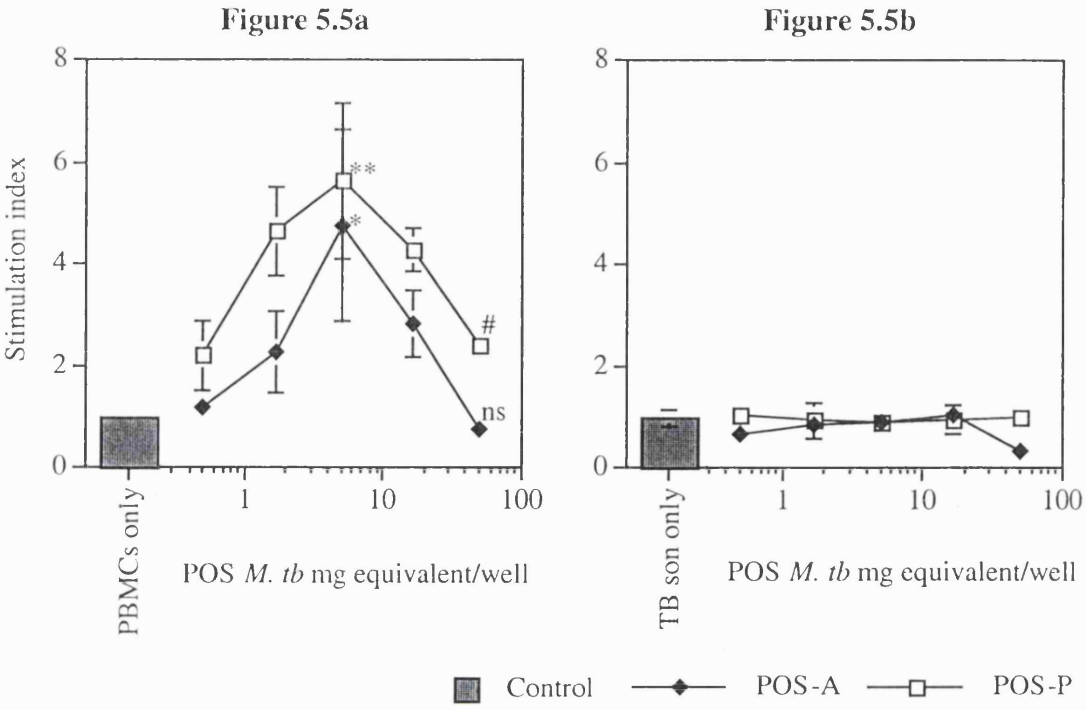


Figure 5.5. Effect of POS from *M. tuberculosis* H37Ra on the proliferation of human peripheral blood mononuclear cells in culture. Human PBMCs (2 x 10^5 cells per well) were cultured for six days in RPMI 10% donor plasma with POS from *M. tuberculosis* H37Ra (figure 5.5a) or with POS and sonicate antigen from *M. tuberculosis* H37Ra (figure 5.5b). PBMCs were pulsed with 0.5μCi per well [^{3}H]-thymidine for the final 18 hours of culture and harvested for liquid scintillation counting. Values shown are stimulation index (fold increase in proliferation over the negative control) calculated from counts per minute after liquid scintillation counting where the negative control (PBMCs only) is assigned a value of 1. Data are the mean stimulation index of triplicate assays +/- one standard deviation of the mean. *M. tuberculosis* H37Ra POS fractions caused significant proliferation at 5mg equivalent/well (* = $p < 0.05$, ** = $p < 0.01$, student's t-test). At the highest dose of POS proliferation in response to POS-P was still significant (*** = $p < 0.005$, student's t-test) but proliferation in response to POS-A was not (ns = not significant). In the presence of sonicate antigen from *M. tuberculosis* proliferation in response to POS was never significantly greater than the negative control (sonicate antigen only). *M. tuberculosis* H37Ra POS fractions are expressed as the mass of organisms from which the amount of POS in each well is derived in mg.

5.4.2 Phospho-oligosaccharide from M. tuberculosis H37Ra is not a gamma delta T cell antigen

M. tuberculosis is known to contain several low molecular weight antigens that contain phosphate and selectively stimulate T cells that bear the γδ T cell receptor rather than the more common αβ T cell receptor, for example the TUB antigens and isoprenyl pyrophosphate (IPP) (Constant *et al.* 1994, Tanaka *et al.* 1995). Moreover, a phosphoantigen that is carbohydrate in nature has been described (Schoel *et al.* 1994). These antigens alone are not sufficient to cause γδ T cell expansion, external help is required, conventionally provided by adding IL-2 or IL-15 to the cell cultures (Wesch *et al.* 1997). Alternatively these experiments may be performed using whole PBMCs and taking advantage of the presence of αβ T cells by adding another antigen that stimulates αβ T cell IL-2 production, thus providing the help required. Any selective effect on γδ T cells may then be examined by selectively staining for the γδ TCR for flow cytometry.

As POS are known to contain phosphate (except the *M. tuberculosis* H37Ra POS-P fraction), the effect of POS from *M. tuberculosis* H37Ra upon γδ T cell numbers was examined by flow cytometry. Material derived from *M. tuberculosis* H37Ra was suitable because the POS-A fraction contains phosphate whereas the POS-P fraction does not (chapter 3), allowing a potential comparison to be drawn between a phosphate containing fraction and a fraction that does not contain phosphate. Purified protein derivative (PPD) of *M. tuberculosis* was added to stimulate αβ T cell production of IL-2. POS produced a very modest increase in the percentage of cells bearing the γδ T cell receptor compared with PPD alone (figure 5.6). This result itself is of doubtful significance as the assay was performed in only single data points (due to lack of available POS material), however this percentage of γδ T cells is far less than that reported in the literature for other known γδ T cell antigens (Pfeffer *et al.* 1992, Garcia *et al.* 1998), so this result is likely to indicate that POS do not function as a γδ T cell antigen and these experiments were not pursued any further. POS from *M. tuberculosis* H37Rv was not examined due to shortage of this material.

