MEDIATORS OF GRAM-POSITIVE SEPTIC SHOCK

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SUMMARY

Septic shock can occur as a result of Gram-negative or Gram-positive infection and involves a complex interaction between bacterial factors and the host immune system producing a systemic inflammatory state that may progress to multiple organ failure and death. Gram-positive bacteria are increasingly becoming more prevalent especially *Staphylococcus epidermidis* in association with indwelling devices. Lipopolysaccaride (LPS) is the key Gram-negative component involved in this process, but it is not clear which components of Gram-positive bacteria are responsible for progression of this often fatal disease.

The aim of this thesis was to investigate the effect of bacterial components on the immune system. Lipid S, a short chain form of lipoteichoic acid (LTA) found to be excreted from bacteria during growth in culture medium was examined along with other Gram-positive cell wall components: LTA, peptidoglycan (PG) and wall teichoic acids (WTA) and LPS from Gram-negative bacteria. Lipid S, LTA, PG and LPS but not WTA all stimulated murine macrophages and cell lines to produce significant amounts of NO, TNF-α, IL-6 and IL-1 and would induce fever and tissue damage seen in inflammatory diseases. Lipid S proved to be the most potent out of the Gram-positive samples tested. IgG antibodies in patients serum were found to bind to and cross react with lipid S and LTA. Anti-inflammatory antibiotics, platelet activating factor (PAF), PAF receptor antagonists and monoclonal antibodies (mAbs) directed to LTA, CD14 and toll-like receptors were utilised to modulate cytokine and NO production. In cell culture the anti-LTA and the anti-CD14 mAbs failed to markedly attenuate the production of NO, TNF-α, IL-6 or IL-1, the anti-TLR4 antibody did greatly inhibit the ability of LPS to stimulate cytokine production but not lipid S. The tetracyclines proved to be the most effective compounds, many were active at low concentrations and showed efficacy to inhibit both lipid S and LPS stimulated macrophages to produce NO.

Key words: septic shock; lipid S; lipoteichoic acid; inflammatory mediators; immune response.

For Kyle and Kieran

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ABBREVIATIONS

Ab	antibody
BSA	bovine serum albumin
CNS	coagulase-negative staphylococci
CVC	central venous catheter
DMEM	Dulbeccos modified eagles medium
DMSO	dimethylsulfoxide
ELISA	enzyme-linked immunosorbent assay
FACScan	fluorescent activated cell scanner
FCS	foetal calf serum
FITC	fluorescein iso-thiocyanate
FSC	forward scatter
GM-CSF	granulocyte-macrophage –colony stimulating factor
Ig	immunoglobulin
IL	interleukin
IU	international unit
LAL	limulus amebocyte lysaite
LBP	LPS binding protein
LTA	lipoteichoic acid
LPS	lipopolysaccharide
LRR	leucine rich repeats
Mφ	macrophage
mAb	monoclonal antibody
MCSF	macrophage colony stimulating factor
MDP	muramyl dipeptide
MHC	major histocompatibility complex
MIF	macrophage inhibitory factor
MNC	mononuclear cell
MOD	major organ dysfunction
NO	nitric oxide
NOS	nitric oxide synthase

NO_2-	nitrite
NO_3-	nitrate
PAF	platelet activation factor
PAMP	pathogen associated molecular patterns
PG	peptidoglycan
PI	propidium iodide
PMB	polymyxin B
PBS	phosphate buffered saline
PMA	phorbol 12-myristate 13-acetate
PMN	polymorphonuclear cell
ROI	reactive oxygen intermediates
RNI	reactive nitrogen intermediates
RPM	revolutions per minute
SEM	standard error of the mean
TLR	toll-like receptor
TNF	tumour necrosis factor
WTA	wall teichoic acid

CHAPTER 1: INTRODUCTION

1.1 Septic Shock

Septic shock is a systemic response to severe infection, the most common cause being contamination of blood by bacteria. There are approximately 500,000 cases of sepsis annually in the USA; 100,000 are fatal. The Intensive Care National Audit and Research Centre (ICNARC) based at Birmingham University reported the mortality rate for severe sepsis in the UK was 44.7% and the incidence of severe sepsis during the first 24 hours in a total of 92 Intensive Care Units (ICU) was 27.7%. This represents about 21,000 cases per year in Britain (ICNARC 2001). Sepsis develops when the initial, appropriate host response to an infection becomes amplified, and then unregulated. This results in a progressive failure of the circulation to provide vital organs with blood and oxygen. It is characterised by severe hypotension, dehydration, myocardial suppression, diffuse capillary leakage and haemorrhagic necrosis of tissues (Gallin and Snyderman, 1999). This leads to dysfunction or failure of major organs such as lungs, liver, kidney and brain, and ultimately death (Cohen, 2002).

Some individuals become prone to septic shock following impairment of host defence mechanisms, for example after trauma, burns, diabetes or treatment with chemotherapeutic drugs (Titheradge, 1999). The more widespread use of immunosuppressants after organ transplants and more invasive catheters and prosthetic devices has also led to an increased incidence of septic shock in intensive care units (Friedman *et al.*, 1998). The clinical manifestations of septic shock are caused by the elevated release of inflammatory mediators and cytokines by host cells upon interaction with bacterial products (Teti, 1999).

1.2 Gram-Positive and Gram-Negative Cell Walls

Both Gram-positive and Gram-negative bacteria can cause septic shock and numerous investigations have examined their cell wall components as possible mediators.

Gram-negative cell walls (Fig 1.1) are thinner than those of Gram-positive bacteria (Fig 1.5), but are structurally more complex.

Figure 1.1: Gram-negative cell wall. Adapted from (Madigan *et al.*, 2000)

Gram-negative cell walls are composed of a thin layer of peptidoglycan (PG), which forms the outer border of the periplasmic space. PG is a polysaccharide composed of alternating repeats of N-acetylglucosamine and N-acetylmuramic acid with the latter in adjacent layers cross-linked by short peptides, as shown in Fig 1.2. Multiple cross-links between the chains makes PG a very strong and rigid structure. A minimum component responsible for the bioactivity of PG is muramyl dipeptide (MDP) (N-acetylmuramyl-L-alanyl-D-isoglutamine) which has been reported to activate the immune system (Yang *et al.*, 2001).

Figure 1.2: Structure of one of the repeating units of PG and the muramyl dipeptide (MDP) fragment (Madigan *et al.*, 2000)

Outside of the thin layer of PG is the outer membrane, comprising of a lipid bilayer in which various other macromolecules are embedded. The outer membrane is attached to PG by lipoproteins. A structural component that is unique to the Gram-negative outer membrane is lipopolysaccharide (LPS) (Figure 1.3). This is composed of a glycolipid portion called lipid A, linked to a carbohydrate core region and a variable polysaccharide 'O' antigen chain. The outer membrane also contains phospholipids and proteins (Koneman *et al.*, 1997; Madigan *et al.*, 2000).

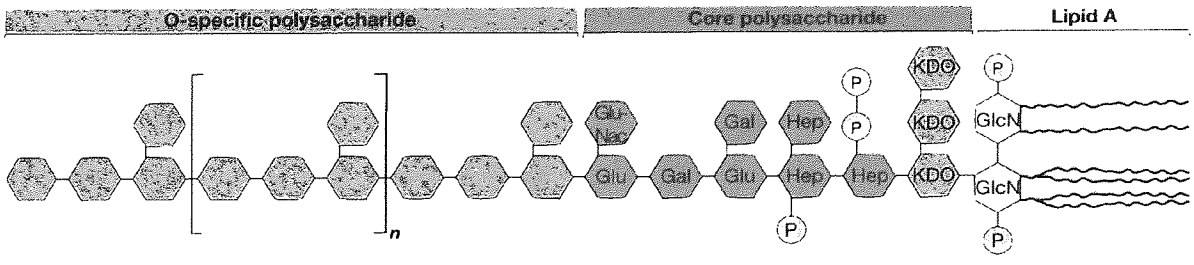


Figure 1.3: Structure of LPS of Gram-negative bacteria.

The sequence of major components is generally uniform but the 'O' specific polysaccharide varies among species. P, phosphate; KDO, ketodeoxyoctonate; Glu, glucose; Hep, heptose; Gal, galactose; GluNac, *N*-acetylglucosamine; GlcN, glucosamine.

During growth of a Gram–negative organism some LPS is released from the cells and it has now been clearly established that LPS is responsible for the initiation of Gram-negative sepsis (Nau and Eiffert, 2002). LPS binding protein (LBP) is the plasma protein that first interacts with and recruits LPS. When LPS is present in the bloodstream it binds to LBP and the complex may then bind to a macrophage surface receptor CD14 (Wright *et al.*, 1990; Ulevitch and Tobias, 1995).

Figure 1.4: Phagocytes bear several different receptors that recognize microbial components (Adapted from (Janeway *et al.*, 2001)

When LPS/LBP binds to CD14 inflammatory mediators are released. However CD14 does not span the membrane of the macrophage to initiate a signal cascade. It requires the presence of Toll-like receptors (TLRs) to do this. In LPS stimulation the TLR has been identified as TLR4 (Akashi *et al.,* 2000b). On the surface of monocytes and macrophage molecules other than CD14 also have the capacity to recognise LPS. Several of these molecules, including the CD11b/CD18 complex, glucan and scavenger receptors bind LPS (Haziot *et al.*, 1994; Janeway *et al.*, 2001) and these may also contribute to the symptoms of septic shock

In addition to the cell surface form of CD14 it may also circulate in plasma as a soluble form. Soluble CD14 (sCD14) can antagonise LPS/LBP induced activation of CD14 positive cells, by competition with membrane CD14 (mCD14) for LPS binding. However this process requires sCD14 in concentrations higher than those normally present in the blood (Haziot *et al.,* 1994). In addition sCD14 also facilitates the transfer of LPS to HDL (high density lipoprotein) resulting in the neutralisation of LPS. This indicates that LBP and CD14 act as shuttle molecules for LPS, mediating both activatory and suppressive effects via mCD14 and HDL respectively (Heumann, 2002). Mice lacking CD14 are highly resistant to LPS –induced shock (Haziot *et al.*, 1994).

The incidence of Gram-negative sepsis has remained fairly constant, despite the use of antibiotics, whereas Gram-positive sepsis has been increasing over the last two decades (Teti, 1999). Gram-positive microorganisms do not contain LPS and much less is known about the causes of shock induced by these organisms.

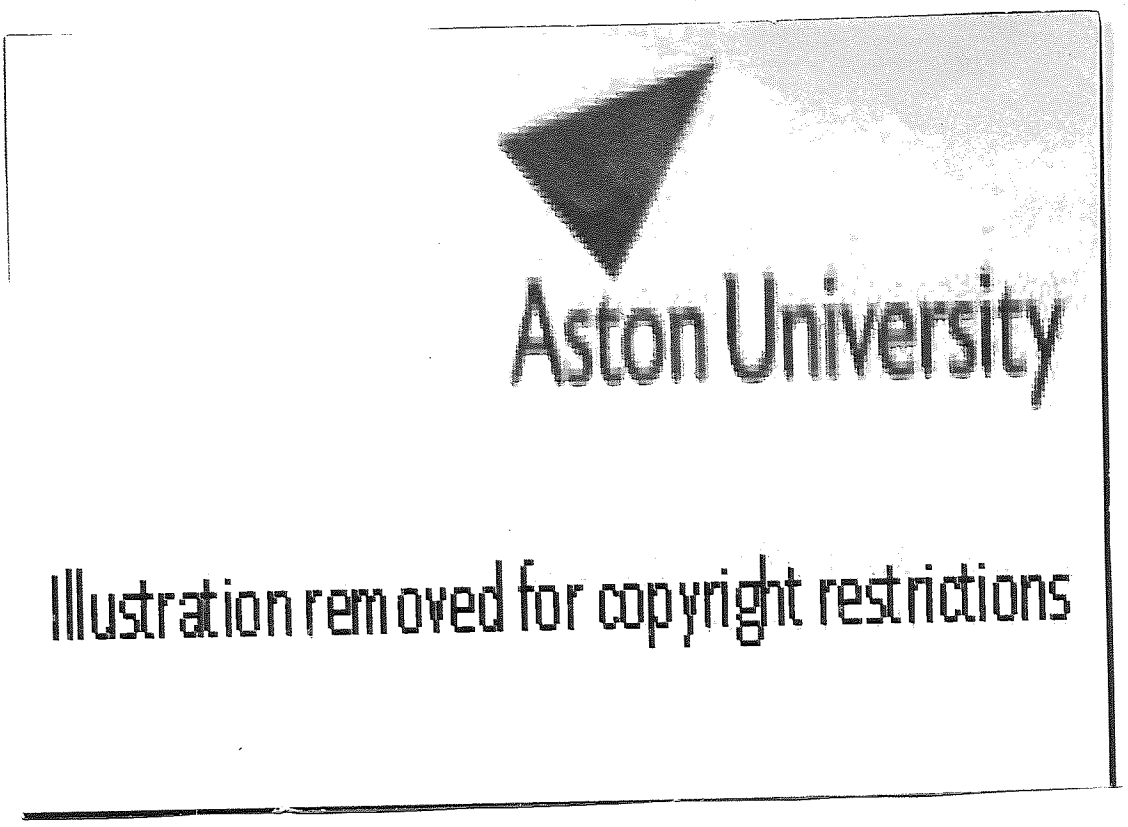


Figure 1.5: Gram-positive cell wall. Adapted from (Madigan *et al.*, 2000)

The Gram-positive cell wall is thicker than the Gram-negative cell wall and is largely composed of a matrix of several layers of PG; within which a variety of proteins, polysaccharides and teichoic acids are embedded. The structure of PG is similar to that found in Gram-negative bacteria (Fig 1.2), the main differences being in the nature of the peptides and the degree of cross-linking, although MDP has the same structure in both types of bacteria. Teichoic acids are polymers of either ribitol or glycerol units joined together by phosphodiester linkages. Ribitol or glycerol teichoic acids when covalently linked to PG in the cell wall are termed wall teichoic acids (WTA). Another form of glycerol acid located in the cytoplasmic membrane and also protrudes through the cell membrane is termed lipoteichoic acid (LTA) (Koneman *et al.*, 1997; Prescott *et al.*, 1996). LTA consists of a unbranched 1,3-linked polyglycerophosphate chain covalently linked to a glycolipid anchor (Fig 1.6). On average the chain is 25 glycerophosphate units in length but can range up to 40-42 units long (Lambert *et al.*, 2000). Other antigens may also be found on the cell surface such as streptococcal proteins and staphylococcal protein A.

Figure 1.6: Structure of LTA (Fischer, 1994)

1.2.1 Staphylococcal Bacteria

One of the common causes of sepsis is systemic infection by staphylococci organisms. Staphylococci are Gram-positive non-motile spherical bacteria 0.5 – 1.5μm diameter, their name is derived from the Greek *staphyle* meaning "bunch of grapes" this is because they divide in more than one plane and form irregular bunches. They are found as commensal flora in humans on the skin and in mucous membranes.

The first evidence that staphylococci are pathogenic was reported in 1880 by Sir Alexander Ogston who identified this organism as the cause of pyogenic abscesses and furuncles in man (Parker and Topley, 1990). In the past pathogenicity in Gram-positive organisms has mostly been associated with *Staphlococcus aureus,* the degree of infection is determined by the virulence of the strain. This organism has been linked with chronic infections such as endocarditis, meningitis, pneumonia and sepsis. *S. aureus* is known to produce a range of toxins, including toxic shock syndrome toxin (TSST) and is also linked with post-operative and post-trauma infections. *S. aureus* is unique and distinct because of its production of the enzyme, coagulase. The *Staphylococcus epidermidis* does not produce coagulase and is a member of the large group of coagulase-negative staphylococci (CNS).

It was first thought that *S. epidermidis* was merely part of the indigenous flora of the skin and incapable of pathogenesis, and was generally disregarded when isolated from clinical specimens (Tierno and Stotzky, 1978). The species remained relatively ill-

defined until the mid-sixties when Baird-Parker attempted to phenotype the taxon (Baird-Parker, 1965). Other studies on Gram-positive bacteria led to other species being assigned to CNS (Kloos and Schleifer, 1975). There are currently over thirty-two recognised species within the CNS taxon (Huebner and Goldmann, 1999) which have been isolated from air, dust, soil, water, food and many animals and plants (Kloos, 1996). *S. epidermidis* is the dominant species on the human cutaneous surface, representing between 65% and 90% of all recovered staphylococci (Archer, 1990) followed by considerably fewer numbers of *S. hominis* (Kloos, 1996). *S. epidermidis* is now recognised as a major cause of prosthetic infection, especially replacement hip joints, heart valves and invasive materials such as catheters (Huebar and Goldman, 1999; Elliott, 1988).

The relatively sudden emergence of cases of CNS-related infections has coincided with the increasing use of relatively new invasive biomedical materials. For example in the 1940s the first plastic catheter was introduced, in the 1950s the first artificial heart valve was used and in the 1960s the indwelling peritoneal catheter was introduced, allowing long-term catheterisation. These devices provide a more frequent opportunity for these usually non-pathogenic bacteria to gain entry into the body and cause serious infections. Colonisation is initiated during surgery or, in the case of catheters, at the point of entry into the skin; organisms are transferred from the patients' skin or from medical staff. *S. epidermidis* have been frequently found colonising central venous catheters and has caused infections ranging from localised sepsis to bacteraemia and septicaemia (Elliott and Tebbs, 1998).

Many actions have been taken to reduce the incidence of these infections. These include using antibiotic ointments such as mupirocin (Hill *et al.*, 1990) or a combination of antibiotics applied to the skin, such as polymyxin, neomycin and bacitracin (Maki and Band, 1981). Catheters have also been coated with antimicrobials such as teicoplanin (Jansen *et al.*, 1992), or benzalkonium chloride (Elliott, 2001). Other methods such as disinfecting the skin with chlorhexidine prior to catheter insertion have also been used (Sheehan *et al.*, 1993). All these have had limited success and are likely to encourage bacterial resistance. Staphylococci have rapidly developed resistance to antibiotics, particularly β-lactams and it is now rare to

find susceptible strains, especially as there are increasing numbers of methicillin resistant coagulase negative staphylococci isolated from prosthetic implants.

Extensive studies have examined materials released by staphylococci which may characterise and possibly aid initiation of chronic infection. Fragments of PG together with WTA and LTA are liberated during bacterial growth. They may also be released by phagocytic or complement-mediated bacterial killing, or by agents that cause bacterial lysis, such as β-lactam antibiotics (van Langevelde *et al.*, 1998).

A variety of Gram-positive organisms are capable of causing sepsis but it has been difficult to pinpoint a single causative agent. LTA isolated from *E. faecalis* can elicit a respiratory burst in human monocytes (Levy *et al.*, 1990), and LTA from *S. pyogenes* can also stimulate these cells to produce TNF-α and IL-1β (Mancuso *et al.*, 1994). Teichoic acids and PG isolated from *S. epidermidis* have both been shown to induce the production of the cytokines TNF-α, IL-1β and IL-6 from human monocytes (Mattsson *et al.*, 1993; Timmerman *et al.*, 1993). Studies on cytokine production induced by LTA from several species of Gram-positive bacteria showed that the magnitude of stimulation varied considerably between species, with some LTAs having no effect (Keller, *et al.* 1992b; Bhakdi, *et al.*, 1991). PG and LTA isolated from *S. aureus* act synergistically to induce the production of proinflammatory mediators TNF-α and IFN-γ in a rat model (Thiemermann, 1997; De Kimpe *et al.*, 1995a). These cell wall components also synergise to enhance the formation of nitric oxide, which can result in circulatory and respiratory failure and liver injury in anaesthetised rats (Kengatharan *et al.*, 1996b). Later (Kengatharan *et al.*, 1998) found that a specific fragment of PG, muramyl dipeptide (MDP) (Fig 1.2) was the moiety within PG that appeared to synergise with LTA to cause the production of nitric oxide, shock and organ injury. MDP can also increase the formation of superoxide anions in macrophages (Pabst *et al.*, 1980).

An exocellular antigen termed lipid S (Fig 1.7) is produced by coagulase-negative staphylococci (CNS) when grown in a chemically defined medium. It is a glycerophosphate glycolipid, closely related to the cellular LTA produced by most pathogenic Gram-positive organisms (Lambert *et al.*, 2000). It is a short chain length

form of LTA with six glycerophosphate units in contrast to 40-42 units in LTA (Lambert *et al., 2000*). Lipid S is released during growth of bacteria, whereas LTA remains within the cell wall and membrane; it too may therefore be a potential contributor to Gram-positive septic shock

Speculation on the origin of lipid S and its relation to LTA is based on consideration of the biosynthetic pathway of LTA. Fischer (1994) has described the biosynthesis of LTA in Gram-positive bacteria, shown in outline in Figure 1.8. The glycolipid diglucosyl-diacylglycerol is the starting point in the biosynthetic pathway; it serves as the lipid backbone to which glycerophosphate units from phosphatidyl glycerol in the cytoplasmic membrane are added sequentially to make up the teichoic acid chain. During the biosynthetic process a range of different length LTA molecules would be synthesised in the cell membrane. Lipid S might be a short chain length intermediate of the full chain cellular LTA, and could be released from the cells during growth (Fig 1.7). The reason why the n = 6 chain length alone is released is not clear. Analysis of culture supernatants show that no other short chain intermediates or the cellular LTA itself are released (Lambert *et al.*, 2000).

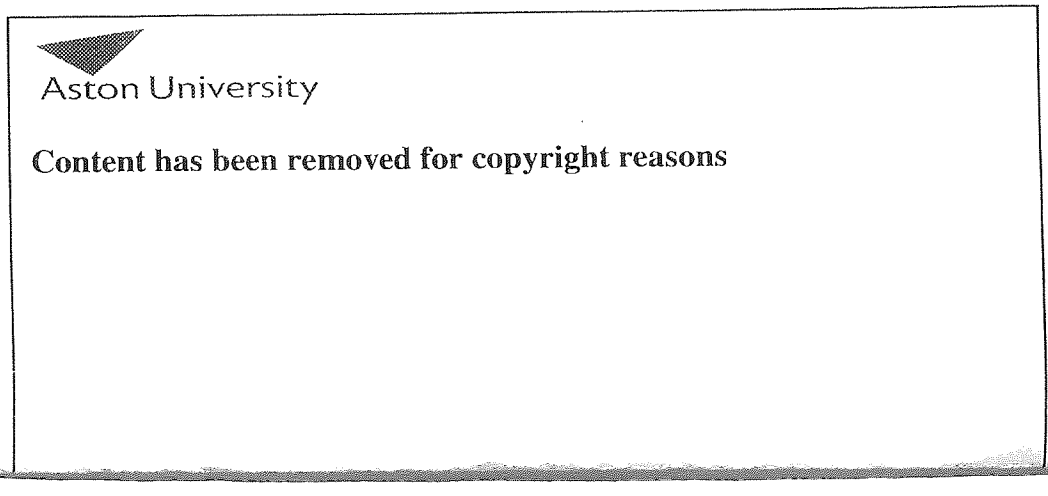


Figure 1.7: Structure of lipid S from *S. epidermidis*. Reproduced from (Lambert *et al.*, 2000).

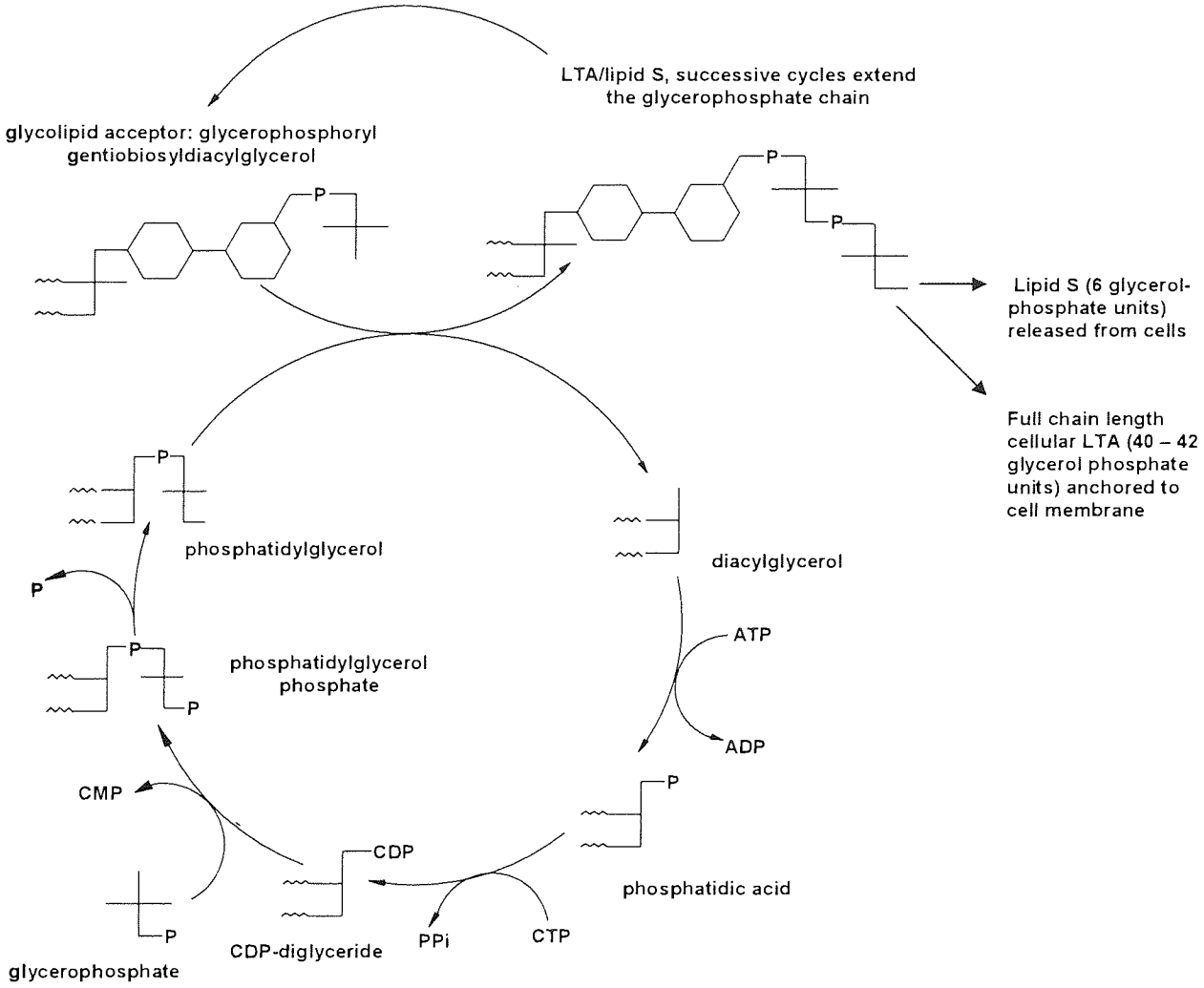


Figure 1.8: Biosynthesis of lipid S/LTA. Adapted from (Fischer, 1994)

Schematic representation

indicates: disaccharide, e.g. gentiobiose

fatty acid, ester linked

P phosphate ester

The identification of a causative agent and therefore a diagnostic tool or antigen for Gram-positive infections and in particular sepsis has remained inconclusive. This is because staphylococci are ubiquitous on the skin surface and many individuals already have basal levels of antibody to the common antigens of staphylococci (Rafiq *et al.*, 2000). Serological tests to diagnose Gram-positive infections utilising lipid S have however recently been developed. Elliott *et al.* (2000) have used lipid S to detect anti-lipid S IgG antibodies in an ELISA test developed to diagnose catheter related sepsis. Furthermore the use of lipid S antigen for serodiagnosis of prosthetic joint infection has also been investigated. Serum IgG levels to lipid S in patients with an infection was significantly higher than in control patients with no signs of infection (Rafiq *et al.*, 2000). It has also been used as a serological test for diagnosing endocarditis caused by Gram-positive cocci (Connaughton *et al.*, 2001). Patients with *S. epidermidis* infection develop high titres of IgG, which can bind to lipid S, but presently it is not known whether this short chain lipid is a contributor to septic shock (Lambert *et al.*, 2000; Elliott *et al.* , 2000).

1.3 Immune Reaction

Whenever tissues are infected a series of vascular and cellular events known as the inflammatory response is set in motion (Fig 1.8). This response is protective of health in that it destroys or wards off injurious influences and paves the way for the restoration of normality. The primary physical effect of the inflammatory response is for blood circulation to increase around the affected area. Gaps appear between the capillary endothelial cells allowing egress of various immunocytes from the blood, i.e. the immune cells, to pass through. As a result of the increased blood flow, the immune presence is thus strengthened. All of the different types of cells that constitute the immune system congregate at the site of inflammation.

Figure 1.9: Potential mediators involved in an immune response (Gallin and Snyderman, 1999)

While an intact host defence system is essential to survival, excessive levels of inflammatory mediators may cause the organ dysfunction and lethality of sepsis.

1.4 Macrophages

Mononuclear phagocytes are cells of the immune system, consisting of macrophages and monocytes. Together with neutrophils and eosinophils, macrophages provide an innate, antigen non-specific, first line of defence against infection before participating in humoral and cell-mediated immunity as a professional antigen-presenting cells and effector cells (Roitt, 1997). Large numbers of macrophages have been found at sites of infection and play an important role in combating foreign antigens.

Macrophages are found extensively throughout the body; some are resident within tissues whereas others are motile and can move freely (Goldsby *et al.*, 1997). They surround all blood vessels in the connective tissue but are more prominent in the spleen, bone marrow, lung (alveolar macrophages), liver (kupffer cells), as well as being resident within the kidney (mesangial cells) and the brain (microglial cells).

Most macrophages in the tissues are derived from circulating monocytes in the bloodstream. Monocytes circulate in the peripheral blood for between 8 and 70 hours before adhering to capillary and venule endothelial cells. They then go through a process called diapedesis where they migrate between the endothelial cells, through the basement membrane and into tissues. The maturation occurs once they are in the tissues (Roitt, 1997). Monocytes increase in size from 10-18μm up to 80μm and the cytoplasm also increases in complexity. The nucleus becomes more prominent and changes its configuration to form indented nuclei, of which there may be more than one. The intracellular organelles also increase in intricacy and number, for example there are greater proportions of both mitochondria and lysosomes in macrophages than monocytes. There is also an increase in the components of the Golgi apparatus and subsequently the production and secretion of soluble factors is augmented (Vernon-Roberts, 1972). The lifespan of the cell is also prolonged from a few weeks as a monocyte to months or years as a tissue macrophage. These tissue macrophages are known as resident macrophages and are relatively quiescent immunologically, having low oxygen consumption, low levels of major histocompatibility complex (MHC) class II gene expression, and little or no cytokine secretion. Resident macrophages are, however, phagocytic and chemotactic and retain some proliferative capacity. The resident macrophages remain in this state until activated by numerous diverse stimuli which may include LPS, heat-killed Gram-positive bacteria, TNF-α, IL-1, IL-2, IL-4, IL-6, yeast glucans, GM-CSF and phorbol esters (Adams and Hamilton, 1992; Goldsby *et al.*, 2000).

Activated macrophages undergo changes that greatly increase their antimicrobial effectiveness and amplify the immune response The transformation results in increased phagocytosis, maximal secretion of mediators of inflammation, including cytokines, cytotoxic oxygen, nitrogen radicals and enhanced release of enzymes

which degrade the extracellular matrix, (Rabinovitch, 1995) complement factors, proteases and coagulation factors (Reeves and Todd, 1996).

Macrophages are highly phagocytic, and they are responsible for ingesting and destroying particulate matter, such as bacteria, viruses and protozoa that enter the body (Rabinovitch, 1995). The process of phagocytosis starts when the macrophage is attracted by chemotaxis to an antigen (Goldsby *et al.*, 2000). Chemotaxis is a reaction whereby the direction of locomotion is determined by chemical substances in the environment. There are many different chemoattractants or mediators that attract macrophages to the site of inflammation, amongst them are macrophage inflammatory proteins and some lipid components of bacterial cell walls. The macrophage moves towards a chemoattractant so that the cell surface eventually makes contact with the antigen (Carr, 1973). Adherence induces membrane protrusions called pseudopodia to extend partially or wholly around the attached antigen. The antigen is engulfed in a membrane-bound structure called a phagosome, which fuses with one or more lysosomes forming a phagolysosome. Lysosomes contain many hydrolytic enzymes that are capable of digesting all kinds of macromolecules that may be present in the engulfed material. The digested contents of the phagolysosome are then eliminated by exocytosis (Kimball, 1996). Recognition of a foreign antigen also activates macrophages to generate reactive oxygen intermediates (ROI), reactive nitrogen species (especially nitric oxide, NO) and cytokines (Marcinkiewicz *et al.*, 1994). Evidence suggests that one of the major antimicrobial mechanisms employed by phagocytes is the production of reactive oxygen metabolites. The susceptibility of an organism to killing is directly related to its ability to trigger the secretion of these species. This is often referred to as the respiratory or oxidative burst. This measurable uptake of oxygen is the end result of the stimulation of the NADPH oxidase system following attachment of the pathogen to phagocytic cell receptors (Patrick and Larkin, 1995). Activation of NADPH oxidase in the cell membrane utilises molecular oxygen to generate a superoxide anion (O_2^-), a toxic ROI.

$$NADPH + O_2 \longrightarrow NADP^+ + O_2^- + H^+$$

This is further transformed into a variety of other active metabolites, such as hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\bullet}$), singlet oxygen ($^1O_2$) and hypochlorous acid (HOCl), which are all extremely bactericidal.

1.5 Nitric Oxide

Nitric oxide (NO) is a small molecule with an unpaired electron and so can be defined as a free radical. It is highly toxic and acts as a mediator of cytotoxicity during host defense (Lincoln *et al.*, 1997). NO is generated by three different isoforms of NO synthase (NOS), two are calcium dependent and are expressed constitutively in endothelium (eNOS) and the nervous system (nNOS), whilst the other isoform is induced in macrophage cells independently of calcium (iNOS) by bacterial products or cytokines. Following an appropriate stimulus, the expression of iNOS can be rapidly induced and maintained for a prolonged period.

Figure 1.10: Synthesis of nitric oxide.
NO is derived from the oxidation of the terminal guanidine nitrogen atom of L-arginine by NO synthase (NOS) (Marcinkiewicz *et al.*, 1994).

Over-production of NO may also contribute to the pathophysiology of septic shock and major organ dysfunction (MOD). NO can act as both a cell messenger and as a cytotoxic agent and maybe the major factor involved in pathological vasodilatation

and tissue damage (Fahmi *et al.*, 1996). NO is known to irreparably damage DNA by deamination and to inhibit respiratory chain activity (Lincoln *et al.*, 1997).

Macrophage activation by Gram-positive and Gram-negative bacterial cell wall components can result in the induction of NOS. LPS from Gram–negative bacteria is the most widely studied in this context and was the first bacterial component to be used to induce iNOS expression (Kroncke *et al.*, 1995) and subsequently the formation of nitrite and nitrate by activated macrophages (Stuehr and Marletta, 1985). However Gram-positive cell wall products have also been shown to be effective. Cunha *et al.* (1993) have demonstrated that murine peritoneal macrophages stimulated *in vitro* with killed Gram-positive bacteria also express high levels of NOS and produce large amounts of NO in a dose-dependent manner. It was then established that LTA is the component responsible for this enhanced release. LTA also stimulates release of cytokines from human blood monocytes (Bhakdi *et al.*, 1991) and murine peritoneal macrophages (Fahmi *et al.*, 1996). In rats LTA causes NO production in vascular smooth muscle as well as macrophages and this is responsible for profound vasodilation (Hattor *et al.*, 1997). The release of NO may also be a regulator of cytokines, by enhancing the release of TNF-α and down regulating IL-6 (Deakin *et al.*, 1995). NO has also been suggested to be a regulator of IL-1 (Hill *et al.*, 1996).

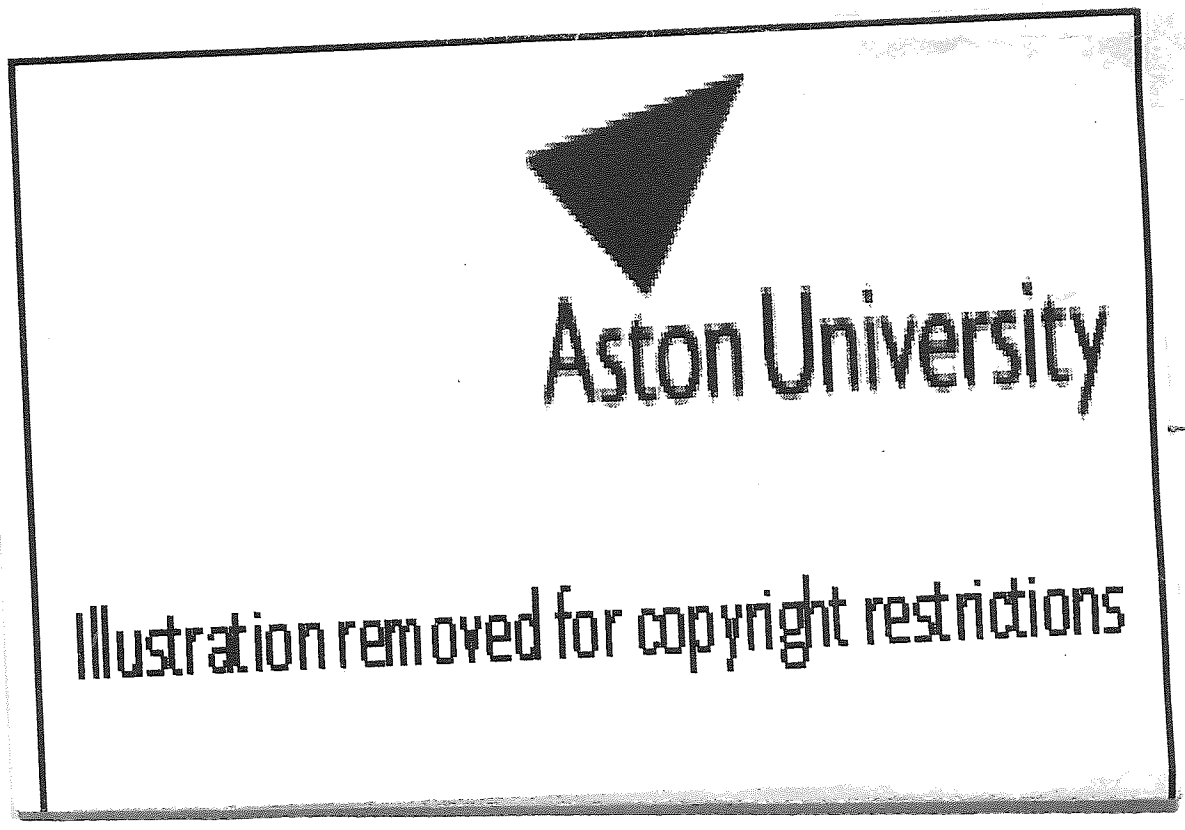


Figure 1.11: Mechanism by which bacterial cell wall fragments induce NO synthase in macrophages. Adapted from (Thiemermann, 1997).

These responses to LTA and LPS are probably all mediated largely via the CD14 receptor. CD14 was originally defined as a specific receptor for LPS, it now appears that common cellular recognition pathways are involved in the response to a variety of pathogens (Pugin *et al.*, 1994). Gram-positive PG has been shown to bind to CD14 (Dziarski *et al.*, 1998) and preparations of LTA can induce IL-12 through CD14 (Cleveland *et al.*, 1996). CD14 was also found to bind LTA and cause a time-and dose-dependent increase in NO production and a marked induction of iNOS mRNA. A monoclonal antibody to mouse CD14 significantly inhibited NO synthesis in response to LTA (Hattor et al., 1997). In addition to LTA, PG also contributes to MOD and septic shock (De Kimpe *et al.*, 1995a; Hattor *et al.*, 1997). Indeed the two substances seem to be synergistic in this respect (Kengatharan *et al.*, 1996b).

NO alone may be insufficient to account for all the symptoms of septic shock and the generation of other mediators such as ROIs, proteases, clotting factors and adhesion molecules may also play a role (Titheradge, 1999). Once formed, NO has the ability to react with superoxide to form peroxynitrite ($OONO^-$), a strong oxidant and nitrating agent, which can promote lipid peroxidation and subsequently may degrade to form the highly cytotoxic hydroxyl radical ($OH^\bullet$) (Miller and Britigan, 1997). Peroxynitrite and hydroxyl radicals are both more toxic than NO itself (Gallin and Snyderman, 1999).

1.6 Cytokines

In response to an invasion of pathogens macrophages synthesise and secrete cytokines which act together to communicate with other immune cells via cytokine receptors and co-ordinate the inflammatory response. A range of these activities is illustrated in figure 1.14.

Cytokines are a group of diverse, small protein or glycoprotein molecules produced and secreted by cells in response to a range of stimuli. Cytokines allow cell to cell communication, they act on cells either in an autocrine or paracrine manner; some also act via the bloodstream. They differ from classical hormones as they can be produced in a range of tissues by more than one type of cell (Clemens, 1991). They are involved in combating bacterial infections and in the regulation of homeostasis. They are not normally stored, but are made and released quickly in response to stimuli. They produce their biological effects by high affinity binding to target cells that bear their specific receptor. This in turn activates intercellular signalling cascades, which lead to gene transcription and the production of a variety of proteins. These can include further production of the stimulating cytokine itself, other cytokines, nitric oxide, acute phase proteins, cell-to-cell adhesion molecules, proteases and lipid mobilising enzymes (Henderson *et al.*, 1996). Over one hundred cytokines have been identified to date all acting in a vast network to orchestrate cell and tissue regulation (Cohen and Segel, 2001). The high affinity binding and low receptor density has

resulted in a very sensitive cell-control system, which is able to respond to cytokine levels in the nano-femtomolar range, many cytokines also overlap in their actions.

The production of cytokines by macrophages can influence both the adaptive and innate immune responses, and they interact with many different cell types. Some of these molecules include tumour necrosis factor (TNF-α) IL-1, IL-6, IL-7 and IL-12 (interleukin) (Janeway *et al.*, 2001).

Figure 1.12: Cytokines secreted by macrophages in response to bacterial products

TNF-α is an inducer of a local inflammatory response that helps to contain infections; it also has systemic effects, many of which are harmful. IL-8 is also involved in the local inflammatory response, helping to attract neutrophils to the site of infection. IL-1, IL-6, and TNF-α have a critical role in inducing the acute-phase response in the liver and induce fever, which favours effective host defence in several ways. IL-12 activates natural killer (NK) cells and favours the differentiation of CD4 T cells into the T_H1 subset during adaptive immunity (Janeway *et al.*, 2001)

A number of cytokines can stimulate their own release and that of other cytokines, which serve to enhance their inflammatory effects. TNF-α increases its own

production by acting on macrophages in an autocrine manner (Blasi *et al.*, 1994). TNF-α and IL-1 can induce production of each other and can also amplify the production of IL-6 (Wang and Tracy, 1999). Thus a positive feedback mechanism exists for increased activation of monocytes and macrophages. The localised inflammatory response would induce the newly-produced mononuclear phagocytes to the site of the infection. In addition to the proinflammatory cytokines that induce the inflammatory mechanism there are also the anti-inflammatory cytokines that act to down-regulate and inhibit inflammatory and immune reactions, these include IL-1ra, IL-4, IL-10, IL-13 and TGFβ. IL-10 has been claimed to be the major deactivator of macrophages in sepsis (Brandtzaeg *et al.*, 1996). Failure to control inflammatory responses can lead to extensive tissue damage (Sundy *et al.*, 1999).

For a successful adaptive immune response other actions of these cytokines are also necessary. For example IL-12 stimulates T helper cells and cytotoxic T cells to proliferate (Ma, 2001). IL-6 stimulates B cell maturation, differentiation and the production of immunoglobulins. It also stimulates thymocytes and T cell activation. TNF-α induces dendritic cells to mature and enhances their ability to stimulate naïve T cells (Sallustro and Lanzavecchia, 1994). TNF-α also upregulates class II MHC molecules on macrophages, which when presented with antigen along with a co-stimulation signal from IL-1, activates T cells (Auger and Ross, 1992).

TNF-α and IL-1 also induce the synthesis and expression of coagulation proteins; also they subdue the activity of the protein C pathway, thereby limiting natural anti-coagulation mechanisms (Auger and Ross, 1992). This results in increased platelet adhesion to capillary walls and activation of a clotting cascade which blocks the blood supply and isolates the infection, preventing spread of the pathogen.

The overall effect of an immune response following invasion of a pathogen is either to destroy the infective agent or to contain it whilst assisting in the preparation of cells involved in the adaptive immune response (Janeway *et al.*, 2001). Thus TNF-α, IL-1 and IL-6 seem to be the most important macrophage-derived proinflammatory agents and are considered in a little more detail below.

1.6.1 TNF-α

TNF-α exists as both a soluble cytokine and in a membrane-bound form. Initially it is produced in a 233 amino acid precursor protein form with a molecular weight of 26 KDa. This pro-TNF is inserted into the plasma membrane (Wang and Tracey, 1999) and then is proteolytically cleaved between residues ala-76 and val-77 to yield the 157 amino acid (17 KDa) soluble TNF-α molecule (Gearing *et al.*, 1994; McGeehan *et al.*, 1994). A matrix metalloproteinase-like enzyme called TNF-converting enzyme (TCE) has been identified as responsible for this cleavage process (Robache-Gallea *et al.*, 1995). The soluble form of TNF is responsible for most of its biological effects, whereas the transmembrane form can interact with TNF receptors (Ma, 2001).