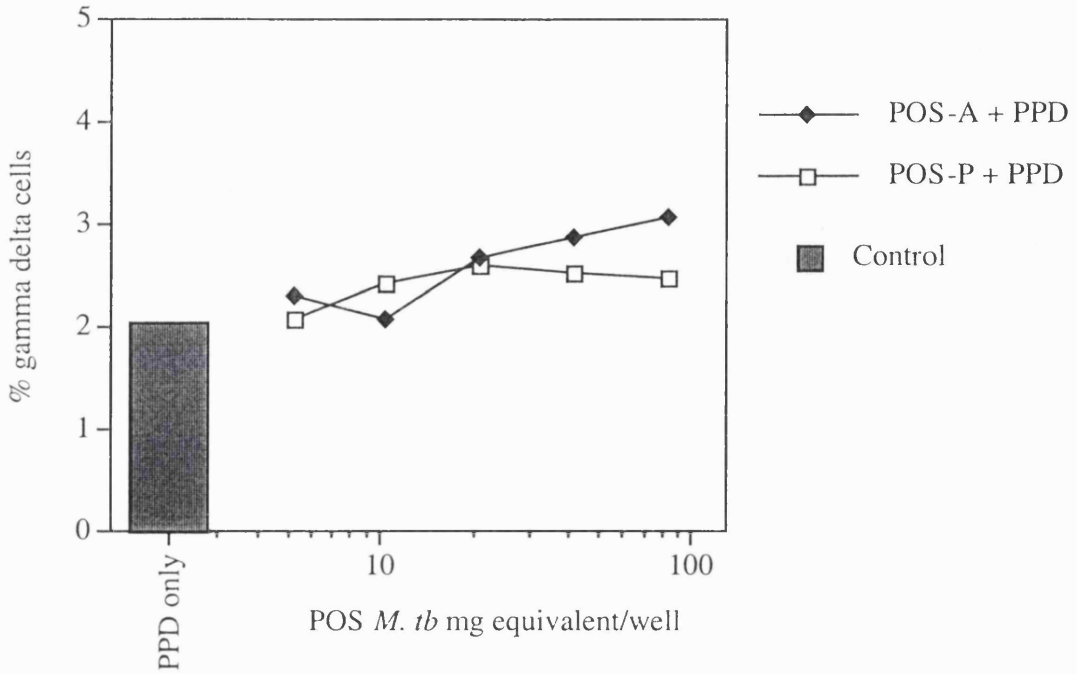


Figure 5.6. POS from *M. tuberculosis* H37Ra does not stimulate gamma delta T cell proliferation. Human PBMCs (2 x 10^5 per well) were cultured for six days in RPMI 10% donor plasma in the presence of 50μg/ml PPD, with and without POS from *M. tuberculosis* H37Ra. Cells stained for the gamma delta T cell receptor chains and analysed by flow cytometry. Data values shown are percentages of total cells in the lymphocyte gate that are gamma delta positive. Neither POS fraction increased the percentage of gamma delta cells significantly above the control value. POS fractions are expressed as the mass of organisms from which the amount of POS in each well is derived in mg.

5.4.3 Effect of phospho-oligosaccharide from M. tuberculosis on B and T lymphocyte populations by cell surface marker expression

Mycobacterial POS induce modest proliferation of human PBMCs, but it was not established which cells were being activated. In order to determine this, the expression of a pan-lymphocytic activation marker, CD25, and a T cell activation marker, CD30 following culture of PBMCs with POS from *M. tuberculosis* H37Ra were examined by flow cytometry. PBMCs were also stained for the T cell marker CD3 and, in later experiments, the B cell marker CD19. The T cell compartment was initially singled out for further study because of the known importance of the T cell response to *M. tuberculosis* in the outcome of infection in both murine models and humans (Rook and Hernandez-Pando 1996, Flynn and Chan 2001). CD25 was selected as a marker of activation partly because it is known to become upregulated on some T cells after stimulation with non-peptide antigen from *M. tuberculosis* (Wesch *et al.* 1997).

POS from *M. tuberculosis* H37Ra had no effect on CD30 expression in human PBMCs (data not shown). There was a modest (approximately 1.5 fold) but reproducible increase in the CD3/CD25 double positive population at the highest dose of POS (figure 5.7a). Total PBMC CD25 expression also showed a very similar increase at the higher doses of POS (data not shown). This was not surprising as T cells (CD3 positive cells) account for most of the cells in the culture, comprising approximately 80% of human PBMCs. The CD25 positive, CD3 negative population, however, (which is much smaller to begin with) was strikingly increased, almost threefold at high POS dose, in a dose dependent manner with mycobacterial POS (figure 5.7b). This effect was swamped when the data were plotted including all the cells in the culture because the CD3 positive population so greatly outnumbered the CD3 negative population.

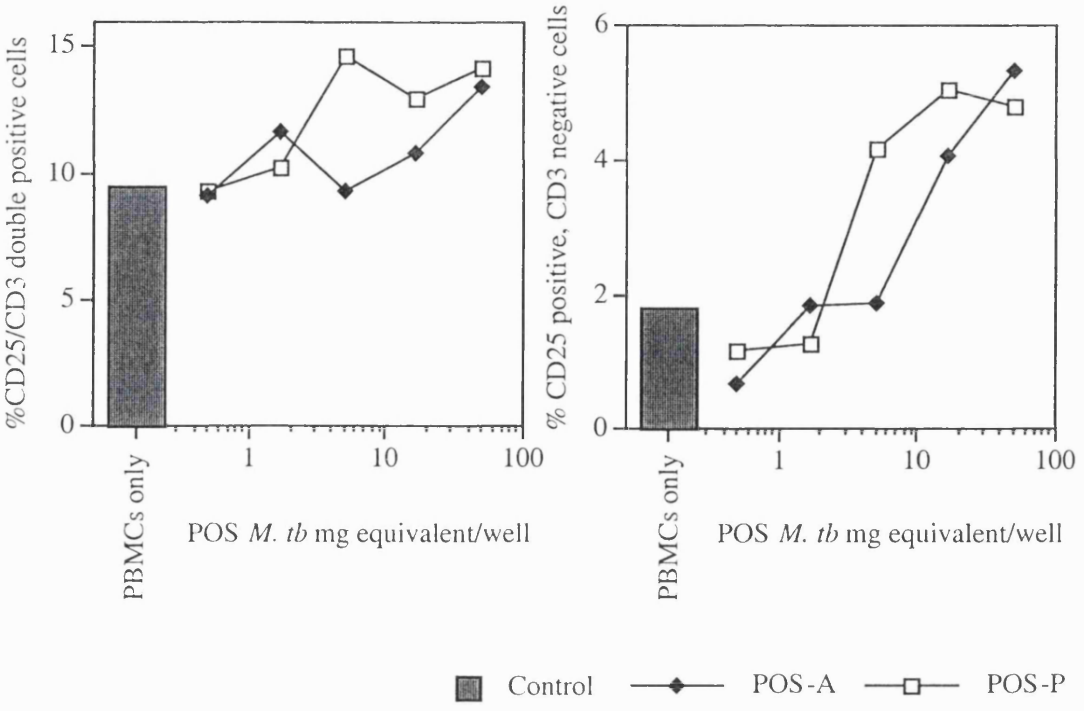


Figure 5.7. POS from *M. tuberculosis* H37Ra induce an increase in CD25 expression on human peripheral blood lymphocytes in culture. Human PBMCs (2 x 10^5 cells per well) were cultured for six days in RPMI 10% donor plasma with POS from *M. tuberculosis* H37Ra. Following culture cells were harvested, stained for CD3 and CD25 and analysed by flow cytometry. Data plotted represent percentages of cells after quadrant analysis. Figure 5.7a, percentage of cells that are CD3/CD25 double positive; figure 5.7b, percentage of cells that are CD3 negative and CD25 positive. Both POS fractions produced an increase in CD25 expression compared with control (culture medium only), this effect was greater within the population of cells that were CD3 negative. POS fractions are expressed as the mass of organisms from which the amount of POS in each well is derived in mg.

Cells were gated for analysis by forward and side scatter to select the lymphocytes, making it likely that the CD3 negative cell population represents the B cell population. Therefore these data suggested that mycobacterial POS affected B cell CD25 expression more than T cell CD25 expression. To confirm this observation and exclude the presence of other small cell populations such as NK cells these experiments were repeated with the addition of a B cell marker stain for flow cytometry, CD19.

POS from *M. tuberculosis* H37Ra caused a dose dependent increase in the proportion of CD25 positive B cells. Figure 5.8a shows a representative experiment. The data are represented as the percentage of all B cells that are CD25 positive; this number is derived in the following way:

> The percentage of CD19 positive cells and CD19/CD25 double positive cells was determined by quadrant analysis of the gated lymphocytes.
> The sum was performed: (%$CD19^{+}$ CD25 double positive÷%$CD19^{+}$) x 100 to give the percentage of all B cells (CD19 positive) that were also CD25 positive.

The percentage of T cells that express CD25 was derived in the same way (figure 5.8b). Proportionally, the increase in T cell CD25 expression was not as great as that of B cells.

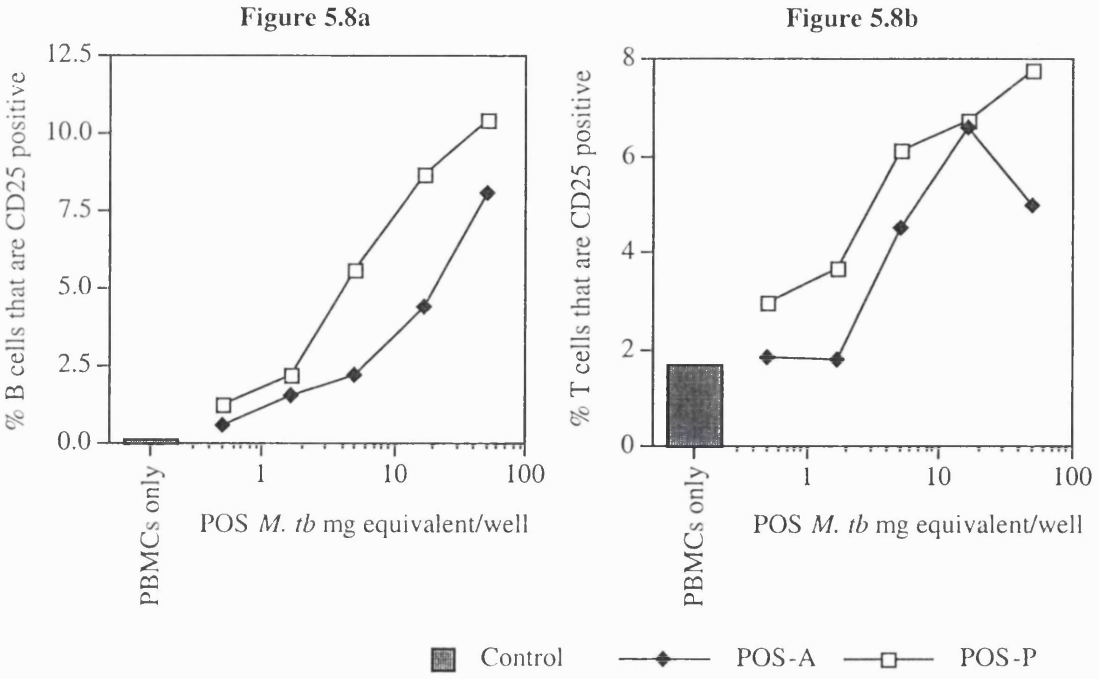


Figure 5.8. Effect of POS from *M. tuberculosis* H37Ra onCD25 expression on human lymphocytes in culture. Human PBMCs (2 x 10^5 cells per well) were cultured for six days in RPMI 10% donor plasma with POS from *M. tuberculosis* H37Ra. Following culture cells were harvested, stained for CD3, CD19 and CD25 and analysed by flow cytometry. Figure 5.8a represents the percentage of B cells that express CD25 and figure 5.8b represents the percentage of T cells that express CD25, derived as described in the text. Both POS fractions produced a dose dependent increase in lymphocyte CD25 expression compared with control (culture medium only), the effect appears to be more pronounced for B cells than for T cells. POS fractions are expressed as the mass of organisms from which the amount of POS in each well is derived in mg.

Expressed in this way the same effect as observed figure 5.7 is seen. If the data are expressed as unmodified quadrant statistics, that is the percentage of CD19/CD25 double positive cells within the gate, again they show exactly the same trend (data not shown). This experiment was repeated a further time. Each repeat gave different results in numerical terms, but in every case the dose dependent effect upon B cell CD25 expression was observed and was qualitatively the same. Moreover the degree of activation of the B cell compartment (expressed as the percentage of all B cells bearing the CD25 surface marker) was always greater than that of the T cell compartment (figure 5.8b). Although an

increase in CD25 expression is also seen on T cells cultured with mycobacterial POS, the relative increase over background is much greater on B cells. The overall relative proportions of B and T cells were not changed (figure 5.9).