TNF-α has a diverse range of biological activities; it functions as a growth factor for some types of cells, promoting proliferation of astroglia and microglia (Ibelgauft, 1999), and appears to play an important role is B cell proliferation, function and immunoglobulin production (Kehrl *et al.*, 1987). TNF-α demonstrates cytotoxic effects on a number of tumour cells in-vitro, causing haemorrhagic necrosis via the destruction of small blood vessels. However, in-vivo TNF-α is a potent promoter of angiogenesis (Ibelgauft, 1999). Its stimulation of epithelial cell motility also suggests a role in epithelial cell migration during wound healing (Rosen *et al.*, 1991). TNF-α also stimulates phagocytosis, neutrophil degranulation, the production of ROIs, enhanced cytotoxicity and increased adherence of neutrophils (Cerami and Beutler, 1988). At low concentrations it amplifies superoxide production by macrophages and also enhances IL-1 production (Gallin and Snyderman, 1999). It confers protection against intracellular bacterial infections such as *Listeria monocytogenes.* It inhibits lipid metabolism in adipocytes and activates osteoclasts (Ibelgauft, 1999). TNF-α also stimulates the production of superoxide dismutase, providing intracellular protection against its own cytotoxic effects (Okamoto *et al.*, 2001).

TNF-α is an important molecule in restraining the spread of pathogenic organisms and its beneficial inflammatory effects are essential for host survival. However, TNF-α can have damaging effects when released systemically. During infections an excessive production or ineffective down-regulation of TNF-α can have deleterious

effects on the host. It has been implicated in a number of disease processes such as Crohns disease, rheumatoid arthritis, cancer and cachexia (Ma, 2001). In particular TNF-α is notorious for the dominant role it plays in the pathophysiology of sepsis and septic shock (Read, 1998).

1.6.2 IL-1

There are two forms of IL-1, IL-1α and IL-1β, they are produced as propeptides of molecular mass 31-33kDa, both have the same activity and both are agonists to IL-1R (the interleukin 1 receptor) (Matsushima *et al.*, 1986). The IL-1α propeptide precursor is biologically active, but the IL-1β propeptide is inactive. Activation of IL-1β is by a specific cysteine proteinase called proIL-1β-converting enzyme (ICE), which cleaves and converts it to the active form. ICE is found in its inactive p45 form in the cytoplasm of stimulated and unstimulated monocytes (Yamin *et al.*, 1996).

IL-1β is produced in significant quantities and is an endogenous pyrogen, which can raise body temperature and induce fever by eliciting the production of prostaglandins (Gallin and Snyderman, 1999). IL-1 has also been shown to induce bone and cartilage resorption, regulate chondrocyte proliferation and enhance the production of other cytokines including IL-6, GM-CSF and prostaglandins (Gallin and Synderman, 1999). On injection IL-1 has been shown to induce hypotension in rabbits, furthermore serum levels of IL-1β are elevated in patients with bacterial infections and levels appear to correlate with the severity of sepsis and the associated fever and hypotension (Okusawa *et al.*, 1998).

The action of IL-1 is blocked by a naturally occurring inhibitor (IL-1 receptor antagonist, IL-1ra). This is a 22-25 kDa protein produced by monocytes and structurally related to IL-1α and IL-1β, with which it shares between 26-30% homology (Eisenberg *et al.*, 1991). It inhibits the action of both IL-1α and IL-1β by competitive inhibition at the cell surface. Hill *et al.* (1996) has suggested that NO regulates IL-1. Thus by using an inhibitor to NOS synthase, the release of IL-1 from activated macrophages was almost completely inhibited.

1.6.3 IL-6

IL-6 is secreted by mammalian cells as a heterogeneous set of proteins with molecular masses ranging from 19 to 30kd (Wang and Tracey, 1999). It is a multifunctional cytokine involved in the induction and activation of many cell types such as B-cells, T-cells and hepatocytes (Gallin and Snyderman, 1999). The effects of IL-6 are mediated through the interaction of IL-6 with its receptor complex (IL-6R) on the surface of target cells, the receptor complex is composed of a 60 kDa ligand-binding subunit and a 130 kDa signal transducing subunit (Wang and Tracey, 1999). Experiments with IL-6 indicate that its production is stimulated by IL-1 and TNF-α (van Snick, 1990). Serum IL-6 levels are elevated in patients with sepsis and high concentrations have also been found in patients with malaria and meningococcal meningitis, furthermore high concentrations found in serum has been correlated to initial outcome in sepsis (Waage and Aasen, 1992).

1.6.4 Cytokines and Sepsis

A number of reports suggest that Gram-positive organisms can prompt the release of inflammatory mediators that may be associated with sepsis (Bone, 1994). Bhakdi and Tranum-Jensen (1991) reported that human monocytes secreted the cytokine IL-1 after stimulation with *S. aureus* α-toxin and produced TNF-α after exposure to enterotoxins. Subsequently Bhakdi *et al,* (1991) demonstrated that LTA from several enterococcal species induced the release of TNF-α, IL-1 and IL-6 from cultivated macrophages at levels similar to those elicited by LPS from Gram-negative organisms. Bone (1994) also found high levels of TNF-α, IL-1 and IL-6 in patients with Gram-positive sepsis. Excessive, high levels of TNF-α are associated with an unfavourable clinical outcome (Lewis and McGee, 1992).

1.7 Toll-like receptors (TLRs)

A family of receptor proteins known as the Toll family were first discovered in the fruit fly, *Drosophila*. These receptors were shown to recognise both bacteria and fungi and activate the immune system against the pathogen (Medzhitov and Janeway, 2000). Toll-like receptors (TLRs) have been highly conserved throughout evolution. The first human homologue of a *Drosophila* Toll protein was identified in 1997 (Means *et al.*, 2000) and to date 10 homologues have been identified in mammals, each of which seems to recognise conserved molecular patterns carried by different pathogens (Garred, 2001). TLRs are predominantly present in monocytes/macrophages, PMNs, umbilical vein endothelial cells and intestinal epithelial cells (Faure *et al.*, 2001). Mammalian TLRs are now thought to play a crucial part of an immune response against microbial pathogens.

Mammalian TLR family members are transmembrane proteins containing leucine rich repeat (LRR) motifs in their extracellular regions, they also contain a cytoplasmic domain which is homologous to the IL-1 receptor (IL-1R) and can trigger intracellular signalling pathways. The extracellular Ig domain of the IL-1R family distinguishes them from TLRs (Krutzik *et al.*, 2001; Takeuchi *et al.*, 2000). LRRs are short protein molecules about 20-29 amino acids in length, they are found in a diverse group of proteins which include CD14 and platelet glycoproteins.

TLRs participate in innate immunity to bacteria in many ways. They participate in the recognition of PAMPs (pathogen associated molecular patterns) which are present on microorganisms. TLRs are expressed at the interface with the environment at the site of microbial invasion; their activation induces expression of co-stimulatory molecules and the release of cytokines. Also the activation of TLRs leads to direct antimicrobial effector pathways that can result in the elimination of foreign pathogens (Krutzik, 2001).

The first evidence that TLRs mediate responsiveness that is dependent on PAMPs was provided by (Yang *et al.*, 1998). They showed that embryonic kidney cells transfected with TLR2 can be activated by LPS, which led to the activation of NFκB and cytokine induction. Further reports using transfected cells and neutralising antibodies have

suggested that both TLR2 and TLR4 can mediate LPS responsiveness (Kirschning *et al.*, 1998).

In contrast other studies demonstrated that TLR2 and not TLR4 is involved in signalling induced by Gram-positive cell wall products, PG and mycobacterial factors (Means *et al.*, 2000; Schwandner *et al.* 1999; Yoshimura, 1999). Takeuchi *et al.* (1999) also showed mice deficient in TLR4 lacked responsiveness to LTA from Gram-positive bacteria indicating that TLR4 also recognises LTA. The findings that both TLR2 and TLR4 respond to LTA and LPS suggest there might be an overlap in the array of ligands whose responses are mediated by TLRs. Later Takeuchi and Akira (2001) also showed by using gene disruption studies that TLR2 maybe responsible for the response to Gram-positive bacteria and TLR4 for Gram-negative bacteria and LPS. TLR4 deficient macrophages lacked a response to LTA and TLR2 deficient macrophages were hyporesponsive to several Gram-positive cell wall products and displayed impaired production of TNF, IL-6 and NO in response to Gram-positive bacteria. Also, TLR2-deficient macrophages responded to LPS to the same extent as wild-type mice.

An explanation for the apparent role of TLR2 in LPS signalling came when Hirschfield (2000) demonstrated that phenol-soluble contaminants in commercial LPS preparations were responsible for signalling through TLR2, while protein-free LPS signals through TLR4 only. This was later confirmed by Tapping (2000), who engineered THP-1 cells to express both CD14 and TLR4. Repurified LPS induced the production of TNF-α, but when an antibody to TLR4 was used this response was blocked, in contrast the effects of PG and heat-killed *S. aureus* were blocked by anti-TLR2 antibody, but not vice versa by anti-TLR4.

The possible role of TLR4 in development of septicaemia was suggested by Lorenz *et al.* (2002). In their study of 203 bone marrow recipients, which were all successfully genotyped for defects in the extracellular domain of TLR4, 24 had at least 1 mutation. Following haemopoietic stem cell transplantation 16.7% that had TLR4 defects developed Gram-negative sepsis in comparison to 6.6% of recipients with normal

TLR4. This suggests that mutations in the extracellular portion of TLR4 may increase the risk of sepsis. However, bone marrow recipients are at high risk of infection due to the administration of immunosuppressive drugs so a much larger population needs to be investigated. Arbour (2000) found genetic evidence that mutations in TLR4 are associated with differences in LPS responsiveness in humans and demonstrated that gene sequence changes can alter the ability of the host to respond to environmental stress.

Another factor implicated in TLRs is MyD88, an adaptor protein which interacts with IL1R and TLR family members. Activation of MyD88 leads to activation of transcription factor NFκB (Medzhitov *et al.*, 1998). Takeuchi *et al,* (2000) has also shown that MyD88 is essential for signalling of the IL-1R/TLR family. They generated MyD88-deficient mice and showed that MyD88 is essential for cellular response to IL-1 and IL-18. MyD88-deficient mice were highly resistant to LPS-induced shock. Macrophages did not secrete TNF, IL-6 or NO in response to LPS or PG. In contrast, TLR2-deficient macrophages produced reduced, but significant, levels of cytokines demonstrating that MyD88 is essential for LPS, PG and LTA. Kawai *et al.* (1999) also showed that inactivation of MyD88 leads to profound LPS unresponsiveness.

Recently Mancek *et al.* (2002) further studied Toll activation pathways and demonstrated that CD14 functions solely as a ligand-binding protein (LPS or LPS–LBP complexes) and does not participate in the generation of a transmembrane signal. The signal is transduced by members of the Toll family. The LPS-LPB-CD14 complex activates TLR4 which in turn relays the signal through a cascade involving MYD88, serine kinase IRAK and TRAF6 to activate transcription factor NFκB, leading to activation of genes encoding for inflammatory cytokines TNF-α, IL-1 and IL-6. This confirmed earlier results by Kirschning *et al.* (1998) and Poltorak *et al.* (1998).

Shimazu *et al.* (1999) identified MD-2, a accessory protein that associates with the extracellular domain of TLRs and can complement the LPS response when co-expressed with TLR4. Later Dziarski *et al,* (2001) showed that the MD-2 protein also has a role in TLR2 signalling. MD-2 can enhance responses of TLR2 to both Gram-

positive and Gram-negative bacteria. TLR4 cannot be activated by any stimuli without MD-2, therefore TLR4 has an absolute requirement for MD-2 for cell activation. Thus the MD-2 complex serves as the LPS signalling receptor (Akashi *et al.*, 2000b; Dobrovolskaia and Vogel, 2002).

LPS, LTA and lipid S contain a number of similar structural features which distinguish them from PG. The presence of negatively-charged groups and lipid chains render them amphiphillic. The MD-2 peptide resembles the cationic amphiphilic regions of LBP and probably recognises the amphiphilic patterns in LPS and LTA (Dziarski *et al.*, 2001). Additional support comes from findings that MD-2 added to medium competes for available LPS and inhibits cellular activation (Viriyakosol *et al.*, 2001). Also LPS is capable of activating cells overexpressing TLR2 in the presence of MD-2 (Dziarski *et al.*, 2001).

Akashi *et al.* (2000b) showed there was a relationship between TLR4–MD-2 and CD14 on PBMCs. Using transfectants and mAbs to soluble CD14 they showed that LPS signalling via TLR4 and MD-2 was about twofold more sensitive in the presence of CD14 and that membrane CD14 may positively regulate TLR4-MD-2 signalling. This is similar in *Drosophila* where the defence program against pathogens requires Toll and a Toll ligand. daSilva Correia *et al.* (2001) showed that LPS is brought into close proximity to TLR4 only when it is present as an LPS-CD14 complex and when TLR4 is co-expressed with MD-2. Bound LPS is transferred from the CD14-LBP complex to a TLR4-MD-2 complex at the cell surface. By using antibody to CD14 the binding of LPS not only to CD14 but also to TLR4 and MD-2 was strongly decreased.

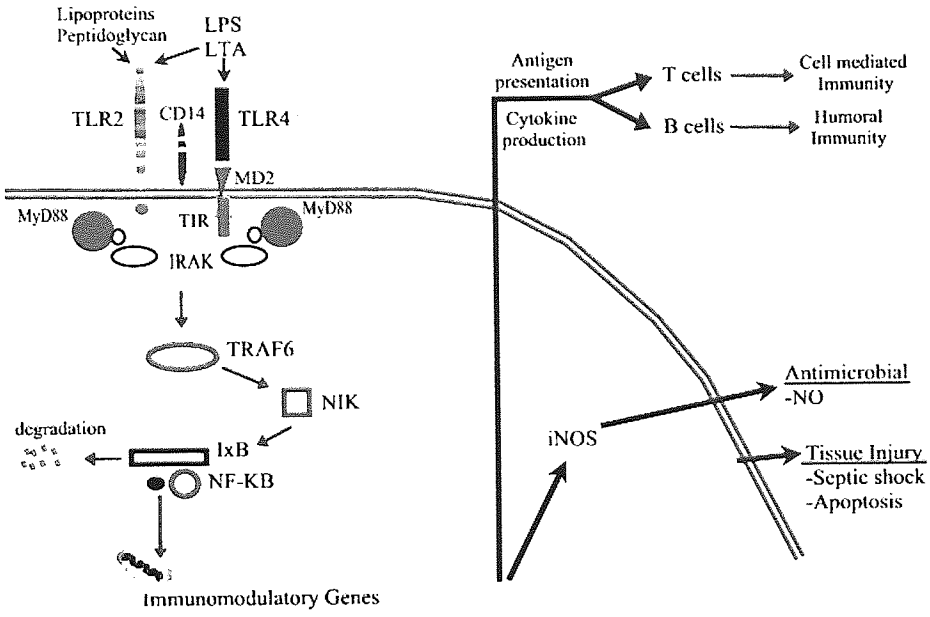


Figure 1.13: Overview of TLR signalling pathways. Adapted from (Brightbill and Modlin, 2000)

1.7.1 Other factors involved in TLRs

In addition to TLR2 and TLR4 other TLRs are reported to be involved in the immune response, for example TLR5 has also been implicated in anti-bacterial host defence (Takeuchi *et al.*, 2001). TLR2 and TLR6 have been shown to work together to coordinate macrophage activation by Gram-positive bacteria and yeast cells (Ozinsky *et al.*, 2000). Also other factors are involved in TLR activation, for example Ohashi *et al.* (2000) reported that heat shock protein (hsp60) is an endogenous TLR4 ligand and Mita *et al.* (2001) demonstrated that both IFN-γ (Interferon gamma) and M-CSF (macrophage colony stimulating factor) modulate the surface expression of TLR2 and TLR4 on human monocytes. Also macrophage inhibitory factor (MIF) regulates immune response though modulation of TLR4, it is also a macrophage cytokine and is an essential regulator of macrophages response to LPS (Roger *et al.*, 2001).

1.8 Endotoxin Contamination

Recently there have been some reports that commercial preparations of LTA from various Gram-positive species are contaminated with LPS. Gao *et al,* (2001) observed the effects on NO production from the macrophage cell line RAW-264, they reported that the normal response of LTA was abrogated when incubated with polymyxin B (PMB). High concentrations of LTA (10 to 30mg/ml) with the addition of PMB resulted in more than 80% inhibition of NO production. To confirm samples of LTA were contaminated with LPS they also used the LAL assay (Limulus amebocyte lysate), a sensitive and specific test which measures endotoxin concentrations in samples.

Polymyxin B is a cyclic polypeptide produced and secreted from the spore-forming soil bacterium *Bacillus polymyxa* (Paulus and Gray, 1964). The bactericidal effect of the antibiotic is based on an increase of the bacterial cell wall membrane permeability. PMB creates channels in the cell membrane, resulting in the leakage of small molecules such as phosphates out of the cell. The peptide amino groups of PMB bind to the anionic phosphate groups present in the core and lipid A of LPS (Nummila *et al.*, 1995). These hydrophobic interactions are the main forces for the association of PMB and LPS (Srimal *et al.*, 1996). PMB disrupts the LPS structure by the insertion of peptides in the fatty acid chain and detoxifies the LPS (Pristovsek and Kidric, 1999). PMBs spectrum includes solely Gram-negative rods, such as *Pseudomonas, E.coli, Enterobacter, Klebsiella, Salmonella* and *Shigella* species. Gram-negative cocci and all Gram-positive bacteria are insensitive to polymyxin B. Therefore when PMB is added to preparations of cultured macrophages exposed to LPS the response is diminished greatly. PMB is incapable of binding to Gram-positive bacteria and hence its use in Gram-positive sepsis is futile. However it does discriminate between endotoxin and Gram-positive cell wall components, and therefore can also be used in endotoxin contamination studies.

1.9 Antibiotics

It is apparent that proinflammatory mediators are involved with septic shock, and several of them are derived from lymphocytes and macrophages. Some antibiotics have been reported to have anti-inflammatory properties and it therefore seems appropriate to examine effects of certain antibiotic compounds to block the release of inflammatory mediators which could possibly prevent the septic shock condition.

There have also been suggestions that antibiotic therapy may further complicate the severity of septic shock. For example, β-lactams attacks the cell wall of bacteria, releasing cellular contents into the body, this could lead to more antigenic molecules being released into the body (Periti and Mazzei, 1998). van Langevelde *et al.* (1998) demonstrated that β-lactams greatly enhance the release of LTA and PG into bacterial supernatants, they also stimulate the production of cytokines TNF-α and IL10 in amount proportional to the LTA and PG released. High titres of antibodies to bacterial antigens may also be detrimental as they may induce increased secretions of proinflammatory cytokines and reactive species.

1.9.1 Tetracyclines

It is well known that inflammatory processes are involved in the aetiology of a number of different disorders including endotoxic shock, stroke, Parkinsons disease, Huntingtons disease, cystic fibrosis (CF), adult respiratory distress syndrome (ARDS) and diabetes (Chen *et al.*, 2000; Ma, 2001; Ryan *et al.*, 1998: Ryan *et al.*, 1999). However, current anti-inflammatory drugs often fail to modulate the inflammatory process sufficiently and yield a positive clinical outcome. This may be because current drugs are unable to modulate the many cellular and humoral pathways of signalling which culminate in the production of enzymatic and free radical mediated tissue damage. The tetracyclines have been shown to exhibit inhibitory activity toward several initiators of the inflammatory cascade (Milano *et al.*, 1997), making them attractive candidates for development as anti-inflammatory agents.

The tetracycline family of drugs is represented mainly by five established compounds: tetracycline, chlortetracycline, doxycycline, oxytetracycline and minocycline. They are active against Gram-positive, Gram-negative bacteria and parasites. These drugs were developed specifically as antibacterial agents and their use for many years provides a clear understanding of their safety and efficacy. Chlortetracycline was the first tetracycline to be discovered in 1948 and along with tetracycline was the first to be used. The discoveries of other tetracyclines such as oxytetracycline led to the development of a number of synthetic tetracyclines such as doxycycline (1967) and minocycline (1972) (Perdue and Standiford, 1999).

Tetracyclines are bacteriostatic, they act by binding to the 30S subunit of the ribosome, preventing the aminoacyl-tRNA binding to the mRNA-ribosome complex, thus blocking protein synthesis (Goldman *et al.*, 1983). Tetracyclines can penetrate through hydrophilic pores in the outer membrane by passive diffusion; active transport then enables further penetration through to the inner membrane. Penetration in to tissue depends on lipid solubility, doxycycline and minocycline are the most active of the tetracyclines and both are highly lipophillic, minocycline being fifteen times more lipophillic than tetracycline (Barza *et al.*, 1975).

However, in addition to their use as anti-bacterials, the tetracyclines demonstrate a number of other clinically useful properties including anti-inflammatory activity. Shapira *et al.* (1996) demonstrated that in the presence of LPS, tetracyclines protected mice against LPS-induced septic shock and inflammatory lesions by reducing TNF-α levels in serum. This group also found that in human monocytes tetracycline was found to inhibit LPS-induced TNF-α and IL-1 production, but not cytokine mRNA accumulation in-vitro, suggesting the mechanism of tetracycline action involves the blocking of post transcriptional events of cytokine production. In addition doxycycline has been shown to inhibit the synthesis of NO in LPS-activated macrophages (D'Agostino *et al.*, 1998a). Therefore the tetracyclines as a class are deemed worthy for further exploration as modulators of inflammatory pathways.

Aims

The continuing high mortality rate caused by septic shock shows the need to increase our understanding of the pathogenesis of this condition in order to develop therapeutic measures.

Both Gram-positive and Gram-negative bacteria can cause sepsis. In Gram-negative sepsis it has been established that LPS can cause macrophage sensitisation, NO release and the production of cytokines. Similar effects are seen in Gram-positive sepsis, however it is unclear which components mediate this reaction. Some candidates from *S. aureus* have been examined for release of NO, TNF-α, IL-1 and IL-6. The results show some positive effects and there was some evidence of synergy. However, *S. epidermidis* is playing an increasingly significant part in sepsis associated with CVC and other prosthetic devices. *S. epidermidis* when multiplying *invitro* releases copious amounts of lipid S which may contribute to the severity of sepsis.

The aim of this project was to purify lipid S and other *S. epidermidis* cell wall components and investigate their ability to bind to antibody in serum from patients with Gram-positive sepsis. Their potential to elicit NO, TNF-α, IL-1 and IL-6 manufacture in primary cultures of peritoneal macrophages and different macrophage-like cell lines was also investigated. Other Gram-positive cell wall components from *S. aureus* and LPS from Gram-negative bacteria were also examined to compare their ability to elicit NO, TNF-α, IL-1 and IL-6 production with components from *S. epidermidis*. LPS has been extensively studied and is a known factor in Gram-negative septic shock and could provide a means for comparison with Gram-positive bacteria.

Tetracyclines have been used in the past to reduce inflammation; a number of new synthetic tetracyclines were examined to assess their capacity to reduce the inflammatory mediators NO, and TNF-α in response to bacterial components.

Since Toll-like receptors are the latest identified components involved in the pathway leading to the upregulation of cytokines and other inflammatory mediators, their

involvement in Gram-positive bacteria will be investigated. The possibility of blocking receptor signalling will also be examined.

CHAPTER 2: MATERIALS AND METHODS

2.1: Serum

Serum (denoted SS) was obtained from The Queen Elizabeth Hospital, Birmingham from a patient with catheter-associated sepsis due to *S. epidermidis*. Control serum (denoted MD) was also obtained from a patient who showed no signs of infection.