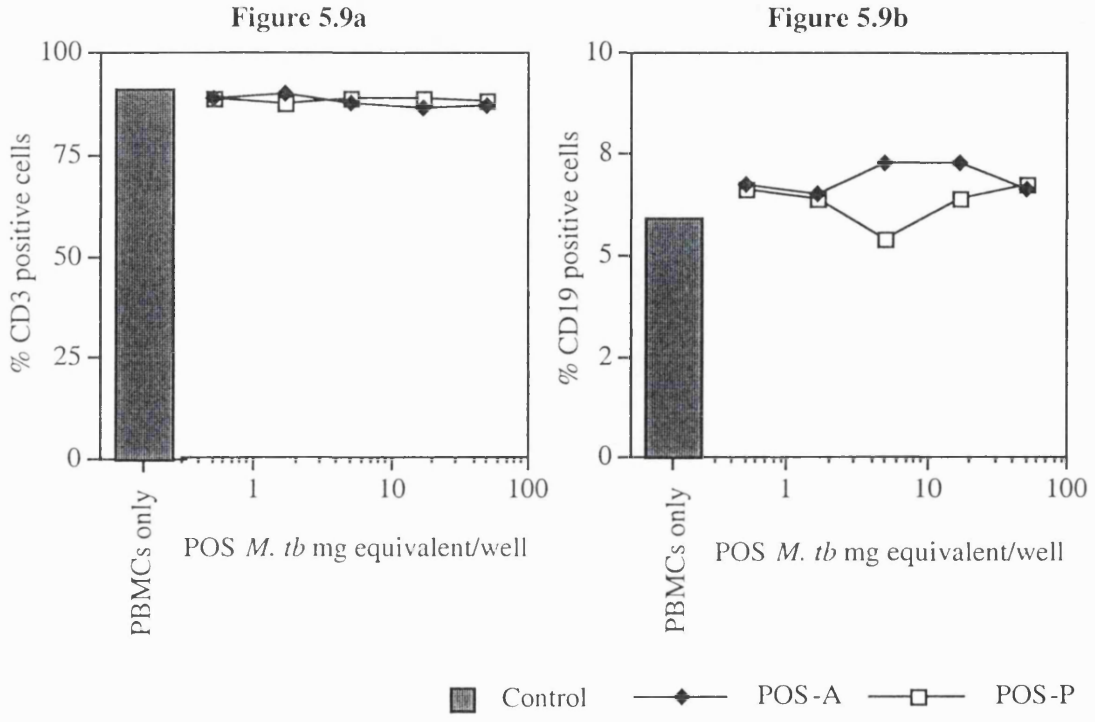


Figure 5.9. POS from *M. tuberculosis* H37Ra have no effect on the overall proportions of human B and T cells derived from peripheral blood in culture. Human PBMCs (2 x 10^5 per well) were cultured for six days in RPMI 10% donor plasma with POS from *M. tuberculosis* H37Ra. Following culture cells were harvested and stained for CD3, CD19 (and CD25, not represented here) and analysed by flow cytometry. Data values plotted are percentages of cells after quadrant analysis. The data shown are from the same experiment as figure H.2.3.2. Figure 5.9a, percentage of total cells that are T cells (CD3 positive); figure 5.9b, percentage of total cells that are B cells (CD19 positive). Neither POS fraction produced any effect on the proportion of overall lymphocyte populations, expressed as percentage of total cells in the lymphocyte gate. POS fractions are expressed as the mass of organisms from which the amount of POS in each well is derived in mg.

5.5 Discussion

5.5.1 Proliferogenic effects of mycobacterial phospho-oligosaccharide

POS from *M. vaccae* R877R and *M. tuberculosis* H37Ra were able to induce proliferation of human PBMCs as measured by [^{3}H]-thymidine uptake (figures 5.1 and 5.5). Although derived from two separate experiments and not from the same occasion, from the available evidence expansion of both T and B cell subsets after culture with POS from *M. tuberculosis* H37Ra occurred in equal proportions. The relative percentages of B and T cells present after six days in culture at least with POS from *M. tuberculosis* H37Ra were the same as control cells (figure 5.9).

Mycobacterial POS may cause this effect through antigen specific receptors, by acting as a T independent type 1 antigen for B cells, or by acting as a growth factor mimetic as the mammalian mediators do. The antigenicity of mycobacterial POS is discussed below. Addition of exogenous mammalian IPG containing medium has been shown to reduce the EC_{50} of IL-2 on a lymphocyte cell line (Eardley and Koshland 1991). Although POS from *M. vaccae* R877R enhanced [^{3}H]-thymidine uptake in response to PHA-P (figures 5.3 and 5.4) this effect did not show a true leftward shift in the PHA-P dose response curve, rather the effect appeared merely to be additive. Moreover in order to show growth factor mimetic (or second messenger like) activity it would be necessary to shown reduction of the EC_{50} of a particular growth factor rather than a non-specific mitogen. There is currently insufficient evidence to firmly conclude that mycobacterial POS are acting as a second messenger homologue in the tissue culture systems used here. Nevertheless mycobacterial POS are clearly having a proliferogenic effect and the evidence that POS are behaving as an antigen in the classical sense is also not entirely conclusive (see below). The similarity in activity, and the fact that some POS fractions from *M. tuberculosis* have the opposing effects to corresponding mammalian column fractions (chapter 1), indicates that there is still much to be learned about the precise activity of this material in lymphocytes.

5.5.2 Antigenicity of mycobacterial phospho-oligosaccharide

In order to demonstrate mycobacterial POS acting as an antigen recognised by lymphocytes it would be necessary to show that it acts through a specific antigen receptor of the adaptive immune system. This is difficult to do experimentally, especially when there is the suggestion that the mycobacterial POS material may act as a growth factor mimetic and cause proliferation in a pharmacological way.

T cell responses

Indirect evidence that adaptive immunoreceptor recognition is taking place comes from depletion of adherent cells, which abolished [^{3}H]-thymidine uptake in response to POS from *M. vaccae* (figure 5.2). The adherent cell fraction of human PBMCs includes the majority of specialised antigen presenting cells present in PBMC culture that are involved in presentation to T cells *via* MHC class II and other co-stimulatory molecules. This suggests that antigen presentation, and therefore T cell receptor recognition, is necessary for this response, although this would be an unusual type of antigen to be presented in this way.

There is, however, an alternative explaination for this result. Macrophages (which are adherent) express a variety of receptors for bacterial carbohydrates that stimulate the release to cytokines and other inflammatory mediators. POS from *M. tuberculosis* have been shown to cause release of IL-1β and NO but not TNFα from J774 murine monocyte/macrophages (Dr. Kiajoo Puan, PhD thesis 1999). IL-1 can contribute to lymphocyte activation, possibly giving the response seen here. T cells are the predominant cell type in PBMCs so any effect upon them will have a greater impact on the amount of radioactivity counted after a [^{3}H]-thymidine uptake assay, although this would apply equally to the antigen presentation argument outlined above. Alternatively another, as yet unknown, soluble or cell associated factor dependent upon the presence of macrophages or other adherent cells may be required for this response.

The latter explanation is favoured on the basis of the current evidence because recognition of a carbohydrate structure by an innate macrophage receptor and not by an adaptive T cell receptor is more likely. There are T cell populations that recognise unusual non-peptide antigens but one of the major subsets of such cells, γδ receptor bearing cells, were shown not to be affected by the presence of POS from *M. tuberculosis* H37Ra (figure 5.6).

Although the existence of other T cell populations that might be recognising POS through specific adaptive receptors has not been formally excluded, such a cell population would be a small fraction of PBMCs and so would be unlikely to greatly impact the [^{3}H]-thymidine uptake of such cultures.

In a thesis that ran in parallel to the current work, the effect of POS upon the J774 monocyte/macrophage cell line was elucidated in more detail. POS induced NO release by a pathway that was CD14 independent. The receptor for POS was not formally identified, but a good candidate is the Toll like receptor (TLR) family. It has already been established that *M. tuberculosis* contains a heat stable, protease resistant factor that acts *via* the TLR family member TLR2 (Means *et al.* 1999b). These properties are entirely compatible with those of mycobacterial POS. This is discussed in more detail in the next chapter.

B cell responses

The most convincing evidence that mycobacterial POS are immunogenic in humans comes from the demonstration that TB patients have anti POS immunoglobulins in their sera. This has been discussed in chapter 4, but may be due to the immunogenicity of some larger precursor molecule, maybe a glycolipid, not POS as such. The same may apply to the antibody responses mounted by rabbits to CFA.