2.2: Bacteria and Growth Conditions

The strain of *S. epidermidis* (NCIMB 40896) was isolated from a patient with a central venous catheter (CVC) infection. This strain was cultured overnight at 37°C on Brain Heart Infusion agar. A single colony was subsequently grown in a chemically defined liquid medium (HHW) developed for *S. epidermidis* (Hussain, *et al.* 1991).

2.2.1: Additional bacteria strains

Staphylococcus: the type strain *S. epidermidis* NCTC 11047; *S. aureus* NCTC 6571 (Oxford strain); *S. hominis* ATCC 35982 (SP-2); *S. aureus* NCTC 12598. These strains were grown as above (2.2)

2.3: HHW Medium

Table 2.1 Composition of HHW Medium (modified from Hussain, *et al.*, 1991)

	mg/L		mg/L
Group 1		L-Threonine	150
$Na_2HPO_4.2H_2O$	10000	L-Tryptophan	100
KH_2PO_4	3000	L-Tyrosine	100
L-Aspartic acid	150	L-Valine	150
L-Alanine	100		
L-Arginine	100	Group 2	
L-Cystine	50	Glucose	10000
Glycine	100	$MgSO_4.7H_2O$	500
L-Glutamic acid	150		
L-Histidine	100	Group 3	
L-Isoleucine	150	Biotin	0.1
L-Lysine	100	Nicotinic acid	2
L-Leucine	150	D-Pantothenic acid Ca	2
L-Methionine	100	Riboflavin	2
L-Phenylalanine	100	Thiamin hydrochloride	2
L-Proline	150	$CaCl_2.6H_2O$	10
L-Serine	100	$MnSO_4$	5

Each group was prepared in distilled water, group 1 in 1600ml; group 2 in 200ml and group 3 in 200ml; all groups were autoclaved and finally mixed together.

Using a chemically defined medium such as HHW reduces the risk of contaminating the antigenic material with high molecular weight ingredients seen in more conventional growth media.

2.4: Cell Culture

2.4.1: Murine Macrophage Isolation

To prepare the cell suspensions adult male, MF1 outbred mice, inbred mice C3H/Hej and C3H/Hen were sacrificed by cervical dislocation under ether anaesthesia. The fur was sprayed with 70% alcohol and then removed using sterile scissors and forceps to reveal the abdominal wall.

The murine peritoneal macrophages were extracted by injecting 10ml of sterile supplemented medium RPMI 1640 (2.9.1) (Sigma) into the peritoneal cavity. The area was massaged for 2-3 min and the injected medium was then extracted. To maximise the yield, a further 10ml of supplemented RPMI 1640 was injected and extracted as before. The sample was then centrifuged for 10 min at 1500 rpg, the resulting pellet, resuspended in 5ml of supplemented RPMI 1640, which was then added to a 10cm petri dish containing 5ml of prewarmed (37°C) medium and incubated at 37°C in an atmosphere of 95% air and 5% CO_2 for 16-24 hours to allow adherence of macrophages to the plastic surface.

In order to remove the spleen the mouse was placed on to its right side and was swabbed with 70% alcohol, sterile scissors and forceps were used to take out the spleen. The spleen was then put into a 10cm petri dish containing 10ml of prewarmed (37°C) RPMI 1640 tissue culture medium. Splenocytes were mechanically dispersed aseptically using forceps to yield a cell suspension containing resident macrophages, follicular dendritic cells and T- and B-cells. The cell mixture was transferred to a sterile 15ml conical tube and clumps were allowed to settle. The single cell suspension was aspirated from the top of the tube and put into a new tube. The cells were then washed twice in RPMI 1640 medium by centrifuging them for 10 minutes at 1000 rpg and decanting the supernatant, replacing it with fresh medium and resuspending the cell pellet. The cells were then placed into a petri dish and left for 6-24 hours to allow the adherence of macrophages to the bottom of the petri dish.

The same technique was used to obtain adherent splenic macrophages and peritoneal macrophages. The cultured cells were routinely inspected using an inverted microscope at low power (× 100) with a dark phase lens to determine their adherence. To remove the non-adherent cells (largely lymphocytes and erythrocytes) the petri dish was washed (twice) with 3ml of supplemented medium. A further 3ml of medium was then added and the macrophages were removed by mechanical scraping using a cell scraper. The contents were then washed twice by centrifugation at 1500 rpg for 10 min. The final macrophage pellet was resuspended in 1ml of medium for counting with a haemocytometer. The cell suspension was finally adjusted to contain 5×10^5 cells/ml.

2.4.2: Murine Cell lines

RAW264.7 macrophages were obtained from the American Type Culture Collection (ATCC) and were originally derived from a tumor induced by Abelson murine leukaemia virus in adult male mice from a strain termed BALB.c.

J774.2 macrophages were also obtained from the European Collection of Cell Cultures (ECACC), they are a semi-adherent cell line and were originally derived from adult female mice BALB.c.

Subculture:

The murine macrophage cell lines RAW264.7 and J774.2 were maintained in supplemented DMEM (2.5.2) because it is a nitrate free medium unlike RPMI 1640. The cell suspension was incubated at 37°C in an atmosphere of 95% air and 5% CO_2. Cultures were passaged at confluence by washing in 3ml PBS and then 3ml DMEM medium. Non-adherent cells were discarded and adherent cells were removed by mechanical scraping using a cell scraper. Adherent cells were split into thirds and placed in petri dishes containing 10ml warm supplemented DMEM. Cell clumps were dissociated by repeatedly pipetting the suspension and cultures were incubated at 37°C in an atmosphere of 95% air and 5% CO_2.

Adherent cells used in assays were removed as above by scraping and centrifuged at 1500 rpg for 10 min. The supernatant was discarded and the pellet was resuspended in DMEM. The cell number was adjusted to an appropriate number (e.g. 1×10^6/ml) using a haemocytometer.

2.4.3: Human cell line

The human monocytes cell line THP-1 (European Collection of Cell Cultures) was maintained in supplemented RPMI 1640 (2.5.1) in 75cm^2 tissue culture flasks. The cell suspension containing 30 mls of THP-1 cells seeded at 1×10^5 was incubated at 37°C in an atmosphere of 95% air and 5% CO_2.

The phorbol ester, phorbol 12-myristate 13-acetate (PMA) was used to activate THP-1 cells. A stock solution of PMA was prepared in dimethyl sulfoxide (DMSO) at a concentration of 10^{-3}M (all Sigma). The solutions were aliquoted in sterile eppendorfs and stored at -20°C. Further dilutions to a working concentration of 10^{-7}M was diluted in supplemented RPMI 1640 medium

They were passaged once a week by decanting the cells into a 50 ml sterile centrifuge tube and were centrifuged for 10 minutes at 1500 rpg. The cell pellet was resuspended in 5mls of prewarmed medium and the cells were counted using a haemocytometer. Cell number was adjusted to 3×10^6 / ml and 1ml was added to 30 mls of medium in a 75 cm^2 tissue culture flask.

2.4.4: Cell count

The cell count of macrophages in cell suspensions was routinely performed twice using a Neubauer haemocytometer (Weber Scientific International Ltd) at a magnification of ×500. The suspension was then adjusted, by the addition of supplemented medium to obtain the desired concentration. In preliminary experiments to determine the accuracy of counting two individuals performed two cell counts with two separate samples of cells from the same batch.

Counts for 1 observer differed by 1.7% and mean count for two observers differed by only 3.3%.

2.4.5: Long term storage of cell lines

Storage of cell lines was achieved by freezing 1×10^9 cells in 1 ml of freezing medium, containing DMSO in a cryovial. This was stored for 24 hours at -70°C then transferred for long term storage to a cell bank at -196°C.

2.4.6: Macrophage assays

A 1ml / 250µl aliquot of the different cell types above were added to individual wells of a 24 or 96 well plate respectively, bacterial components were added and the suspension was incubated at 37°C in an atmosphere of 95% air and 5% CO_2. After 24 and 48 hours, supernatants were collected and stored at -20° C ready for analysis of NO or cytokines.

2.5: Cell culture media

Cell culture media were both prepared aseptically and stored at 2 - 8°C.

2.5.1: RPMI 1640

Mouse macrophages and THP-1 cells were incubated in RPMI 1640 (Invitrogen Corporation), this was supplemented with 10% fetal bovine serum (Invitrogen Corporation); 1% glutamine (Invitrogen Corporation) penicillin 100 U/ml and streptomycin 100 µg/ml.

2.5.2: Dulbecco's Minimal Essential Medium (DMEM)

The RAW264.7 and J774.2 cell lines were incubated in DMEM containing glutamax-1, sodium pyruvate, glucose and pyridoxine (Invitrogen Corporation). This was supplemented with 10% heat inactivated fetal bovine serum (Invitrogen Corporation)

2.6: Buffers and Reagents

2.6.1: Tris-buffered saline and Tween 20 (TBS-Tween)

0.01M Tris-HCl pH 7.4
0.9% (w/v) NaCl
0.3% (v/v) Tween 20

2.6.2: Chromogenic Substrate (ELISA)

10mg of 3,3',5,5'-tetramethybenzidine (Sigma) dissolved in 1ml DMSO and diluted in 100ml sodium acetate/citrate buffer (0.1M pH 6.0) containing 20µl of 6% (w/v) hydrogen peroxide.

2.6.3: Phosphate Buffered Saline (Oxoid)

10 tablets were dissolved in 1 litre of distilled water and autoclaved

2.7: Bacterial cell wall components

2.7.1: Lipoteichoic Acid (LTA) from *Staphlycoccus aureus* (Sigma)

5 mg LTA
Dissolved in 1ml supplemented medium

2.7.2: Adjuvant Peptide Muramyl dipeptide (MDP), (Sigma)

1 mg adjuvant peptide (N-acetylmuramyl-L-alanyl-D-isoglutamine)
Dissolved in 1ml supplemented medium

2.7.3: Lipopolysaccharide (LPS), (Sigma)

5mg lipopolysaccharide (*E. coli*, serotype O111:B4)
Dissolved in 1ml supplemented medium

2.8: Statistical Analysis

Unless specified all data is presented as the mean +/-standard error of the mean (SEM) of independent experiments, using triplicate samples.

Statistical analysis was performed using a two-tailed, unpaired students t-test when comparing the difference between two group means. When comparing column means from more than two samples the one-way analysis of variance (ANOVA), followed by Tukeys Multiple Comparison test was used. P values of < 0.05 were considered significant. A 95% confidence interval was used for the difference between all selected pairs of means in the t-test and the ANOVA/Tukeys post test. Statistics were performed using the software package, Graphpad Prism version 3.02.

CHAPTER 3: IDENTIFYING ANTIGENIC COMPONENTS FROM GRAM-POSITIVE BACTERIA

3.1: INTRODUCTION

Sepsis and septic shock are caused by a systemic response to bacterial toxins or antigens which can be initiated by both Gram-positive and Gram-negative bacteria (Bluethmann *et al.*, 1994). The bacterial factors involved in the induction of septic shock have been identified in Gram-negative bacteria but it is unclear which factors are responsible for causing Gram-positive shock (Teti, 1999). However, both Gram types similarly activate the innate immune system, which is largely responsible for the clinical symptoms observed in sepsis (Cui *et al.*, 2000). In recent years nosocomial acquired Gram-positive infections have increased and presently between 30 -50% of all cases of sepsis are caused by Gram-positive bacteria (Kengatharan *et al.*, 1998). Coagulase negative staphylococci are responsible for many cases of sepsis; they are the commonest resident organisms on the skin surface and are generally not harmful to individuals (Kloos, 1996). However in hospital patients, they are the most common causative organism of catheter infections, intravenous and peritoneal dialysis infections, prosthetic valve and joint infections. This is often the consequence of the host immune system being compromised after surgical invasive procedures have been undertaken, (Mims *et al.*, 2001) and by the ability of certain strains to grow on biomaterials, invade adjacent tissues and contribute to the pathogenesis of bacteraemia and sepsis (Karamanos *et al.*, 1997).

In addition to known antigenic factors PG and LTA, it was recently discovered that coagulase negative *Staphylococcus epidermidis* cultures secreted a soluble short chain length form of the cellular LTA, (a glycerophosphoglycolipid antigen) termed lipid S (Lambert *et al.*, 2000). High titres of IgG which can bind lipid S were found in patients with sepsis due to catheter related staphylococcal infections. However it is not known whether lipid S is responsible for the induction of Gram-positive septic shock (Lambert *et al.*, 2000), it is also unclear if other strains of Gram-positive bacteria also secrete lipid S.

3.2: MATERIALS AND METHODS

To investigate the potential mediators involved in Gram-positive septic shock, the major cell wall components (PG, WTA and LTA) were isolated and purified. Experiments were conducted to investigate their ability to react with antiserum from blood of a patient with Gram-positive infection.

3.2.1: Recovery of Lipid S from Culture Supernatants

The method for preparation of lipid S was as described by Lambert, *et al.* (2000); briefly *S. epidermidis* (strain NCIMB 40896) was grown in 2L of HHW (section 2.3) for 24 h at 37°C with shaking at 200rpg. Cells were harvested by centrifugation (10,000 rpg, 10 min). The supernatant was concentrated ten-fold by freeze-drying and resuspension in one-tenth of the original volume of distilled water. An aliquot of 1ml of the concentrated supernatant was injected into a Superose 12 gel permeation FPLC column (30cm x 1cm) and eluted with water. Fifty fractions were collected at 2 min intervals at a flow rate of 0.4ml/min. Fractions 10–15 (previously shown to contain lipid S, Lambert *et al.* (2000)) were collected and pooled together, freeze dried, weighed and resuspended in water to 10mg/ml.

3.2.2: Extraction of Bacterial Cell Wall Components

The pellet of cells from 2L of HHW culture was resuspended in 60ml water and split into two fractions, 20ml for extraction of PG and WTA, and 40ml for extraction of LTA.

3.2.3: Extraction of LTA (Coley *et al.*, 1975)

The aqueous suspension of cells was freeze-dried, resuspended in 100ml of chloroform:methanol (2:1) and stirred overnight to extract lipids. The cells and chloroform:methanol mixture were passed through filter paper and the solvent was allowed to evaporate from the cell residue leaving de-fatted cells. The de-fatted cell residue was removed from the filter paper, resuspended in a mixture of 40ml water and 40ml of 80% (w/v) phenol and stirred for 40 min at 20°C. Centrifugation at 7000 rpg for 10 min left an upper layer of water saturated with phenol, which contained LTA, DNA and RNA. This phase was removed and transferred into dialysis tubing where the phenol was removed by overnight dialysis against distilled water. A combination of DNase (0.1mg/ml Sigma), RNase (0.1mg/ml Sigma), sodium azide 0.01% (w/v) (Sigma) and 10mM magnesium chloride (Sigma) was added to the remaining solution before incubation overnight at 37°C to digest DNA and RNA and stop contamination by microbes. An equal volume of 80% (w/v) phenol was added and stirred at 20°C for 40 min. Phenol extraction and dialysis was repeated and the resulting solution of LTA was freeze dried, weighed and resuspended in water to 10mg/ml.

3.2.4: Extraction of PG and WTA (modified from (Hancock and Poxton, 1988))

The stock of cells extracted from the HHW medium was freeze-dried, weighed and resuspended in ice-cold water to 2mg/ml. The suspension was added dropwise into an equal volume of boiling 8% sodium dodecylsulphate (SDS) to lyse any remaining cells and release associated proteins from the PG / WTA sacculus. The mixture was boiled for a further 30 min, stirring continuously. The solution was allowed to cool before centrifugation at 50,000g (Beckman Ti 50 rotor) for 15 min. The supernatant was discarded and the pellet containing PG and WTA was resuspended in 100ml water. The extraction with boiling 4% SDS was carried out on the insoluble material twice more to remove any traces of soluble extracts. The pellet was then resuspended in water (washing to remove SDS) and centrifuged for 15 min at 30,000 rpg. Washing

was repeated four times in water, twice in 2M sodium chloride to remove any residual SDS and four more times in water. The final pellet of WTA and PG was resuspended in 5ml water. To release WTA which is covalently linked to PG, 0.1M sodium hydroxide was added to the solution, stirred for 30 min at 20°C and centrifuged at 30,000 rpg for 30 min. The supernatant, which contained the WTA was removed and neutralised with hydrochloric acid. The final solution of WTA was freeze-dried and resuspended to 10mg/ml in water. The pellet, which contained the PG was washed in distilled water to remove any sodium hydroxide present, centrifuged at 30,000 rpg for 30 min and resuspended in 1ml of distilled water. A 40µl volume of lysostaphin (20 U/ml, Sigma) was added to the solution and left overnight at 37°C. This enzyme hydrolyses the peptide bond in the pentaglycine cross-link in staphylococcal cell wall PG to release soluble PG. After centrifugation at 10,000 rpg for 10 min the supernatant was freeze-dried, weighed and resuspended to a concentration of 10mg/ml.

The purified components from *S. epidermidis*; (PG, WTA and LTA) were diluted in water to 1 mg/ml and each component individually was injected into a Superose 12 gel permeation FPLC column and eluted with water. Fifty fractions were collected at 2 min intervals at a flow rate of 0.4ml/min.

3.2.5: Analysis of Eluted Fractions

FPLC gel permeation fractions from lipid S, LTA, WTA and PG were assayed for antigenic content by dot blotting, western blotting and ELISA, using serum from a patient with confirmed *S. epidermidis* CVC sepsis (serum SS). These three techniques were used to assay fractions for their ability to bind IgG in the patient serum. Several techniques were employed because it was not known in advance whether antigenic material in the fractions would bind to polystyrene ELISA plates or to nitrocellulose in dot and western blotting.

Samples from individual fractions were diluted with 100 volumes of sodium carbonate / sodium bicarbonate buffer (0.05M, pH 9.6). To coat wells with antigen each diluted sample was applied in a 100µl aliquot into separate wells of a microtitre plate (Immulon 2HB, Dynex Technologies) and left in the dark at 4°C for 18 hours. Unbound antigen was discarded and the wells were washed four times with TBS-Tween (2.6.1) to remove any remaining residual soluble antigen. Unbound sites in the wells were blocked by incubation for 3 hours at 4°C with TBS-Tween. The blocking agent was then discarded and 100µl of serum diluted in TBS-Tween was added to the wells and incubated at 4°C for 18 hours to allow antibody to bind to antigen. The serum was then discarded and wells were washed four times in TBS-Tween. To detect bound IgG, a 100µl volume of protein A-horseradish peroxidase conjugate (0.5 µg/ml in TBS-Tween) was added to each well and left for 2 hours at 4°C. Following removal of conjugate and washing four times in TBS-Tween, 100µl of chromogenic substrate (2.6.2) was added to each well. After approximately 5 min at 20°C when the colour had developed sufficiently, the reaction was stopped by the addition of 50µl 1M sulphuric acid to each well. The developed product, yellow in colour, was measured at 450nm with an Anthos 2001 plate reader.

3.2.7: Dot Blotting

A 1µl sample of each individual fraction was applied to a piece of nitrocellulose membrane paper (Whatmans 3MM) (divided into 50 squares) and left to air dry for 5min. The nitrocellulose membrane was then immersed in blocking solution 5% (w/v) skimmed milk (Marvel) in phosphate buffered saline (PBS) (supplemented with 0.05% Tween 20) for 1 hour with gentle shaking. The blocking solution was discarded and the membrane was washed three times with PBS-Tween. The membrane was then immersed in SS serum diluted in TBS-Tween (1:400) and left for 18 hours at 4°C. The serum was then discarded and the membrane was washed three times in TBS-Tween ready for immunoscreening. To detect any bound IgG the membrane was soaked in Protein A-horseradish peroxidase conjugate (dissolved in TBS-Tween 0.25µg/ml) and left for 2 hours on shaker. Following removal of conjugate and

washing four times in TBS-Tween, chloronaphthol substrate (10mg 4 chloronaphthol dissolved in 1ml methanol and diluted in 100ml of Tris-HCl 0.01M pH 7.4, containing 40µl H_2O_2) was added to the membrane, which detects bound conjugate by production of an insoluble coloured product. After approximately 30 min, or until the colour had developed the reaction was stopped by washing the nitrocellulose membrane in distilled water.

3.2.8: SDS-PAGE, Western blot analysis

SDS-PAGE was based on the gel method of Lugtenberg *et al.* (1975). Eluted fractions from FPLC that were found to be antigenic by ELISA were analysed further by western blotting using a method by Towbin *et al.* (1979) (see table 3.1 for composition of gels and buffers).

SDS-PAGE

An 11% (w/v) separating gel (80 × 70 × 1mm) (table 3.1) was cast using the PAGE casting apparatus (BioRad)

Table 3.1: Composition of the SDS-PAGE separating and stacking gels, sample buffer and electrode buffer

	Separating gel 11% (w/v)	Stacking gel 5% (w/v)	Sample buffer	Electrode Buffer (pH 8.0)
Acrylamide stock1	5ml	-	-	-
Acrylamide stock II	–	2.5ml	-	-
10% (w/v) SDS	0.5ml	0.15ml	5ml	10ml
1.5M Tris-HCI (pH 8.8)	6ml	-	-	-
0.5M Tris-HCI (pH 6.8)	-	3.75ml	2.5ml	-
Distilled water	8ml	8ml	5ml	1L
TEMED (N,N,N^1N^1-tetramethyl-ethylenediamine)	50µl	40µl	-	-
10% (w/v) ammonium persulphate	70µl	50µl	-	-
Glycerol	-	-	2.5ml	-
2-mercaptoethanol	-	-	0.25ml	-
5% (w/v) bromophenol blue	-	-	0.2ml	-
Tris	-	-	-	3g
Glycine	-	-	-	14.4g

Acrylamide stock I – 44% (w/v) acrylamide and 0.8% (w/v) Bis(N,N^1,-methylene-bis-acrylamide) (Severn Biotech Ltd, UK).
Acrylamide stock II – 30% (w/v) acrylamide and 0.8% (w/v) Bis(N,N^1,-methylene-bis-acrylamide).

Western blot analysis

To 20µl of each sample an equal volume of SDS-PAGE sample buffer was added before denaturation for 10 min at 100°C. A 10µl volume of prestained protein marker ladder (Broad Range Biolabs Inc. New England) was added to lane 1 of an 11% polyacrylamide gel and 20µl of denatured samples were loaded to individual lanes and electrophoresed in SDS electrode buffer at 200V for 45 min using BioRad

mini protean apparatus. Gels were then transferred to a nitrocellulose membrane (Whatmans 3MM) and electrophoresed in Transblot buffer (25mM Tris, 192mM glycine, 20% (v/v) methanol, pH8.3) at 100V for 45 min using a BioRad mini transblot apparatus. On completion of transfer the membrane was blocked with blocking solution (PBS supplemented with 5% (w/v) skimmed milk powder (Marvel, Sainsbury Plc.) and 0.05% (v/v) Tween 20) for 1 hour with agitation at room temperature. The membrane was then rinsed with PBS supplemented with 0.05% (v/v) Tween 20 ready for immunoscreening as for membranes in the dot blotting process (3.2.7)

3.3: RESULTS

3.3.1: FPLC Profiles of HHW Medium Alone and Medium Supernatant after Growth of *S. epidermidis*

A chemically defined medium was used to establish if any antigenic substances were released into the medium during growth of this *S. epidermidis* (strain NCIMB 40896). After 24 hours the culture medium was centrifuged and the supernatant was analysed using a Superose 12 gel permeation column, which separates molecules based on their mass. Use of HHW chemically defined medium ensured that no high molecular weight components present in complex media would obscure the antigen detection and analysis.

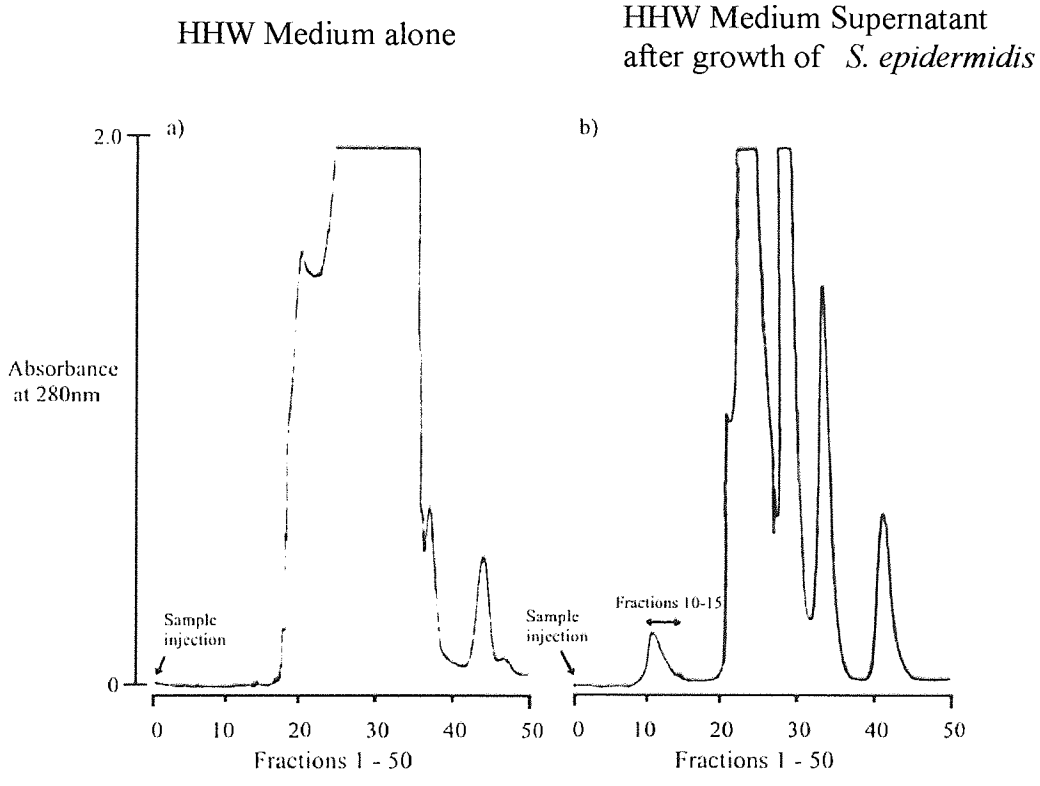


Figure 3.1: FPLC Superose 12 gel permeation elution profiles

FPLC profiles of: a) chemically defined medium HHW alone and b) the HHW supernatant after growth of *S. epidermidis*. A 1ml volume of 10-fold concentrated medium was injected onto the Superose 12 column and fifty fractions were collected at 2 min intervals at a flow rate of 0.4ml/min, absorbance was measured at 280nm.

As can be seen from Figure 3.1, there is a marked difference between profiles, which is due both to nutrients in the medium being used up and microbial products being released into the medium during growth of the organism. Additional material was present in fractions 10 – 15 (Fig 3.1b), which was not seen in the profile of medium alone (Fig 3.1a). This presumably represented high molecular weight material released from the organism during growth.

3.3.2: ELISA Profiles of Fractions Collected from FPLC Column

To analyse the fractions derived from the *S. epidermidis* culture supernatant an indirect ELISA was set up using human serum from a patient with *S. epidermidis* sepsis (SS) to detect the presence of antigens. Serum from a normal (non-infected) patient (MD) was included as a control.

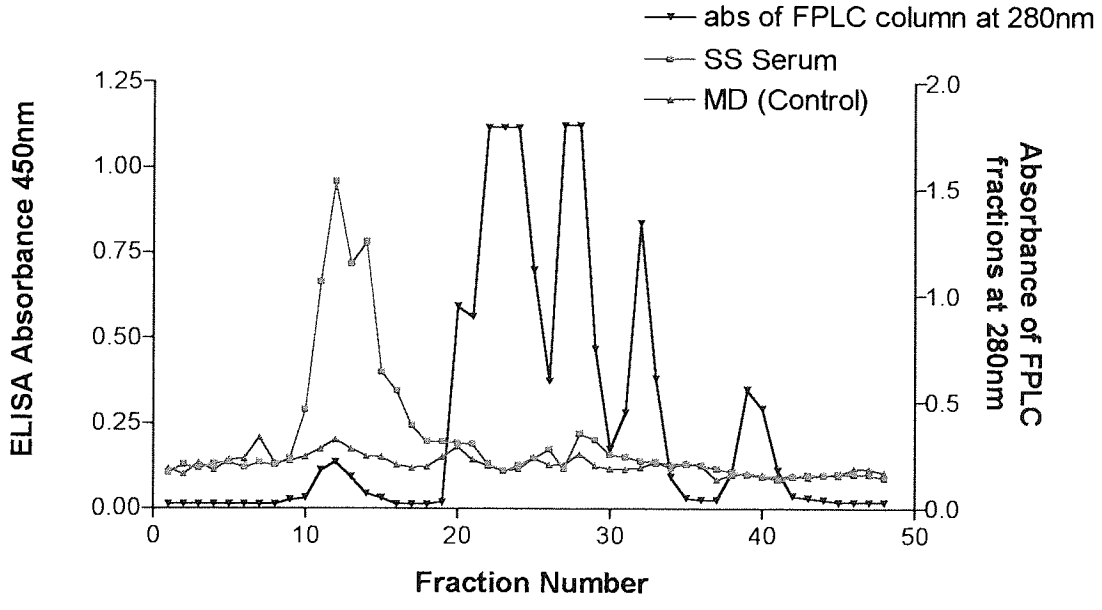


Figure 3.2: ELISA profiles of eluted fraction

Fractions were diluted 100 fold in bicarbonate buffer and coated onto a 96 well ELISA plate Human serum SS was from a patient with CVC *S. epidermidis* infection. Serum MD was from a patient with no signs of infection. The HRP-protein A conjugate for the ELISA assay was used to detect bound IgG. The FPLC profile (3.3.1b) was plotted onto the graph for comparison purposes.

The immune serum (SS) bound to material in fractions 10 - 18 of the culture supernatant. These fractions contain antigenic material, which can clearly bind to IgG released during a *S. epidermidis* infection. Little reaction was obtained when the fractions were analysed using non-immune human serum (MD). None of the other fractions (1–9 and 19-50) contained material capable of binding to the antibodies in the SS serum.

3.3.3: ELISA Analysis of FPLC Fractions from S. epidermidis culture supernatant (Lipid S), LTA and HHW alone

In a separate series of experiments FPLC fractions from HHW medium only, HHW culture supernatant (containing lipid S) and LTA (extracted from whole cells) were examined to compare their elution profiles and capacity to bind antibody in human serum SS (patient with *S. epidermidis*, CVC sepsis). To obtain a direct comparison of the antigenic activity equal amounts of material were applied to the column (1ml of a 10mg/ml solution in water).

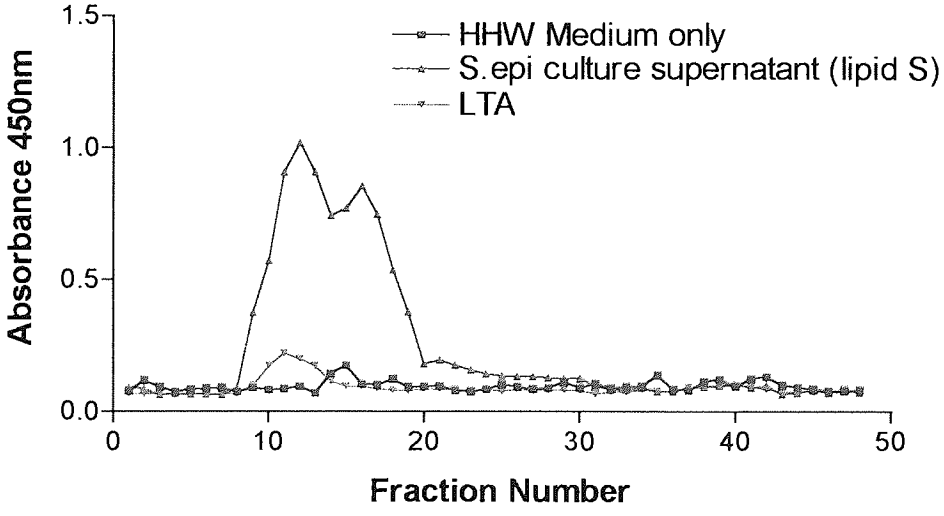


Figure 3.3: ELISA profiles of eluted FPLC fractions 1 – 48

Fractions from HHW alone, culture supernatant from *S. epidermidis* and LTA from whole cells of *S. epidermidis*. Human serum SS, (1:6400 dilution) and HRP Protein A were employed to bind and detect antigens.

When purified preparations of LTA and lipid S are subjected to FPLC (1ml of a 10mg/ml solution in each case) once again fractions 8-20 demonstrated antibody-binding capacity with lipid S apparently having greater activity. However it should be noted that comparisons were not made on a molar basis.

3.3.4: Dot Blotting Analysis

To analyse fractions further dot blotting on both LTA and lipid S was performed. Because material was deposited directly onto a nitrocellulose membrane, any antigen in the fractions would be detected through binding of IgG in serum and is unlikely to be washed away in the process

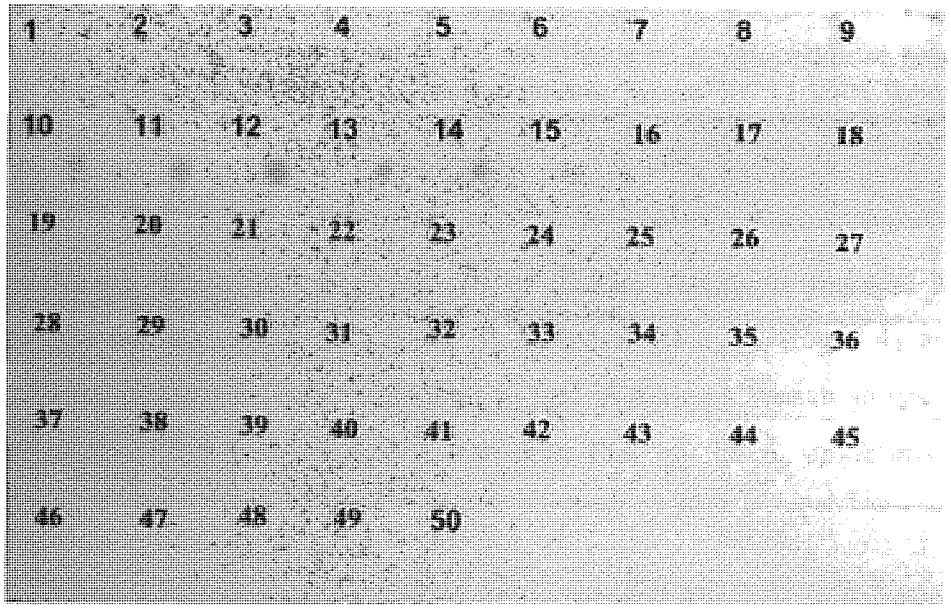


Figure 3.4: Profile from dot blotting of *S. epidermidis* culture supernatant (Lipid S) fractions onto nitrocellulose.

S. epidermidis supernatant (Lipid S) fractions 1 – 50 from the experiment shown in Fig 3.3 were administered in 1µl samples onto nitrocellulose membrane. Antigenic fractions were detected by using human serum SS (1:800) followed by HRP-protein A.

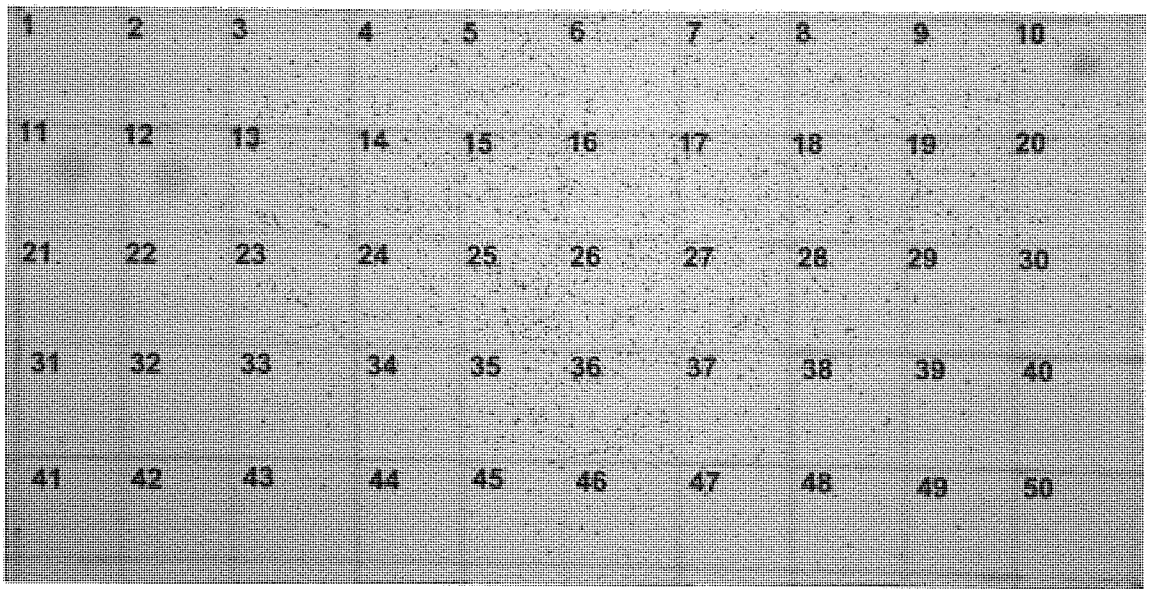


Figure 3.5: Profile from dot blotting LTA fractions onto nitrocellulose.

LTA fractions 1 – 50 were deposited in 1µl samples onto a nitrocellulose membrane. Antigenic fractions were detected by using human serum SS (1:800) followed by HRP-protein A/4 CN

These results are similar to those obtained by ELISA. Antigenic material found in *S. epidermidis* culture supernatant (lipid S) fractions 10 – 15 (Fig 3.4) and LTA fractions 10 – 12 (Fig 3.5), which can bind to IgG in immune human antiserum. The reactivity of the LTA was markedly less than that of the *S. epidermis* culture supernatant.

3.3.5: Western Blotting

To establish whether there were any differences in the electrophoretic properties of lipid S and LTA, the FPLC fractions were examined by western blotting.

In western blotting, samples are electrophoresed on a polyacrylamide gel containing the anionic detergent, SDS. Proteins are denatured by binding SDS and migrate as

negatively charged linear polypeptides. Anionic molecules such as LTA and Lipid S also migrate towards the positive electrode at rates presumed to reflect their size and charge. Separated antigen profiles are then electrophoretically transferred to a solid membrane for analysis. To detect the antigen blotted on the membrane, serum (SS 1:800) is added and incubated with the membrane. If there are any antibodies present, which are directed against one or more of the blotted antigens, those antibodies will bind. The final result is visible bands where the antibody has bound to the antigen. Successful detection of antigen by this method requires them to bind to the nitrocellulose after transfer from the gel.

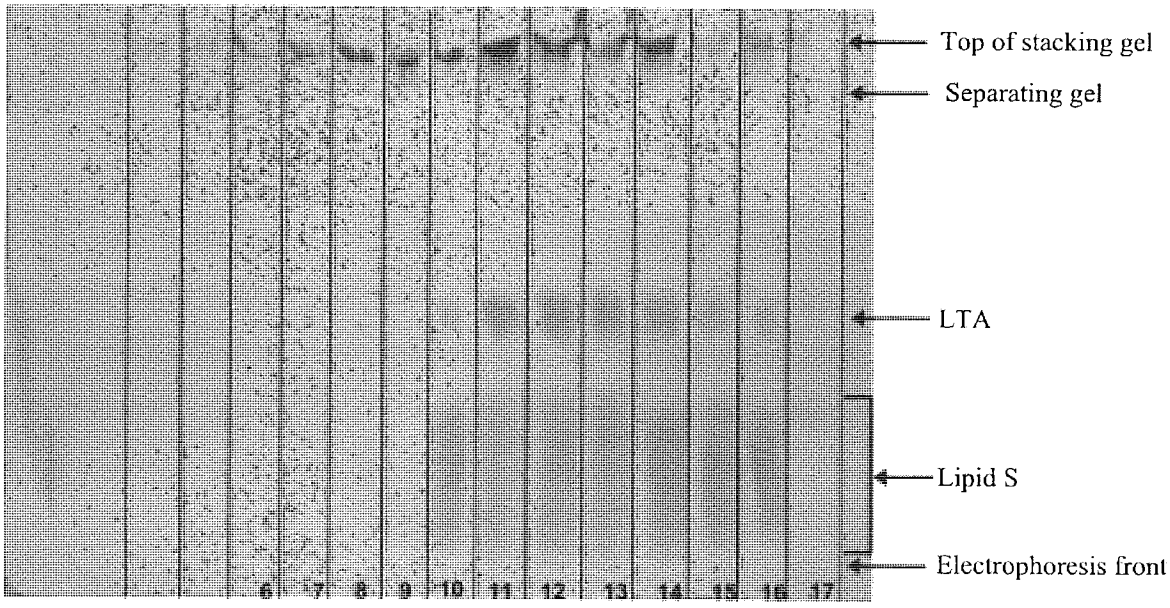


Figure 3.6: Profile of *S. epidermidis* supernatant fractions 6 – 17 on a nitrocellulose membrane, detected by a reaction with immune human serum (SS) and protein A-HRP/4 CN

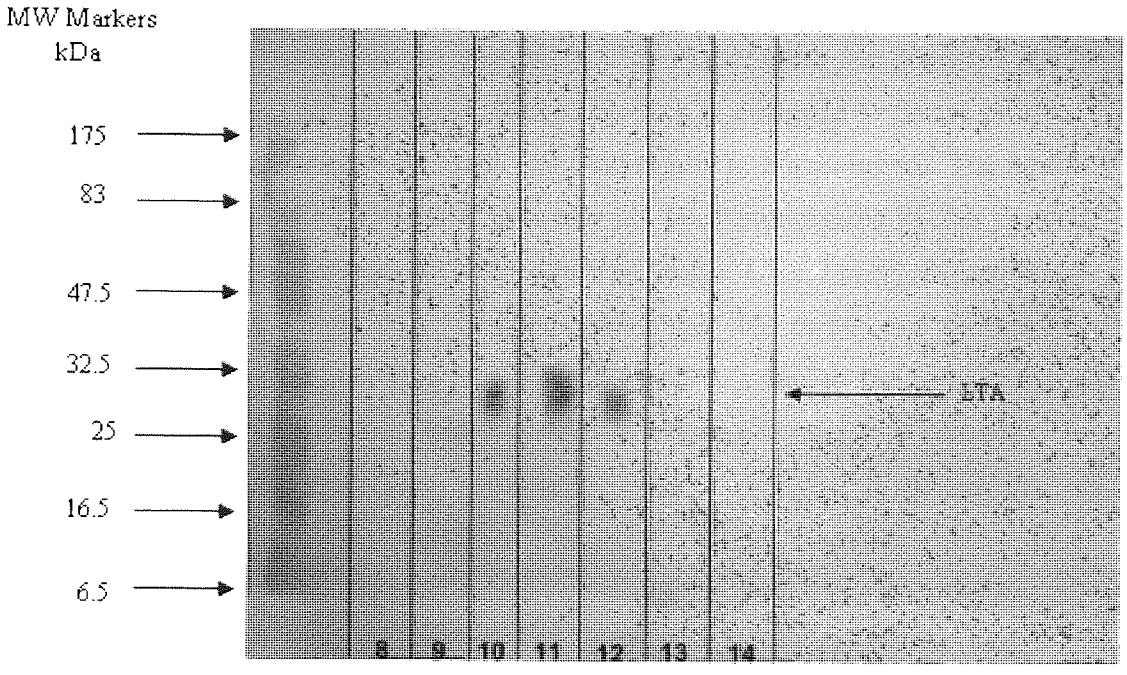


Figure 3.7: Profile of LTA fractions 8 – 14 on nitrocellulose membrane, detected by a reaction with immune human serum (SS) and protein A-HRP/4 CN

LTA was detected as a distinct central band in fractions 10 – 12 (Fig 3.7). *S. epidermidis* culture supernatant fractions showed diffuse bands at the lower portion of the nitrocellulose paper indicating it has a higher mobility than LTA (Fig 3.6). There is also a distinct band (Fig 3.6) that is in a similar position to that detected from LTA fractions, indicating that LTA is also present in the culture supernatant.

It is evident from these results that culture supernatant fractions 10 – 15 contain antigenic material. These fractions were then pooled together and freeze dried for comparison against other bacterial cell wall components. Previous work has utilised this material for measurement of patient antibody response in a range of infections. Following analysis it was shown to contain predominantly short chain length LTA and has been referred to as Lipid S. This terminology will be used throughout the thesis to denote the antigen material pooled from fractions 10 – 15.

Other cell wall components, PG and WTA, were extracted from the cell wall and analysed by ELISA, dot blotting and western blotting in the same way as LTA and

lipid S. IgG in the immune serum failed to bind to any of these components (results not shown). It should be noted that western blotting is designed to detect proteins, which normally migrate as sharp bands and bind tightly to nitrocellulose. The lipid S and LTA antigens studied here have generally not been investigated by this method and characteristic profiles have not been reported. Neither LTA, lipid S, PG nor WTA are thought to bind tightly to nitrocellulose either by direct dot blotting or electrophoretic transfer following SDS-Page. Therefore although the technique has indicated a difference between lipid S and LTA it does not provide a separation or detection suitable for further characterisation.

3.3.6: Comparison of IgG Binding to *S. epidermidis* Cell Wall Products and Lipid S by ELISA

A quantitative comparison of the antigenic activity of S. epidermidis cell wall components (PG, WTA and LTA) with Lipid S was made using ELISA.