In vitro, POS from *M. tuberculosis* H37Ra had a more profound effect upon B cells than T cells in terms of CD25 expression, which reflects cellular activation. B cell proliferation was no greater then T cell proliferation in as far as it was examined (figure 5.9), but there may be other more appropriate measurements of B cell mediated immunity. An attempt to raise antibodies to mycobacterial POS *in vitro* was successful on one occasion but was found not to be reproducible so has not been included. It may be that the apparent presence of anti POS Ig observed on one occasion was due to polyclonal activation of B cells, not specific antigen receptor mediated interactions. *In vitro* immunisation with the postulated precursor of mycobacterial POS, the glycolipid described in chapter 2, was not attempted due to lack of available material but might reveal interesting information in the light of the data presented here.

5.5.3 Mycobacterial phospho-oligosaccharide and polyclonal lymphocyte activation

Although antibodies against mycobacterial POS have been found in both rabbits and humans, *in vitro* it has not been possible to formally demonstrate recognition of mycobacterial POS as an antigen. Some experiments were performed in the presence of other mycobacterial antigens derived from sonicated *M. tuberculosis*, in an attempt to more closely mimic the situation of a real infection. In the case of [^{3}H]-thymidine uptake the data suggest that the effect of POS from *M. tuberculosis* H37Ra was no longer discernable in the presence of other antigens. The data for CD25 expression have not been included, as they did not give entirely reproducible results, but they also showed the same general pattern, that the effect of POS is swamped by the presence of a mixture of other antigens.

In the absence of firm evidence that POS from mycobacteria are acting as a growth factor mimetic or are being specifically recognised as an antigen the most reasonable conclusion to draw from the available data is that POS are functioning as a polyclonal lymphocyte activator. The polyclonal activation, certainly in the case of [^{3}H]-thymidine uptake induced by *M. vaccae* POS, is also dependent upon the presence of some factor associated with, or secreted from, adherent cells. Polyclonal activation, therefore, is not the only property of this material. Bacterial LPS, another material that acts as a lymphocyte (B cell) polyclonal activator, shows similarity in that it too acts on macrophages (adherent cells) to cause cytokine release in addition to B cell mitogenic activity. By drawing a parallel with LPS, therefore, it may be seen that mycobacterial POS may have more than one distinct activity *in vitro*. However, unlike LPS, for the proliferogenic effects of mycobacterial POS to occur both effects may need to be taking place at the same time. The question of whether or not there are two effects taking place could be answered by identifying and then adding back the adherent cell derived factor to adherent cell depleted PBMCs with and without mycobacterial POS.

This explanation is also consistent with other data that have been discussed but not presented here. The addition of other antigens of *M. tuberculosis* along with POS for flow cytometry studies did not, in general, give reliable results. Certainly interpretation of the results of such experiments is difficult as there may be many effects occurring. However all of the results obtained are at least consistent with this hypothesis. The "masking" of

proliferative responses and of CD25 expression by the addition of other antigens suggests that POS are not potent polyclonal activators of human lymphocytes, although the meaning of "potent" remains to be defined as there is currently no way of expressing POS as a molar concentration, allowing comparison with other material. PBMCs may become unresponsive to polyclonal stimulation by POS when co-cultured with other antigens, as shown by the failure to achieve maximal POS induced proliferation in the presence of *M. tuberculosis* sonicate antigen.

The unrepeated induction of immunoglobulin *in vitro* on one occasion can also be explained because polyclonal B cell activation can induce immunoglobulin synthesis by all the B cells present in the culture, whatever their specificity. This explanation does not require recourse to the induction of a primary *in vitro* immune response; such responses are well known to be very difficult to generate. The more marked effect of *M. tuberculosis* POS on B cell CD25 expression lends some weight to this argument. Interestingly, PPD at high concentration can also act as a polyclonal B cell activator (Fleming and Rook 1982), and despite is name does contain carbohydrate. It is possible that POS contributes to this activity of PPD.

5.5.4 Conclusion

The conclusion that POS from mycobacteria are acting as a polyclonal lymphocyte activator is a tentative one. Although this seems the best explanation for the present results, future work may change this theory radically. In addition there are many other observations pertaining to mycobacterial POS that have immunological relevance. POS-A from *M. tuberculosis* has opposing activity to rat liver IPG-A and it is conceivable that such a POS fraction may be able to inhibit some mammalian IPG functions *in vivo*. Moreover lipoarabinomannan from some mycobacterial species is able to inhibit mammalian GPI-PLD (Dr. Kiajoo Puan, PhD thesis 1999). These observations suggest that mycobacteria may be able to inhibit endogenous IPG production within macrophages and take over some functions with their own putative version of IPG, POS. Macrophages, therefore, as opposed to lymphocytes, may be the major target of mycobacterial POS.

As described in chapter one, a mycobacterial POS fraction reduced the concentration of recombinant TNFα required to achieve 50% killing of a fibroblast cell line. This was a

property of a POS fraction from *M. vaccae* R877R not *M. tuberculosis*. The presence of *M. tuberculosis* within a macrophage tends in fact to prevent the cell undergoing apoptosis (at least if the number of intracellular organisms remains low), indicating the existence of another immunologically relevant activity. The small the number of donors used in this study makes firm conclusions difficult to draw, but it does show that POS from mycobacteria has significant immunological effects. Also of note is the intrinsic variability associated with use of human PBMCs. The response of fresh PBMCs from a human donor to a given stimulus may vary considerably between different samplings of the same donor, explaining why the dose responses seen vary between occasions and the same effect may not always occur at the same POS concentration. Despite this, however, the existence of so many other activities means that mycobacterial POS (or lipidic precursor molecules if they truly exist) cannot be discounted as making a significant contribution to the immunologically active components of mycobacteria.

Chapter 6

General discussion

6.1 Chemical characterisation of phospho-oligosaccharide and glycophospholipid

Early observations in the field of IPG signaling in mammals described a structurally ill defined bio-activity (Larner *et al.* 1979, Jarett and Seals 1979). Subsequently the glycolipid now referred to as free membrane GPI was identified (Saltiel and Cuatrecasas 1986, Saltiel *et al.* 1986, Mato *et al.* 1987a). The protocol described in chapter 3 was designed later after many of the chemical properties of IPG were known (Nestler *et al.* 1991). Observations in the case of mycobacterial POS have, however, gone the other way round. The original reasoning behind the examination of mycobacterial cells for IPG-like biological activity was that mycobacteria contain phosphoinositol glycolipids and phospholipase activity, however experimentally it was the POS fraction, homologous to mammalian IPG, that was examined first.

Although the presence of biological activity is strongly suggestive of the existence of IPG homologues in mycobacteria, this is far from being experimental proof. The assays that have been used to ascribe IPG-like biological activity to POS are not absolutely specific, for example certain divalent cations can alter PDH phosphatase activation, and cellular responses such as proliferation and lipogenesis may be affected metabolically by the addition of extra substrates to cell cultures, for example in the form of purified low molecular weight carbohydrates.