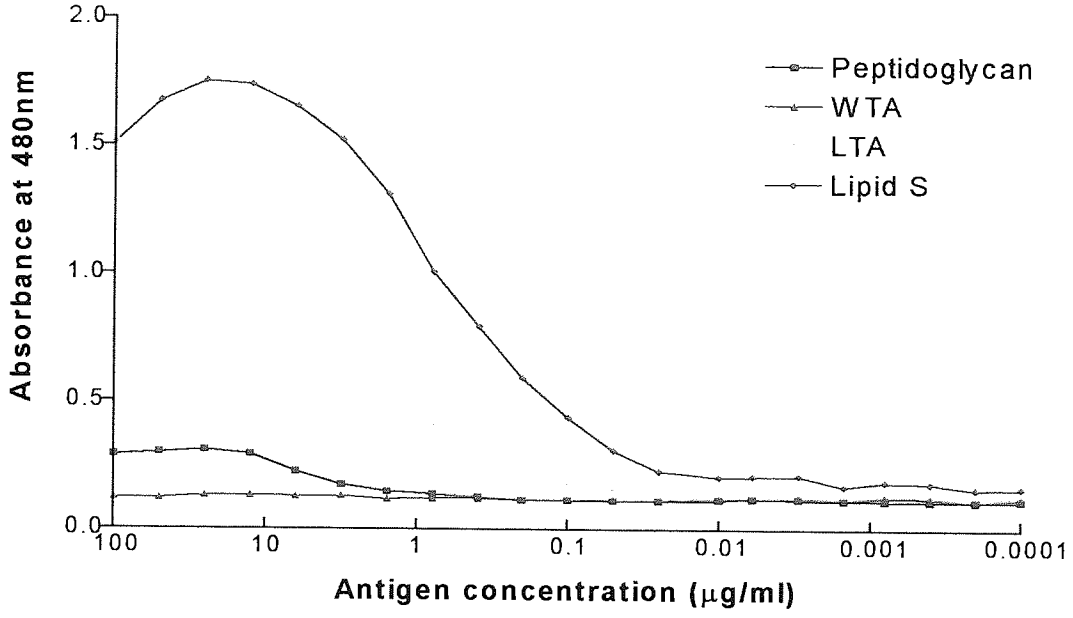


Figure 3.8: IgG antibodies in patients (SS) serum (1:3200) able to bind to LTA, WTA, PG and lipid S.

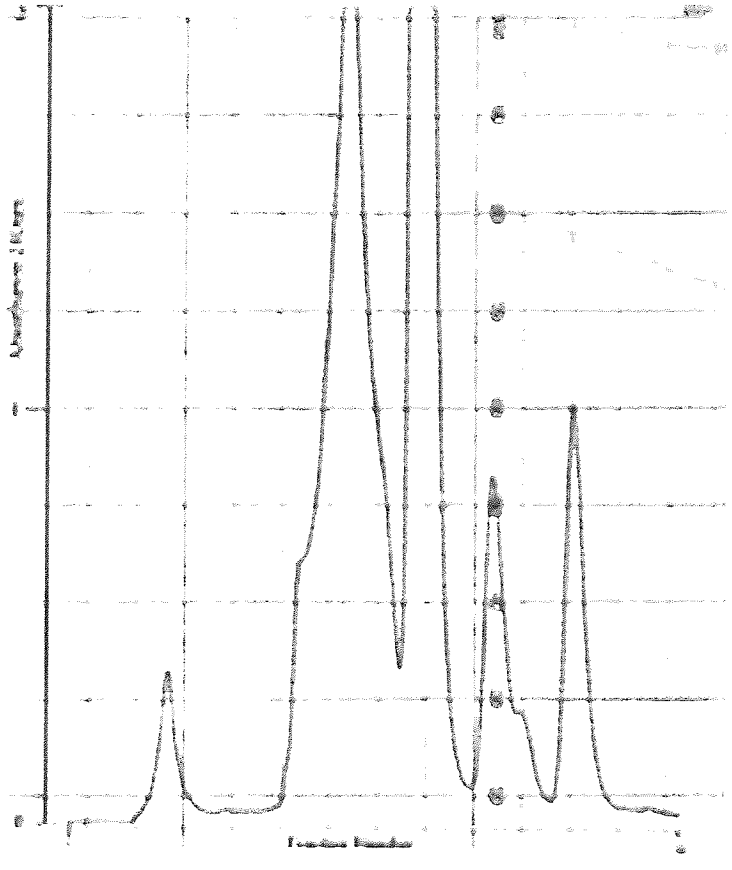
Polystyrene plates were coated with the antigens extracted from *S. epidermidis* cell wall and from the culture supernatant at concentrations ranging from 100 to 0.00001µg/ml. After blocking the wells were reacted with immune patient serum (SS) at 1:3200 dilution, followed by protein A HRP and TMB.

As found by western blotting and Dot blotting the WTA showed no evidence of antigenic material at any concentration, whereas PG showed a small reaction with patient serum at concentrations from 6-100 µg/ml. Both LTA and lipid S at high concentrations (0.1-25 µg/ml) produced high levels of reaction with IgG found in patients' sera and, as the concentration decreased, the absorbance also decreased. Superficially it seems that lipid S is more antigenic than LTA because weight for weight lipid S bound more antibody in serum than did LTA. However, the lipid S molecule is approximately six times smaller than LTA and on a molar basis the molecules have similar antigenic activity.

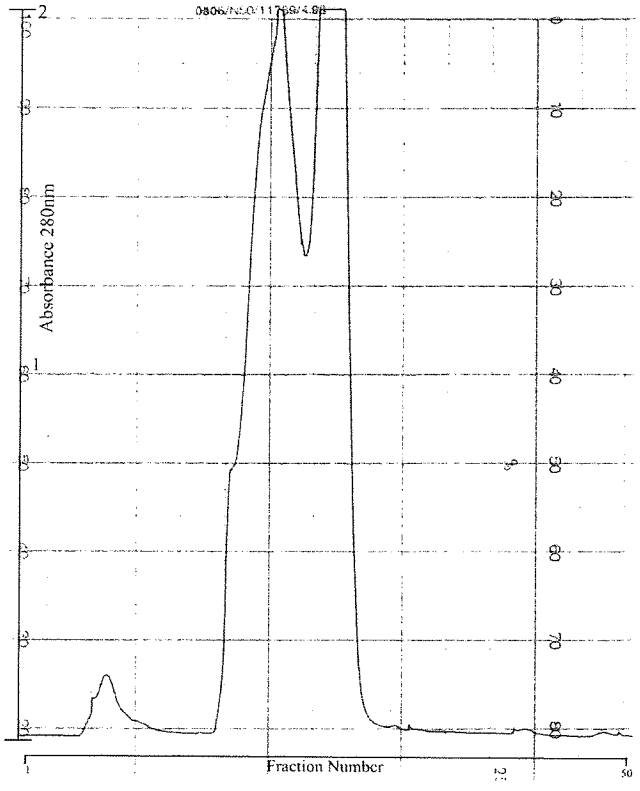
3.3.7: Analysis of antigens produced by other Gram-positive bacterial strains

To establish whether other Gram-positive bacteria could also produce extracellular antigens, *S. epidermidis* NCTC 11047; *S. aureus* NCTC 6571 *S. hominis* K ATCC 35982 and *S. aureus* NCTC 12598 were employed. These strains were grown identically to *S. epidermidis* NCIMB 40896 in HHW. Culture supernatants were analysed by FPLC, ELISA and Western blotting in the same manner as above.

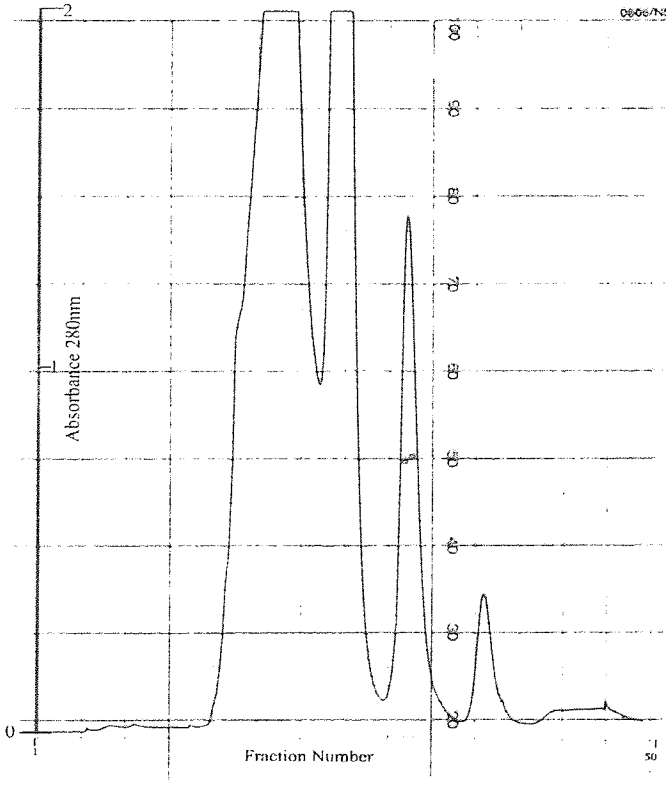
Figure 3.9: FPLC Superose 12 gel permeation elution profiles



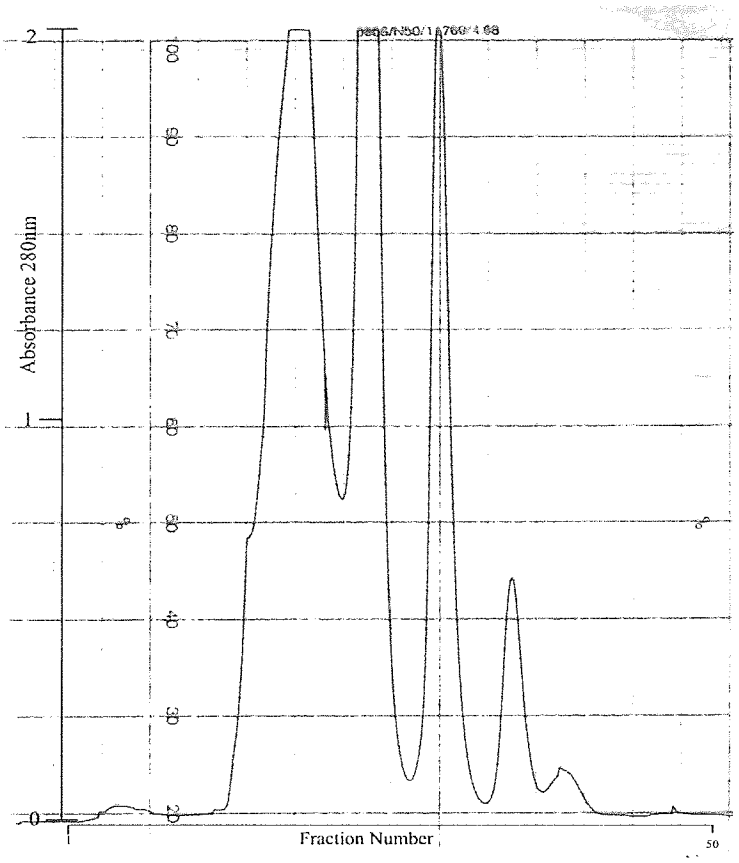
a) *S. epidermidis* NCTC 11047



b) *S. hominis* K ATCC 35982



c) *S. aureus* NCTC 6571

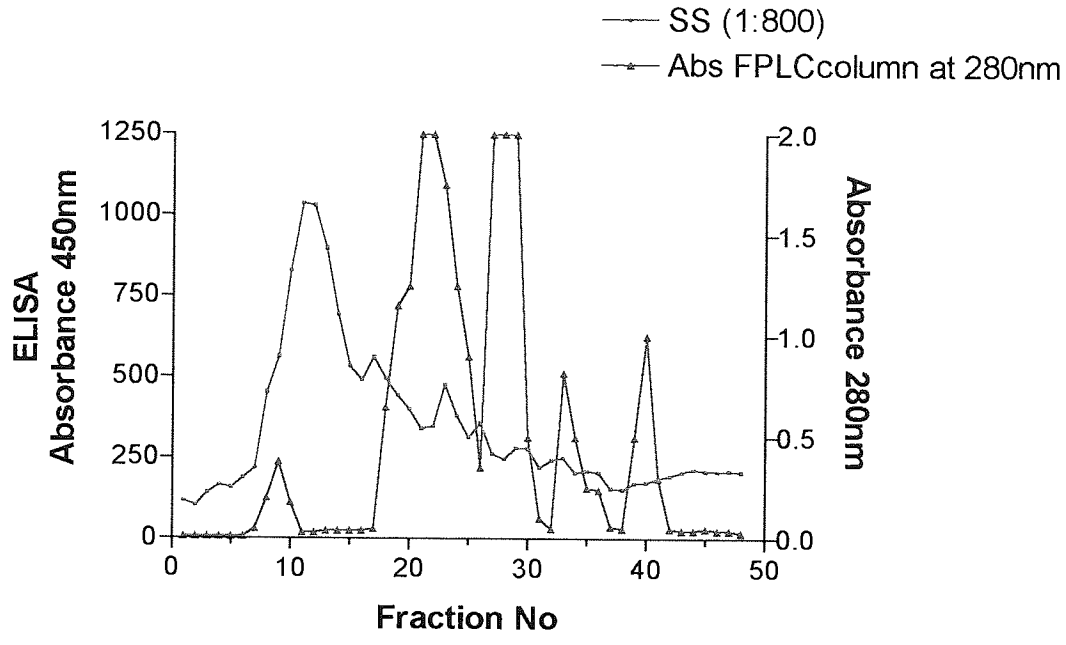


d) *S. aureus* 12598

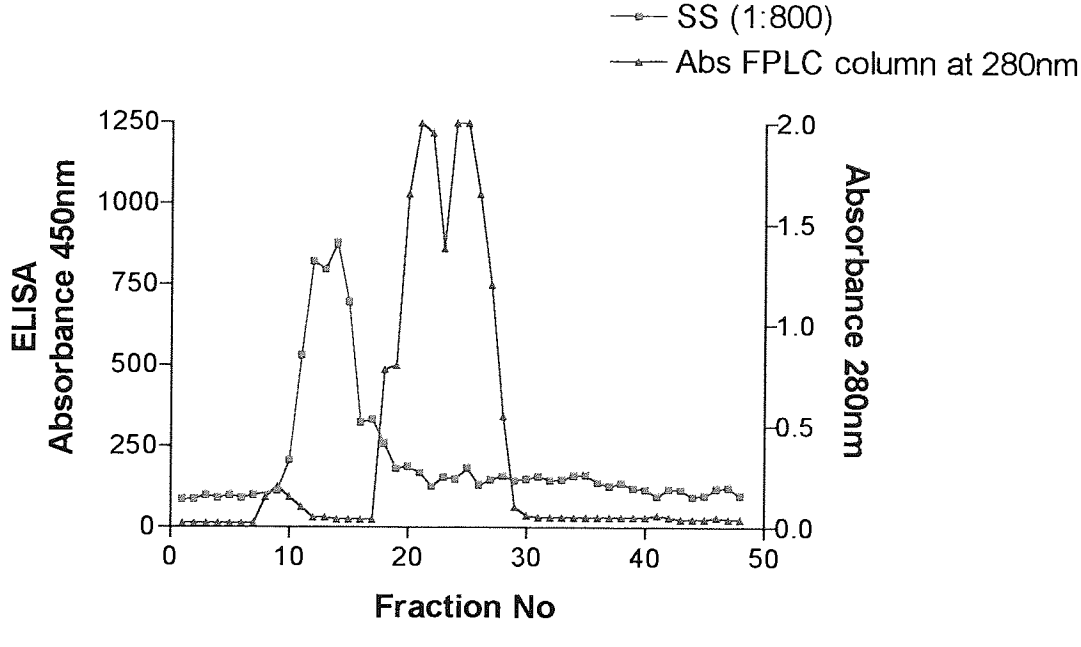
a) *S. epidermidis* NCTC 11047, b) *S. hominis* K ATCC 35982, c) *S. aureus* NCTC 6571 and d) *S. aureus* 12598 supernatant after growth in HHW medium. A 1ml volume of medium was injected onto the column and fifty samples were taken at 2 min intervals at a flow rate of 0.4ml/min and absorbance was measured at 280nm.

Although all the profiles differed visually both the strains *S. epidermidis* NCTC 11047 and *S. hominis* K ATCC 35982 had a similar small peak of UV - absorbing material to that of *S. epidermidis* NCIMB 40896 which was evident in fractions 10 – 15 (Fig 3.9 a and b). This peak was not seen in the profiles of *S. aureus* NCTC 6571 and *S. aureus* 12598 (Fig 3.9 c and d). Since LTA should not absorb at 280nm this did not reflect the presence or absence of LTA or Lipid S. To determine if there was any antigenic material in the fractions they were then analysed by an ELISA assay.

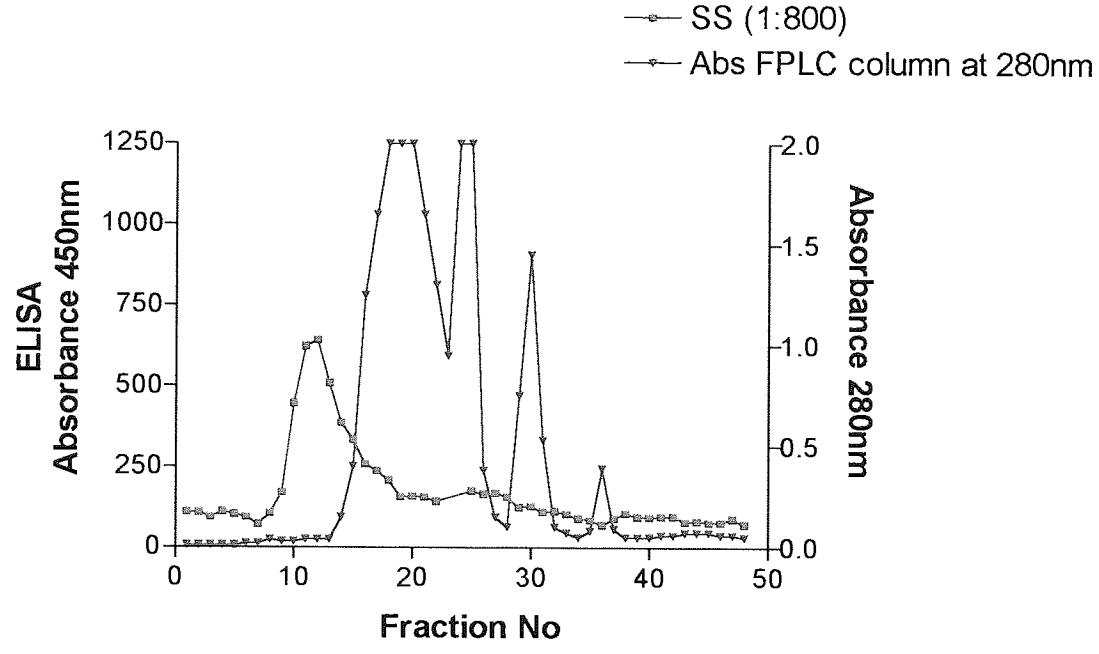
Figure 3.10: ELISA profiles of eluted FPLC fractions 1 - 48.



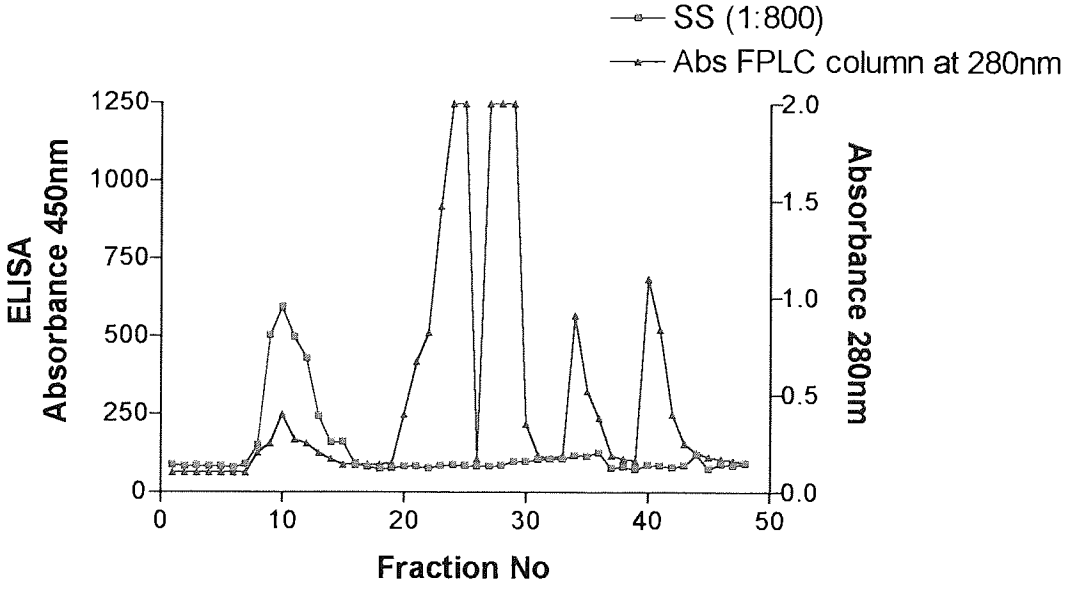
a) *S. epidermidis* NCTC 11047



b) *S. hominis* K ATCC 35982



c) *S. aureus* NCTC 6571



d) *S. aureus* 12598

Fractions were diluted 100 fold in bicarbonate buffer (pH 8.6) and coated onto a 96 well ELISA plate and reacted with human serum SS was from a patient with CVC *S. epidermidis* infection. The bound IgG was detected with Protein A –HRP in DMB.

These results indicate that the Gram-positive strains tested appear to produce material in fractions 10 - 15, which could bind to the IgG antibody in human (SS) serum which was generated during a *S. epidermidis* infectious episode.

3.4: DISCUSSION

Superose 12 gel permeation profiles of the chemically defined medium HHW and supernatant after growth of *S. epidermidis* show distinct differences in positions of peaks (Fig 3.1). To confirm reports from Lambert *et al.* (2000) that this organism produces a short chain length form of LTA (named lipid S) these fractions were analysed by an indirect ELISA system that measures IgG in serum bound to antigen (Fig 3.2). The results show that fractions 10 – 15 did contain antigenic material. Only the material in these fractions had the capacity to bind to antibody directed against *S. epidermidis* in human convalescent sera (patient SS), other fractions did not contain any immunoreactive material. In contrast, serum obtained from control patient MD (with an unrelated infection) did not contain significant levels of IgG directed against any fractions. The difference in IgG levels between serum (SS) and the control serum (MD) indicates that patients who have been exposed to *S. epidermidis* antigens produced high levels of antibody IgG against it. Fractions of HHW medium alone were also analysed (Fig 3.3), the results show that antibody in serum was not directed to any of the components of the medium.

Results obtained from gel permeation chromatography suggest that lipid S behaves as a high molecular weight material. Fractions 10 – 15, which contain the antigen immediately follow the void volume (fraction 9) and therefore contain the higher molecular weight material within the supernatant. Lipid S has previously been shown to be a short chain version of LTA having only 6 glycerophosphate units compared to 42 found in LTA (Lambert *et al.*, 2000) and therefore it would be expected to have a lower molecular weight than LTA (2,400 compared with approximately 13000 for LTA). It has been suggested that individual molecules of LTA aggregate together to form micelles which behave as large molecules on gel permeation chromatography. (Lambert *et al.*, 2000; Wicken *et al.*, 1986). Critical micelle concentrations for LTA have been reported in the range 28-60μg/ml (Courtney *et al.*, 1986). The current results suggest that the short chain length form of LTA also forms micelles at the concentration used in FPLC. The 10mg/ml solution applied to the column was equivalent to ~ 4mM (assuming molecular weight of 2400). Thus assuming the

critical micelle concentration to be in a similar range to LTA, Lipid S would migrate as micellular clusters and appear to have a high molecular weight.