Although there may be similarities in activity between mycobacterial POS and mammlian IPG, the antibody studies described in chapter 4 show that mycobacterial POS and mammalian IPG must have some structural difference. This has been discussed already in chapter 4, the conclusion being that although these data do not preclude some structural similarity, there is definitely significant structural difference. The work undertaken here has provided confirmation of the presence of biological activity homologous to IPG in mycobacteria (named POS) and has demonstrated some basic chemical characteristics of the material (chapter 3).

6.1.1 Is there a relationship between phospho-oligosaccharide and glycophospholipid?

The isolation of the glycolipid described in chapter 2 took place after the observations that *M. vaccae* R877R, *M. tuberculosis* H37Rv and *M. tuberculosis* H37Ra contained biologically active POS. It remains, therefore, to show a relationship between the glycan fragments with and without the lipid before it may be concluded that each separate extraction method, that used to purify POS and that used to purify GPL, is essentially extracting the same material with and without a lipid moiety. Complete structural characterisation would have achieved this aim, but that is beyond the scope of this thesis. What has been attempted is to show similarity based upon some chemical properties and activity.

Compositional analysis

Both GPL and POS contain sugar residues, as shown by anisaldehyde staining in the case of the glycolipid (chapter 2) and the combination of retention at the origin in solvent VI and detection by pulsed amperometric detection (PAD) for carbohydrate by HPLC in the case of POS (chapter 3, Dr. Alan Jaques, unpublished observations). GPL was shown to contain phosphate, but on the basis of the small amount detected and the behaviour of phosphatidylinositol compared with potassium phosphate in the assay it was concluded that all or most of the phosphate detected was as a phosphodiester, not a phosphomonoester. This predicts that the glycan group formed from this glycolipid by the action of a phospholipase would only possess a phosphate if it were the result of PLC hydrolysis, not PLD hydrolysis. All mycobacterial POS fractions except *M. tuberculosis* H37Ra POS-P were shown to contain phosphate (chapter 3). The total phosphate content of both POS fractions from *M. tuberculosis* H37Ra and *M. vaccae* R877R was of a very similar order of magnitude to that of GPL from each respective strain, distributed unevenly between the POS fractions. The relationship between the amount of phosphate in the glycan and GPL, respectively, may be influenced by several different factors. This ratio may represent the normal steady state of turnover between GPL and POS. As POS-P from *M. tuberculosis* H37Ra was found not to contain any detectable phosphate, POS-A may be a PLC product, POS-P may be a PLD product, and the two together account for the total phosphate measured in the GPL. This infers that GPL is hydrolysed at the point of extraction and that

either one, or the other is extracted, in a manner analogous to the trypanosome sVSG, which is unlikely, but remains undetemined. Both of these explanations assume that POS is a cleavage product of GPL, as is the case with GPI and IPG in mammalian cells. In the case of *M. vaccae* R877R in fact slightly more phosphate was found in the total POS fractions than was found in GPL, per gram of starting material in each extraction.

Biological activity

Perhaps the most intriguing evidence that GPL and POS are related comes from their biological activities. As described in chapter 2, an attempt was made to cleave the glycolipid with PI-PLC and to show that the cleavage product had the biological activity of an IPG structure. This experiment was confounded by the serendipitous observation that the glycolipid itself was active in the cell free PDH phosphatase activation assay. IPG-like structures still covalently attached to lipid have previously been shown to be active in this assay, both in the form of synthetic compounds (Figure 2.23 and Dr. S. Kunjara, unpublished observations) and GPI derived from human placenta (Dr. Sylvie Deborde, PhD thesis, 2000). For mycobacterial GPL and POS, both purified using methods well known to selectively isolate such second messengers and their precursors in mammalian tissues, to both show activity that is an artefact is not likely. For example, one of the potential contaminant that is active in the PDH phosphatase assay, free divalent cations, cannot be present in GPL (excepting cations bound within the structure) as GPL was purified by TLC as hydrophobic material.

Notably, the ability of POS derived from *M. vaccae* R877R to induce NO production by J774 cells was minimal (chapter 3), whereas the *M. vaccae* R877R GPL GPI-PLC reaction product was much better at inducing NO production by these cells (figure 2.15), although this was not the case in every experiment (figure 2.14). Nevertheless, the similarities in biological activity suggest that although POS and GPL from *M. vaccae* R877R are not identical, but may be related. The respective biological activities of *M. tuberculosis* derived POS and GPL were also not identical. For example, *M. tuberculosis* H37Ra GPL was much more active in the PDH phosphatase activation assay (figure 2.12) than POS from the same species (figure 3.3). Direct numerical comparison between these two experiments is not possible because it is not known how much of the 500mg organism mass equivalent of GPL was in the aqueous phase after enzyme or mock treatment and was

accessible to the assay, but assuming that all of it was, then 500mg organism mass equivalent of GPL was able to induce a 3.5 fold increase in enzyme activity, whereas 1g organism mass equivalent of POS-P produced only a very modest increase in activity of a few percent. NO inducing activity was of a similar order of magnitude by GPL and POS from *M. tuberculosis* H37Rv (figures 2.18 and 3.2). Again, these data suggest that GPL and POS from *M. tuberculosis* may be related, but are not identical in their glycan portion.

Reaction with fluorescamine and ninhydrin

Although GPL appeared to give a fluorescent product after reaction with fluorescamine, the unexpectedly high fluorescence in these experiments taken together with the failure of GPL to stain with ninhydrin was interpreted to indicate that GPL does not contain amino groups. Following reaction of POS with fluorescamine very high fluorescence was also measured and approximation of the amount of amino groups present showed that most or all of this fluorescence must be artefact. By ninhydrin staining, however, POS was shown to have free amino groups (figures 3.6 and 3.7). Therefore, GPL does not have a free amino group, and POS does. The reason for this discrepancy between fluorescamine and ninhydrin reactivity is not clear.

Although it is conceivable that POS, if it were a cleavage product of GPL, could lose the amino group in the process of cleavage (for example if the generation of POS was a multi step process involving removal of an amino sugar), it is inconceivable that it could gain an amino group in the process of cleavage. It may be that the glycan portion of GPL is further modified after removal, but then this would represent a pathway of biosynthesis rather than homology to the mammalian GPI/IPG signalling mechanism. Taken together, these data indicate that GPL and POS do not have the same relationship as GPI and IPG in mammalian cells. Nevertheless, the suggestion that not all of the material within the *M tuberculosis* H37Ra POS-A fraction contains amino groups, indicated by the observation that some immunoreactivity was not retained on AG50W X-12 resin, leaves the possibility that POS are a mixture of species, some of which are amino group containing and some of which are not. POS could be a collection of species from different sources, some being derived from GPL and some not. As mentioned in chapter 2, inositol glycan structures have been described in plants that do not contain phosphate or free amino groups. Such

molecules are also thought to be involved in signalling in plant cells and to be derived from a lipidic parent molecule (Smith and Fry 1999, Smith *et al.* 1999).