IgG in patients serum (SS) reacts with lipid S and LTA (Fig 3.3), though the reaction with lipid S was markedly greater than LTA. This suggests that lipid S might be more antigenic than LTA. However, lipid S is a smaller molecule than LTA (by 6-7 fold) and activities judged by ELISA on a molar basis are roughly equivalent.

Results obtained from dot blotting confirmed those obtained from the ELISA tests, lipid S fractions 10 – 15 (Fig 3.4) and LTA fractions 10 – 12 (Fig 3.5) contained antigenic material, this was detected by antibody in serum SS binding to fractions that were recognised as antigenic. When samples were analysed by western blotting, which separates samples based upon their size and charge, LTA was detected as a single band (Fig 3.7) whereas lipid S appeared as two bands (Fig 3.6) - one band very diffuse with a fast migration rate and one sharper band higher in the gel at approximately the same position to that found in LTA. This suggests that lipid S contains two components, possibly some LTA is a contaminant of lipid S. Conditions under which electrophoretic transfer of lipid S and LTA is made to nitrocellulose are critical in determining the western blot profiles. Lipid S in particular did not bind well to the nitrocellulose membrane on transfer. Possibly the lower molecular weight enabled it to migrate through the nitrocellulose membrane.

All major components of the *S. epidermidis* cell wall, PG, WTA, LTA and lipid S in various concentrations were analysed by an ELISA test (Fig 3.8). Results showed that WTA was not detected by IgG in serum SS, whereas PG at high concentrations 12.5 - 100μg/ml showed a low level reaction, suggesting that it does have some antigenic properties. At 100μg/ml both LTA and lipid S exhibited maximum capacity to bind to antibodies in the assay. At a concentration of approximately 0.1μg/ml for LTA and 1.5ng/ml for lipid S insignificant amounts of IgG were detected.

The production of lipid S as an exocellular antigen was also demonstrated in other Gram-positive cocci. The four reference collection strains (*S. epidermidis* NCTC 11047, *S. hominis* K ATCC 35982, *S. aureus* NCTC 6571 and *S. aureus* 12598) each

showed activity in the ELISA assay. This demonstrates that the ELISA test using patient's serum can identify antibodies to an infection from a number of different strains of Gram-positive bacteria.

Previous reports investigating the possibility of a serodiagnostic test for Gram-positive infections have concluded that the high levels of pre-existing antibodies in the general population would prevent development (Plaunt and Patrick, 1991). However a serological test for the diagnosis of CVC-associated sepsis has been developed by measuring titres of antibody in patients serum bound to the lipid S antigen (Elliott, *et al.*, 2000). This test offers a diagnosis of infection, however it does have some disadvantages in that unless a patient has had a previous infection by the same organism then IgG will not be detected in serum until 10 - 14 days after the onset of infection and during this period an infection would be established and immunocompromised patients would not show significant levels of antibody. During this period where antibody is being manufactured the innate immune system is the host's primary defence against pathogens (Goldsby *et al.*, 1997). A rapid diagnosis of infection could possibly involve specific markers produced by macrophages, which play a major role in a primary infection.

Further investigations to monitor titres of IgG during the course of an infection may establish when levels fall or are heightened and if this coincided with the recovery or escalation of the infection. Therefore by monitoring IgG levels in a patient's serum may give an indication on the severity of the infection and allow assessment of treatment.

CHAPTER 4: NITRIC OXIDE AND REACTIVE OXYGEN INTERMEDIATE PRODUCTION

4.1 INTRODUCTION

Nitric oxide (NO) is a major mammalian secretory product that initiates host defence, homeostatic, and developmental functions by either direct action or intercellular signalling. NO is the product of a five-electron oxidation of the amino acid L-arginine to citrulline, mediated by nitric oxide synthase.

NO can be either cytotoxic or protective. In macrophages the biological purpose of NO is to contain and eliminate invading organisms (Parratt, 1998). It appears that constitutively produced NO from endothelial cells may offer protection against inflammatory insults whereas expression of iNOS is associated with widespread tissue damage and inflammation (Moilanen *et al.*, 1999).

The role of NO in inflammation is not completely understood however NO is highly reactive and is cytotoxic to a variety of cell types. It can cause inhibition of key enzymes in the mitochondrial respiratory chain, such as glyceraldehyde phosphate dehydrogenase and can also cause irreparable damage to DNA, hence blocking microbial proliferation and energy flow (Thiemermann, 1997). In addition, it increases vascular permeability and suppresses apoptosis in a number of cell types (Titheradge, 1999). Nitrate and nitrite are the end products of short-lived NO *in-vivo*, NO can also react with other molecules to form reactive nitrogen intermediates (RNI's) such as peroxynitrite and peryoxynitrous acid. Both these RNI's are formed by the reaction of NO with the superoxide anion (O_2^-) produced during the respiratory burst and it has been suggested that these factors are the major source of tissue damage seen during inflammation (Miles *et al.*, 1996).

After establishing that some microbial components have strong antigenic properties, experiments to determine their capacity to elicit NO production and ROI's in murine peritoneal macrophages and murine macrophage cell lines were devised. In Gram-negative septic shock LPS is known to induce NO synthase (Moncada *et al.*, 1991),

which contributes to vascular hypotension and hyporeactivity to vasoconstriction. Although it is recognised that LTA induces macrophages to produce NO which probably contributes to the symptoms of septic shock, the role of NO in Gram-positive septic shock and the nature of the several bacterium cell wall components possibly responsible are not as well known.

4.2 MATERIALS AND METHODS

4.2.1: Measurement of nitrite

One means to investigate nitric oxide formation is to measure nitrite (NO_2^-), which is one of two primary, stable and non-volatile breakdown products of NO. Nitrite was determined spectrophotometrically using the Griess reagent system (Griess, 1879). For accurate quantitation of nitrite levels, a standard sodium nitrite reference curve in the range of 0 – 200μM was prepared for each assay. Samples to be analysed were applied to a 96 microtitre plate in 50μl aliquots, 50μl of 1% w/v sulphanilamide in 5% v/v phosphoric acid was added to all wells and left to incubate at 20°C, protected from light for 10 min. Following incubation, 50μl of 0.1% N-1-naphthylethylenediamine dihydrochloride (NED) was added to all wells and again left to incubate for 10 min in the dark. The developed product, an azo compound (Fig 4.1) purple/magenta in colour, was measured at 550nm with an Anthos 2001 plate reader.

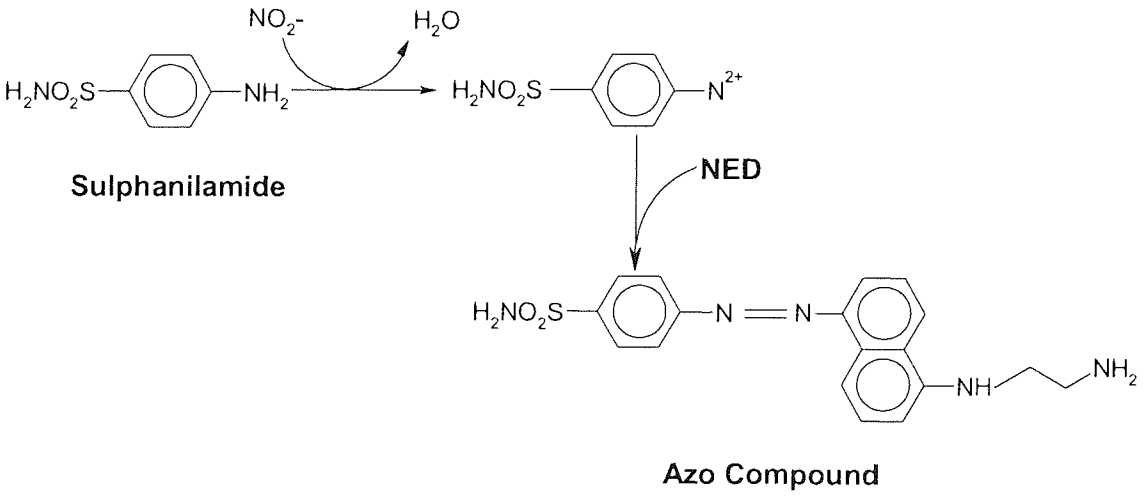


Figure 4.1: Chemical reactions involved in the measurement of NO_2^-

4.2.2: Measurement of nitrate

The other primary, stable and non-volatile breakdown product of NO is nitrate (NO_3^-). This was measured along side nitrite using a method modified from Robbins *et al.* (1994). Total NO was determined spectrophotometrically by converting nitrate to nitrite by nitrate reductase and then measuring nitrite using the Griess reagent system (4.2.1).

Before the assay could commence two solutions were prepared, the assay buffer and the assay mix. Assay buffer (14 mM sodium phosphate pH 7.4) was used to prepare the assay mix (2.5 mM glucose-6-phosphate, 400 U/L glucose-6-phosphate dehydrogenase, 200 U/L NADPH-dependent nitrate reductase).

Table 4.1: Concentration of reagents in nitrite assay

Assay constituent	Stock solution conc.	Concentration in well
Nitrate reductase	200 units/litre	80 units/litre
Glucose-6-phosphate-dehydrogenase	400 units/litre	160 units/litre
Glucose-6-phosphate	2.5mM	1mM
NADPH	10μM	1μM

Three sets of eight standards were also prepared, one for nitrite and the other for nitrate, both in the presence and absence of NADPH dependent nitrate reductase, in the range 0 -200μM. Using a 96 well microtitre plate this required 48 wells for standards, leaving the other 48 wells for test samples. Where more than one 96 well plate was used then only one set of standards was used per experiment and this determined nitrite concentrations in all plates. The assay was carried out in the following manner: nitrate and nitrite standards were prepared in 50μl aliquots on a fresh 96 well plate, and 50μl of sample culture medium was also transferred to the

plate. To half the standards and all the samples, 40µl assay mix and 10µl of 10µM NADPH was added. In wells where only nitrite was being measured then NADPH dependent nitrate reductase was omitted and 40µl assay buffer was substituted for assay mix, see 4.2.4.2

Table 4.2: Preparation of Nitrite and Nitrate Assay on 96 well plate

Assay constituent	**Nitrite Determinations**	**Nitrate Determinations**
Sample / Standard	50µl	50µl
Assay mix (4.2.4.2)	Omitted	40µl
Assay buffer (4.2.4.1)	40µl	Omitted
NADPH	10µl	10µl
Incubate 45min at room temperature, away from light, shake gently.		
Griess Reagent		
Sulfanilamide	50µl	50µl
Incubate 5 - 10min at room temperature away from light		
NED	50µl	50µl
Incubate 5 – 10 min at room temperature away from light Read absorbance 550nm		

The plate was left to incubate at room temperature for 45 minutes with gentle shaking, following incubation period sequentially Greiss reagents was added to all wells, see section 4.2.1.

4.2.3: Nitrate/ Nitrite reagents

4.2.3.1: Sodium Nitrate (Sigma)

0.1M solution in Tissue culture medium (section 2.5)

4.2.3.2: Sodium Nitrite (Sigma)

0.1M solution in Tissue culture medium (section 2.5)

4.2.4: Nitrate Assay Solutions

4.2.4.1: Assay Buffer

14mM sodium phosphate buffer pH 7.4

Made up from stock solution of:

0.1M di-sodium hydrogen orthophosphate (Na_2HPO_4) (Sigma)

0.1M sodium di-hydrogen Orthophosphate (NaH_2PO_4) (Sigma)

Mix together; adjust pH7.4 and store at 4°C. Dilute to working concentration with H_2O before use.

4.2.4.2: Assay Mix

Nitrate Reductase 200 U/L (*Aspergillus niger*, Sigma)

Store -20°C

Glucose-6-Phosphate Dehydrogenase 400 U/L (Sigma)

Store -20°C

Glucose-6-Phosphate 2.5mM (Sigma)

Store -20°C

NADPH 10μM (Sigma) freshly prepared

4.2.5: Griess reagents

4.2.5.1: Sulphanilamide Solution (Sigma)

1% sulphanilamide in 5% phosphoric acid

4.2.5.2: NED Solution (Sigma)

0.1% *N*-1-naphthylethylenediamine dihydrochloride in water

4.2.6: Luminometry - Measurement of Oxidative burst

Phagocytosis by peritoneal macrophages was assessed by measuring the oxidative burst via enhanced chemiluminescence, using a luminometer (1240 Victor, Wallac). Superoxide anions are converted by superoxide dismutase to hydrogen peroxide (H_2O_2) which reacts with lucigenin to generate light.

General equation

$$\text{Reduced lucigenin} + H_2O_2 \longrightarrow \text{Oxidised lucigenin} + \text{light}$$

A photomultiplier converts the light to an electrical signal which is expressed as relative light units (RLU).

4.2.6.1: Treatment of cells

Macrophage suspensions 5 x 10^5cells / ml were prepared in tissue culture medium (2.4.1). 50µl was added to reagents below to make a final suspension of 250µl per well in a 96 microtitre plate. The contents were allowed to equilibrate at 37°C before the test compound was added. After equilibration test compounds were added and readings taken at 5 minute intervals over a 60-minute period, or as appropriate.

4.2.6.2: Contents of wells for oxidative burst assay

The final volume in all wells contained 250µl of the following reagents:

50µl of supplemented medium (2.5.1)

50µl of 5×10^5 macrophages/ml cell suspension

50µl opsonised zymosan (4.2.9.1)

50µl lucigenin (10^{-4}M) (4.2.9.2)

50µl test compound

A well containing 50µl of 10^{-4}M lucigenin and 200µl of supplemented medium served as a blank. In control wells the test compound and opsonised zymosan was substituted with supplemented medium and a positive control included opsonised zymosan but no test compound, this was also substituted with an extra 50µl supplemented medium.

4.2.6.3: Reagents and Buffers

Supplemented medium, see section 2.5.1

4.2.6.4: Opsonised Zymosan (OZ)

50 mg zymosan A from *Saccharomyces cerevisae* (Sigma)
5 ml guinea pig complement
The two components are mixed together and incubated for 30 minutes at 37°C. The mixture was spun down (1500 rpg, 5 min), supernatant was discarded and zymosan was now coated with C3b. The pellet was resuspended in PBS to a final stock concentration of 10mg/ml and stored at 4°C.

4.2.6.5: Bis-N-methylacridinium Nitrate (Lucigenin)

Stock prepared at 2.5 x 10^{-3} M in PBS
Final concentration in wells 5 x 10^{-4} M (1:5 dilution)
Diluted in PBS for use as required
Stock stored at 4°C and protected from light.

4.3 RESULTS

4.3.1: Nitric oxide standard curves

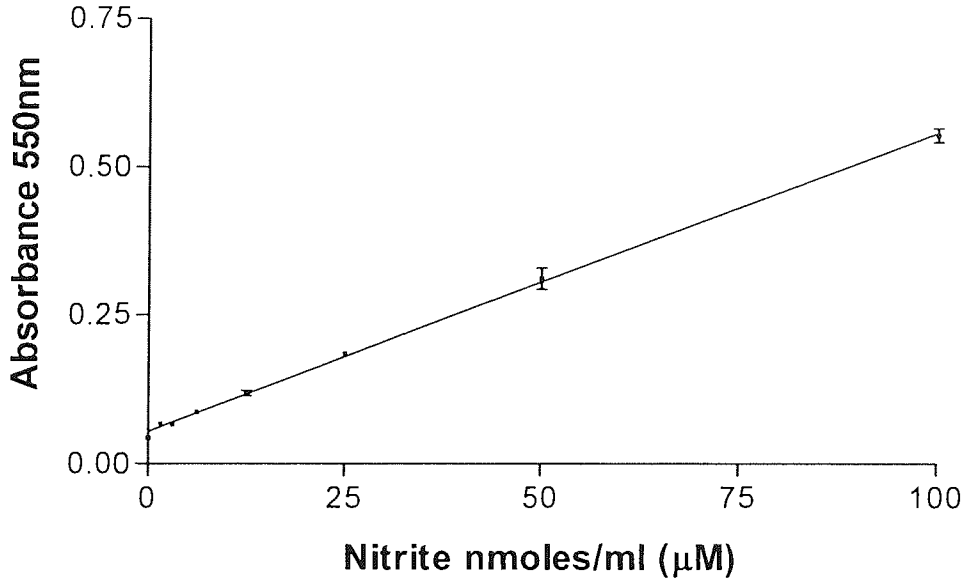


Figure 4.2: Nitrite Standard Curve

This represents a typical nitrite standard curve derived by linear dilution of a known standard nitrite solution. The standard curve was used to determine nitrite production in macrophage supernatant samples. A standard curve was generated for each set of samples assayed. Standards were performed in triplicate over the range 0 to 100μM and error bars represent the standard error of the mean.

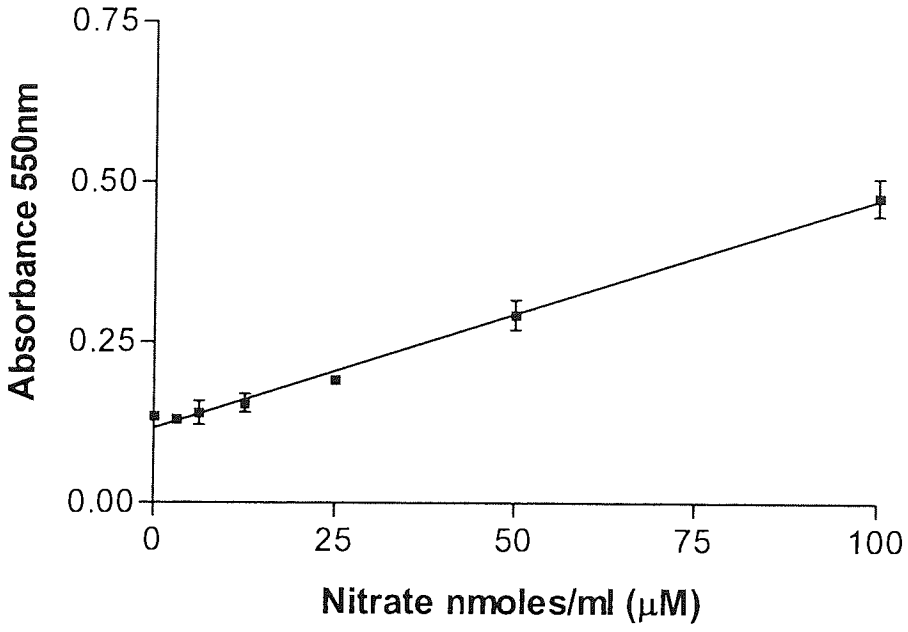


Figure 4.3: Nitrate Standard Curve

This represents a typical standard curve derived by linear dilution of a known standard nitrate solution. The standard curve was used to determine nitrate production in macrophage supernatant samples. A standard curve was generated for each set of samples assayed. Standards were performed in triplicate over the range 0 to 100μM and error bars represent the standard error of the mean.

4.3.2: Effects of bacterial cell components on nitrite production

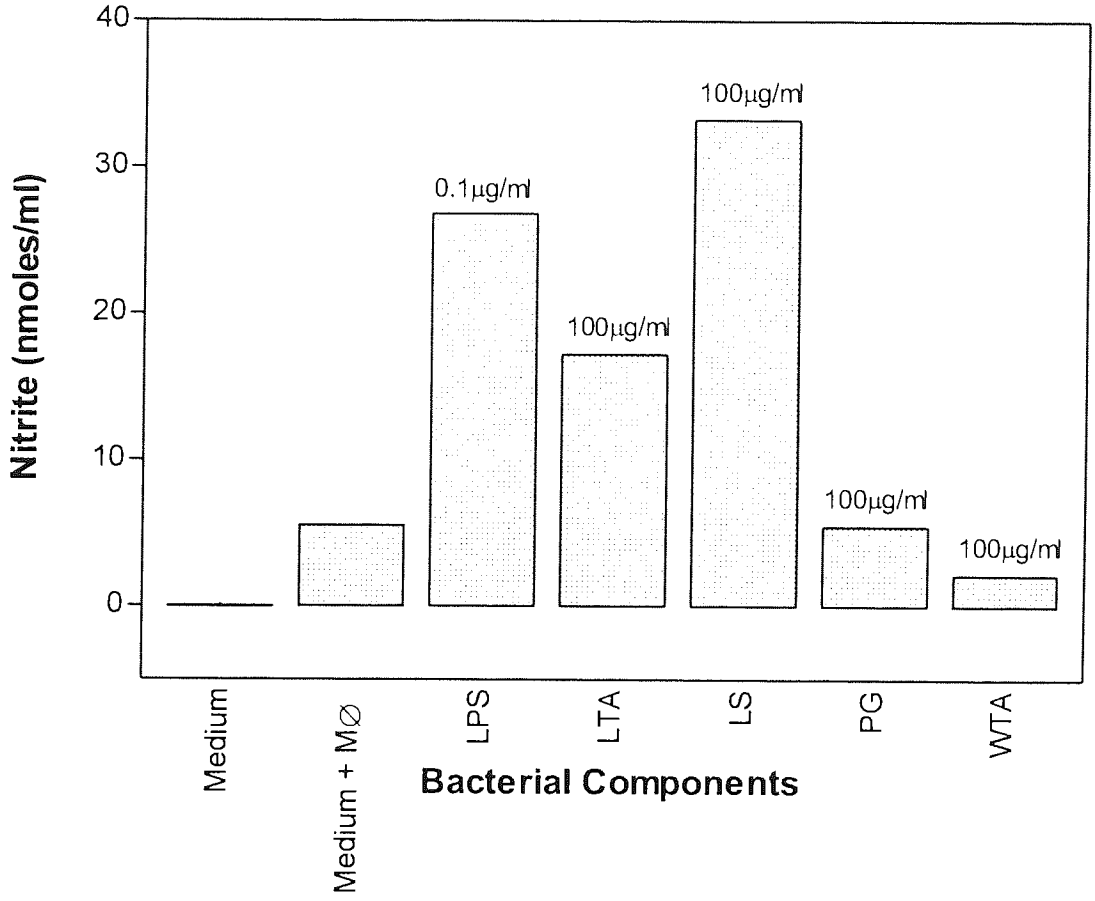


Figure 4.4: Effect of bacterial cell wall components on nitrite production by murine peritoneal macrophages.

A count of 5×10^5 peritoneal macrophages per ml, isolated by adherence were incubated at 37°C in 95% air, 5% CO_2 for 24 hours in 1 ml of supplemented medium. Cell wall components WTA, PG, LTA, lipid S from *S. epidermidis* and LPS from *E. coli* were added to wells at time 0. Controls of medium alone and medium with macrophages were assayed for comparison. Supernatants were removed after 24 hour incubation and assayed for nitrite production using the Griess reaction. Results are from a single mouse experiment.

LPS is known to be a potent inducer of NO synthesis in macrophages and was used here as a positive control at a concentration 100-fold less than the other components

to ensure the assay was working. Neither WTA nor PG induced a response above control levels. Clearly both lipid S and LTA can stimulate NO production in primary cultures of murine peritoneal macrophages. Further experiments were conducted to observe NO formation with different concentrations of antigen.