Combining the available data indicates that GPL and POS are not related, shown principally by the presence of an amino group in POS but not in GPL. However, there are some very interesting similarities in biological activity, particularly in the PDH phosphatase activation assay. In the face of the compositional data these similarities in activity do not indicate similarity in structure, so mycobacteria may contain more than one type of material that has IPG-like biological activity. As GPL and POS are not related, then the question arises as to whether POS is derived from some other lipidic preursor and if so what is the nature of the precursor, or whether it is not derived from a lipidic precursor at all but is made directly in a water soluble form. POS is not derived from LAM becasue LAM does not contain amino sugars.

6.2 Immunological characterisation of phospho-oligosaccharide and glycophospholipid

Although the immunological properties of the material described in the present work are currently incompletely characterized, there are a number of interesting observations that have been made.

Polyclonal lymphocyte activation

POS from *M. tuberculosis*, and possibly from *M. vaccae* may cause polyclonal activation of human lymphocytes in culture. The proportion of activated B cells (though not the proportional number of B cells) in such cultures is also increased, as shown by CD25 expression. There has been some suggestion of *in vitro* immunoglobulin induction. Taken together these data suggest that POS, at least from *M. tuberculosis* H37Ra, do act as a polyclonal activator of B cells. However, POS are also immunogenic to B cells, as shown by the presence of anti-POS antibodies in human tuberculosis patients and by the appearance of anti-POS antibodies after immnuisation with CFA in rabbits. The possibility that POS is mediating its affect on B cells *in vitro* through specific antigen receptors therefore cannnot be excluded.

Phospho-oligosaccharide and toll-like receptors

Despite the effect of mycobacterial POS on human lymphocytes summarised above, within the context of active mycobacterial infection *in vivo* macrophages are likely to be exposed to much more mycobacterial POS or GPL than lymphocytes because of the micro-anatomical characteristics of tuberculous lesions. Both the mycobacterial POS and glycolipid described here have been shown to have effects on macrophages *in vitro*, inducing release of inflammatory mediators. As described in chapter 1, one of the classes of macrophage receptor that participates in such macrophage responses is the Toll like receptors (TLRs). A water soluble, heat stable, proteinase resistant factor of *M. tuberculosis* designated soluble tuberculosis factor (STF) mediated NF-κB activation through TLR2 (Means *et al.* 1999b). These properties are compatible with those of the POS material purified from mycobacteria. In a subsequent report, the same group found that a lipid-A like TLR4 antagonist E5531 did not inhibit *M. tuberculosis* induced NO production, but did inhibit TNFα production (Means *et al.* 2001). *M. tuberculosis* mediated effects on inducible NO synthase gene transcription also appeared not to be TLR dependent, as shown by over expression of a double negative mutant MyD88, the TLR intracellular signaling protein. However, STF induced iNOS promoter activity was abrogated by MyD88 double negative mutant over expression, indicating that although STF is not a dominant contributor to *M. tuberculosis* induced NO production, it is nevertheless TLR dependent. There remains a possibility that mycobacterial POS is at least a component of STF and acts as a TLR2 ligand, although if this is so POS is perhaps not a very significant part of all NO inducing activity of *M. tuberculosis*. The situation *in vivo*, however, might be very different. It may also be that POS act through other macrophage receptors as well as TLRs.

Glycophospholipid, toll-like receptors and inflammatory mediator production

It has also been shown that a heat sensitive, cell associated factor mediated NF-κB activation *via* TLR4 (Means *et al.* 1999b). Whether or not the mycobacterial glycolipid described here is heat sensitive is unknown, but as the system used by Means *et al.* involved examining culture supernatants versus cells the glycolipid would almost certainly be expected to be present in the cellular fraction. In addition, the glycolipid (at least from

M. tuberculosis H37Ra) causes IL-12 release from human THP-1 monocyte/macrophages (figure 2.19). Detergent soluble cell wall proteins of *M. tuberculosis* have previously been shown to also cause IL-12 release from human macrophages in a TLR2 dependent manner (Brightbill *et al.* 1999). GPL might be a component of the same fraction, as it too is detergent soluble. The mycobacterial glycolipid described here is, therefore, a potential candidate for a TLR ligand. Although the cell associated factor described by Means *et al.* was a TLR4 ligand, there are a number of lipidic molecules derived from bacteria that have been described that signal through TLR2 as well, including some mycobacterial products (Brightbill *et al.* 1999, Lien *et al.* 1999, Means *et al.* 1999a). It is possible, therefore, GPL could signal through either TLR2 or TLR4, or both.

GPL from *M. vaccae* R877R and *M. tuberculosis* H37Rv both induced NO production from the murine monocyte/macrophage cell line, J774. Expressed in terms of starting mass of bacterial cells in each extraction, GPL from *M. tuberculosis* H37Rv was four times more active in this assay then GPL from *M. vaccae* R877R (figure 2.18). Perhaps expression of amount in terms of phosphate content is more accurate, but in this case GPL from *M. tuberculosis* H37Rv is still nearly twice as active as GPL from *M. vaccae* R877R. Therefore, GPL from a pathogenic species, *M. tuberculosis* H37Rv, induced more NO production than GPL from a non-pathogenic species, *M. vaccae* R877R. The same appears to be true for POS (chapter 3). Interestingly, this is the opposite to what has been observed for LAM, where manLAM from a pathogenic species, *M. tuberculosis* Erdman, did not elicit RNI and TNFα production from murine macrophages, whereas araLAM from a non-pathogenic species (then mistakenly believed to be *M. tuberculosis* H37Ra) did elicit both RNI and TNFα production (Roach *et al.* 1993). What the significance of this observation might be is difficult to predict, because mycobacteria probably contain more LAM then they do GPL.

6.3 Tuberculosis, metabolism and leptin

One of the most striking features of tuberculosis is the profound weight loss that accompanies the condition. Weight loss with a concomitant increase in energy expenditure accompanies many chronic inflammatory conditions, and under such circumstances is considered to contribute to the pathological state. Weight loss as a result of nutrient

deprivation, on the other hand, induces appropriate metabolic responses such as reduction of energy expenditure and preferential utilization of adipose tissue in order to conserve vital tissues. Experiments investigating whole body protein metabolism in tuberculosis patients, however, identified that protein turnover was not accelerated and nor was energy expenditure raised (Macallan *et al.* 1998). Moreover, upon feeding, tuberculosis patients oxidized relatively more protein than healthy or malnourished controls, providing evidence for anabolic block in tuberculosis. This conclusion is borne out by other observations on the body composition of HIV+ men with and without tuberculosis. The group suffering from tuberculosis had decreased body cell mass compared with the group with HIV only, indicating loss of vital lean tissues (Paton *et al.* 1999).

The mechanism of anabolic block in tuberculosis is unknown, but Macallan *et al.* suggested that insensitivity to some the actions of insulin might provide a putative explanation. As mycobacterial POS appear to be inhibitory in some assays of biological activity where insulin treated liver derived IPG are stimulatory, it is intriguing to speculate whether or not POS may be (at least partly) responsible for preventing the anabolic actions of insulin and inducing anabolic block in lean tissues. Such a hypothesis would probably require that mycobacterial POS are able to leave the focus of infection and gain access to the circulation, acting like a bacterial toxin, a novel concept for tuberculosis infection. Perhaps a more realistic hypothesis is that *in vivo* mycobacterial POS act locally within tuberculosis lesions preventing the accumulated leucocytes from effectively utilizing the metabolic substrates that the wasting occurring in inflammatory conditions presumably serves to generate.

Interestingly, although tuberculosis patients have lower percentage body fat than normal controls (Macallan *et al.* 1998), they have higher circulating leptin concentrations (Cakir *et al.* 1999). This is the opposite of what would be expected, as leptin is released from adipocytes, acts in a feedback loop to regulate food intake over the long term preventing excessive weight gain and in sufficiently low concentrations appears to signal a state of starvation (Friedman and Halaas 1998). Leptin receptors are expressed on CD4+ T cells and leptin has some profound immunological effects, including reversal of starvation induced immunosuppression and polarizing Th responses towards the Th1 type, appropriate for immunity to *M. tuberculosis* (Lord *et al.* 1998). The effect of raised leptin in

tuberculosis, therefore, would be beneficial for the T cell response but detrimental to the food intake required to antagonize cachexia. Experiments in an animal model show that several inflammatory cytokines induce leptin gene transcription, the most potent being TNFα (Sarraf *et al.* 1997). This provides an explanation for the inappropriately high level of leptin in tuberculosis, and may also represent a more general mechanism for maintaining short-term immunity in the face of malnutrition. In isolated rat adipocytes insulin and IPG can modulate leptin release (Kunjara *et al.* 2000), and in mice abnormal leptin levels or leptin sensitivity modulates the tissue response to insulin; both *ob/ob* (leptin deficient) and *db/db* (leptin receptor deficient) mice are diabetic. Precisely what relationship (if any) exists between insulin, leptin, weight loss in tuberculosis and POS is completely unknown, but may represent a fascinating area for further study. Such studies would depend upon the availability of large amounts of pure, well-characterised POS from pathogenic mycobacteria for use in animal models.

6.4 Future work

The experiments described in this thesis were continually hampered by the small amount of material that can be derived from mycobacteria by the isolation procedures described and the length of time it takes to grow enough organisms on the poor but non-antigenic Sauton's medium. Despite that fact that POS and GPL are not related, each alone is sufficiently interesting to justify further study. There are many interesting observations that have been made using either POS or GPL from one of the three strains of mycobacterium used in the present work that have not been performed on all three strains. Examples include IL-12 production by THP-1 cells in response to GPL, retention of POS on C8 columns, the requirement of adherent cells for the PBMC proliferative response to POS and the animal immunisations. The most obvious place to start in terms of future work is to repeat many of the experiments that have been described in this thesis on POS or GPL from the mycobacterial strains used within the present work, and on other mycobacterial species that have not been studied in this regard. It would also be interesting to attempt to determine if there is a lipidic precursor to POS, or to determine the nature of biosynthesisof POS.

Because our laboratory does not have the facility to safely contain shaking broth cultures of biohazard category 3 organisms, radiolabelling of material from *M. tuberculosis* has not been performed. Simple experiments such as these would be most informative of the composition of GPL or POS from pathogenic mycobacteria. There are many other simple chemical experiments that can be performed to provide more information on the materials described here. Glycosidase digestion of POS or GPL would enable the identification of some of the glycan components of these materials and may also reveal their linkage conformation. Mild alkali treatment of POS could establish the presence of acyl groups and their relation to biological activity, as described in chapter 3. Nitrous acid deamination of POS would define whether there is an amino sugar in covalent linkage with an inositol ring, and would provide evidence for the presence of inositol, which has not been formally determined. Full structural characterisation represents the ideal of such chemical investigations, but can be very complex and difficult to perform, especially on biological samples that are a mixture of different structures. Despite the first isolation of IPG 14 years ago, still no complete structure of an IPG molecule has been described. Such experiments on mycobacterial material would probably need to involve two-dimensional NMR, mass spectrometry with a variety of different chemical derivatisation and ionization conditions and many different chromatographic techniques.

Many immunological experiments can be done which do not require such detailed chemical analysis. Model systems already exists for the examination of the ability of any given material to act as a ligand for TLR2 or TLR4 (Means *et al.* 1999a, Means *et al.* 1999b). As described above, there are good reasons for investigating the role of POS and GPL as potential TLR agonists.

The potential immunogenicity of mycobacterial POS and GPL is of considerable interest given the immunological properties described here. GPL, being a lipidic structure, has the potential to function as a CD1 ligand and be presented to specific T cells. The present work has shed no light on this matter, but again model systems for the investigation of mycobacterial glycolipids as CD1 restricted T cell antigens are well described (Sieling *et al.* 1995, Porcelli and Modlin 1999, Moody *et al.* 2000a, Moody *et al.* 2000b). The determination of whether or not GPL (or indeed the component of POS that is acylated) acts as a CD1 restricted T cell antigen would be very significant. If GPL proved to be a T

cell antigen, then determining the nature of the T cell response to GPL would be crucial. The observation of an antibody response to POS in humans warrants further investigation. If POS is derived from a lipidic precursor then the possibility exists that the immune response is directed against the lipidic form through its presentation on CD1 molecules (perhaps presented on B cells after interaction of the glycan potion with B cell receptors) and then cross reacts with POS. Investigations of this nature would be immensely aided by more complete structural information. Expanding the small serum study presented herein has the potential to confirm the presence of an anti-POS antibody response, combined with structural data to determine whether this is merely cross reaction with antibody responses already known to exists in mycobacterial infection, such as anti-arabinan or anti-mannan. The (as yet speculative) implications of the anti-POS antibody response in the serological diagnosis of tuberculosis have been discussed in chapter 4.

POS have activity characteristics strikingly similar to mammalian IPG which are also strongly implicated in some signaling pathways in T cells (Gaulton *et al.* 1988, Eardley and Koshland 1991). The potential effects of POS on signaling cascades in human T cells have not been investigated here. This also may turn out to be an interesting area of study, especially with respect to the influence of POS on T cell cytokine production, or the effect of POS on sensitivity to certain forms of apoptosis.

The most speculative, but perhaps the most intriguing, effects of POS are those effects on metabolism that were touched upon earlier in this chapter. It should be stressed that any effect of POS on whole body protein metabolism in tuberculosis is entirely hypothetical, but nevertheless the putative existence of such an effect is very interesting. Investigation of any such effect would most likely be experimentally difficult, and should come after the majority of the simpler work, particularly chemical characterisation, outlined above has taken place.

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