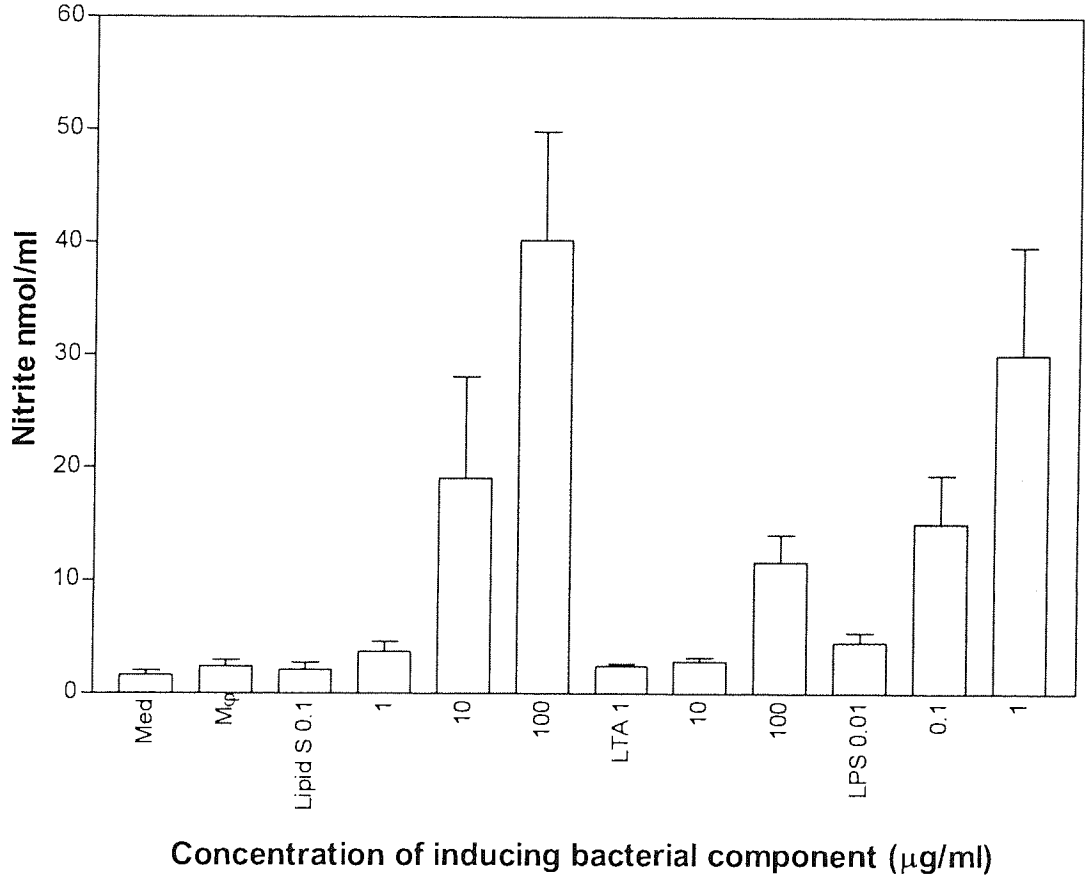


Figure 4.5: Effect of bacterial cell wall components on nitrite production by murine peritoneal macrophages.

A count of 5×10^5 peritoneal macrophages per ml, isolated by adherence were incubated at 37°C in 95% air, 5% CO_2 for 48 hours in 1 ml of supplemented medium. Cell wall components, LTA and lipid S from *S. epidermidis* and LPS from *E. coli* were added to wells at time 0. Controls of medium alone and medium with macrophages were assayed for comparison. Supernatants were removed after 48 hour incubation and assayed for nitrite production using the Griess reaction. Bars are standard error of the mean of 4 samples from 4 MFI mice.

Significant stimulatory effects on the production of nitrite compared to the macrophage control were observed for Lipid S 100μg/ml ($p = 0.008$), LTA 100μg/ml ($p = 0.01$) and LPS 0.1μg/ml ($p = 0.026$) and LPS 1μg/ml ($p = 0.0027$). No significant changes were seen for all other doses.

Because cell yields were relatively low, limiting the number of assays which could be achieved from a single mouse, two murine cell lines were examined for their capacity to produce NO in response to bacterial cell wall components.

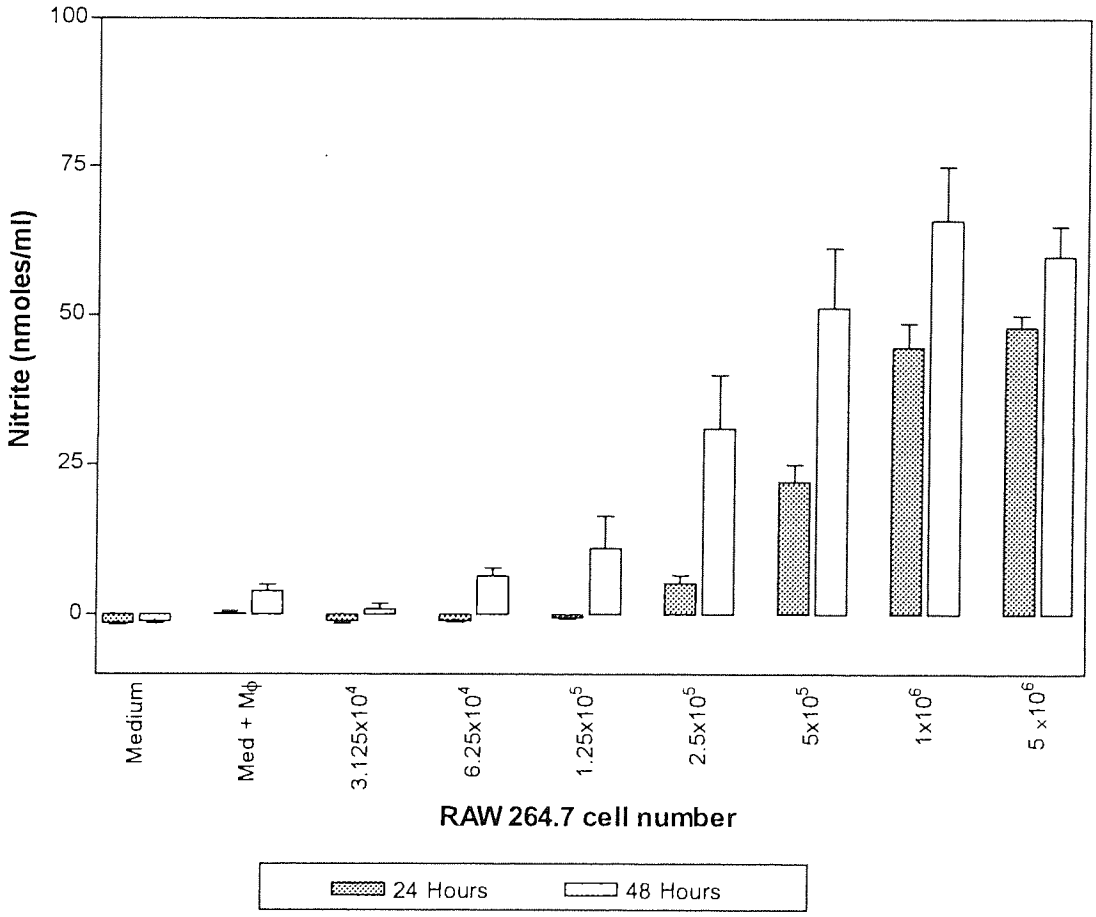


Figure 4.6: Effect of LPS on nitrite production by RAW 264.7.

Cells were incubated at 37°C in 95% air, 5% CO_2 in 1ml of supplemented medium with the addition of LPS 100ng/ml. Supernatant was collected 24 and 48 hours after incubation and assayed using the Griess reaction. Bars are standard error of the mean of 3 samples

LPS (100ng/ml) clearly elicited NO production by RAW 264.7 cells. With increasing cell number output of nitrite at 24 or 48 hours increased proportionately. Slight inhibition was observed at the highest density of 5×10^6, possibly due to contact inhibition of cells. Significant values after 48 hours incubation were obtained for cell densitys above 2.5 × 105 and after 24 hours for cell density 5×10^5 and above ($p = <0.05$) Using 1×10^6 cells/ml, high levels of nitrite could be detected after 24 hours of culture so this density was employed in all subsequent experiments.

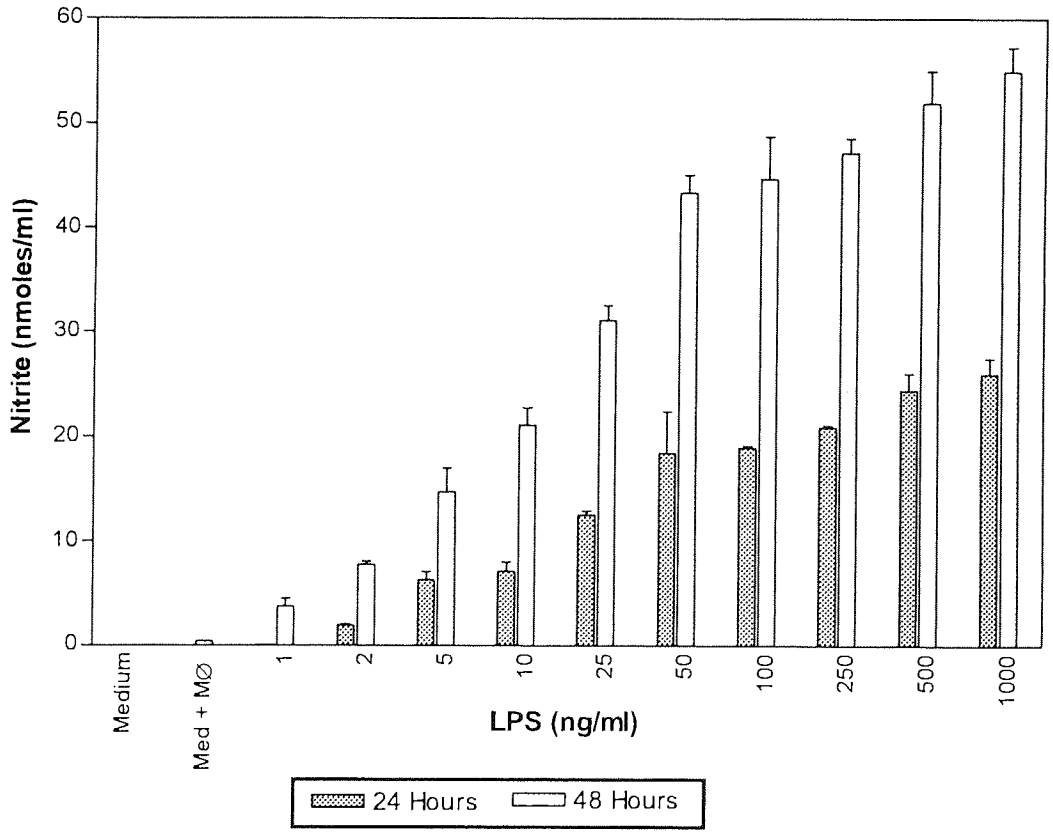


Figure 4.7: Effect of LPS from *E .coli* on nitrite production by RAW 264.7 cells (1×10^6/ml)

Nitrite was measured 24 and 48 hours after incubation with various concentrations of LPS. Bars are standard error of the mean of 3 samples

Results clearly show that there is a linear relationship between the concentration of LPS and nitrite produced from RAW 264.7 cells. Peak production was achieved after 48 hours with the highest dose of LPS compared to the macrophage control ($p = <0.001$). A similar pattern was produced using both adherent and non-adherent cells of the J774.2 cell line (Figure 4.8).

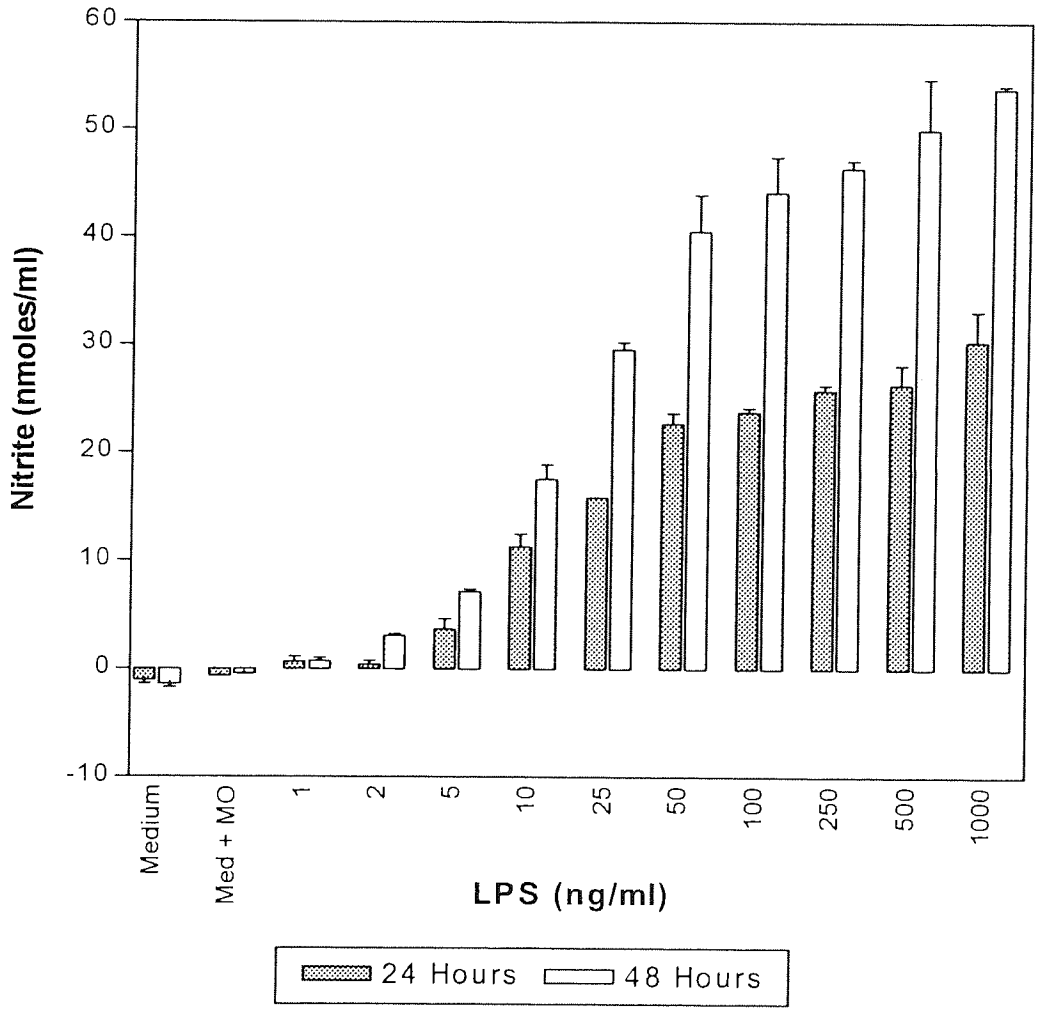


Figure 4.8: Effect of LPS from *E. coli* on nitrite production by J774.2 cells (1×10^6/ml).

Nitrite was measured 24 and 48 hours after incubation with various concentrations of LPS. Results are in triplicate and bars are standard errors of the mean.

After establishing the experimental procedures with LPS, products of Gram-positive bacteria were investigated for their capacity to elicit nitrite production.

For figures 4.9 – 4.13, assays were performed and observed under the same conditions; RAW 264.7 (1×10^6) cells in 1 ml of supplemented medium were incubated with antigens at 37°C in 95% air, 5% CO_2 for 24 and 48 hours. LPS 100ng/ml was used as a positive control, medium alone and with macrophages served as negative controls. Bars are standard error of the mean of three samples.
WTA did not induce nitrite release at any concentration (results not shown).

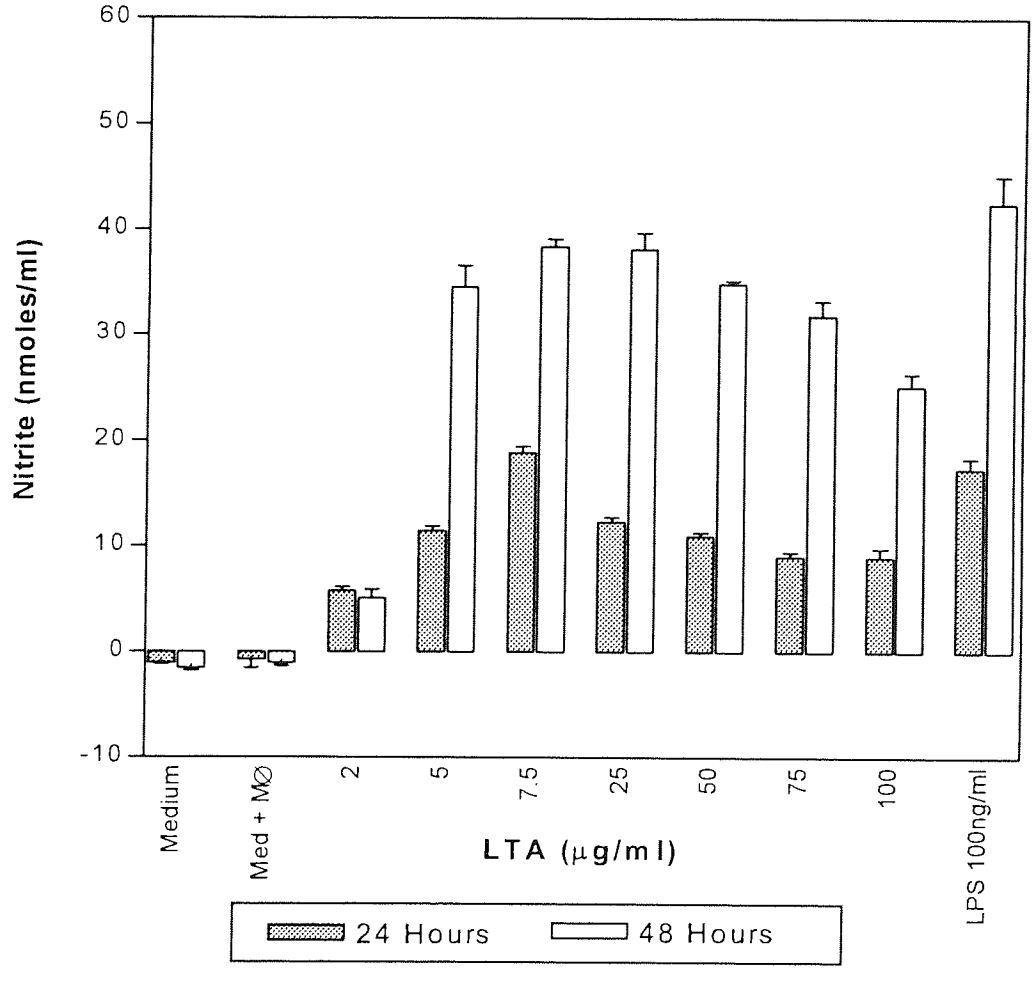


Figure 4.9: Effect of LTA from *S. epidermidis* on nitrite production from RAW 264.7 cells (1×10^6/ml).

Nitrite production increased with increasing doses of LTA, peak production was achieved at 48 hours with a dose of 25μg/ml compared to the macrophage control (p = <0.001). After 24 hours peak production was observed at a lower LTA concentration of 7.5μg/ml (p = <0.001). There is evidence of inhibition at higher concentrations, possibly as a result of micelle formation of the LTA solution or toxicity to cells.

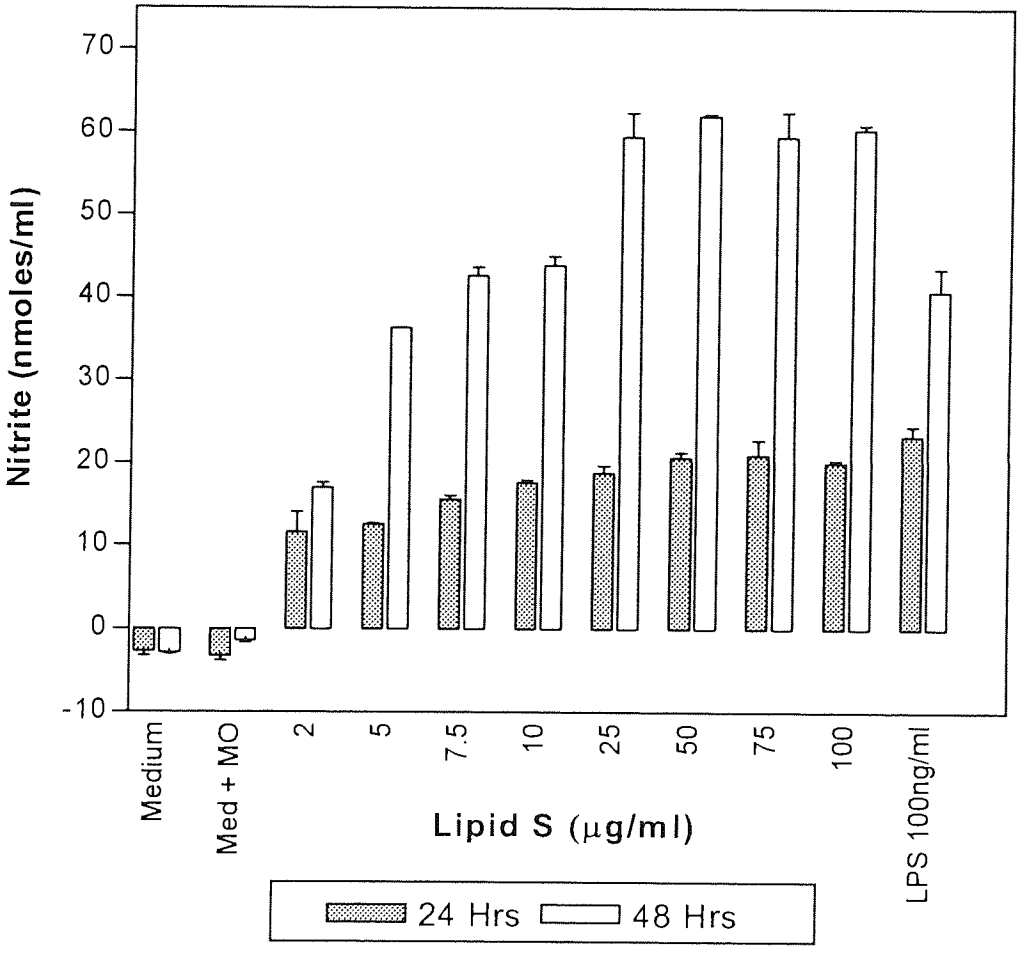


Figure 4.10: Effect of lipid S from *S. epidermidis* on nitrite production by RAW 264.7 cells (1×10^6/ml).

Low doses of lipid S (2 - 25μg/ml) significantly increased nitrite output compared to the macrophage control (p = <0.001). Higher doses of lipid S (50 - 100μg/ml) were

also significant (p = <0.001) but elicited no further increase possibly because receptors for the lipid were fully saturated.

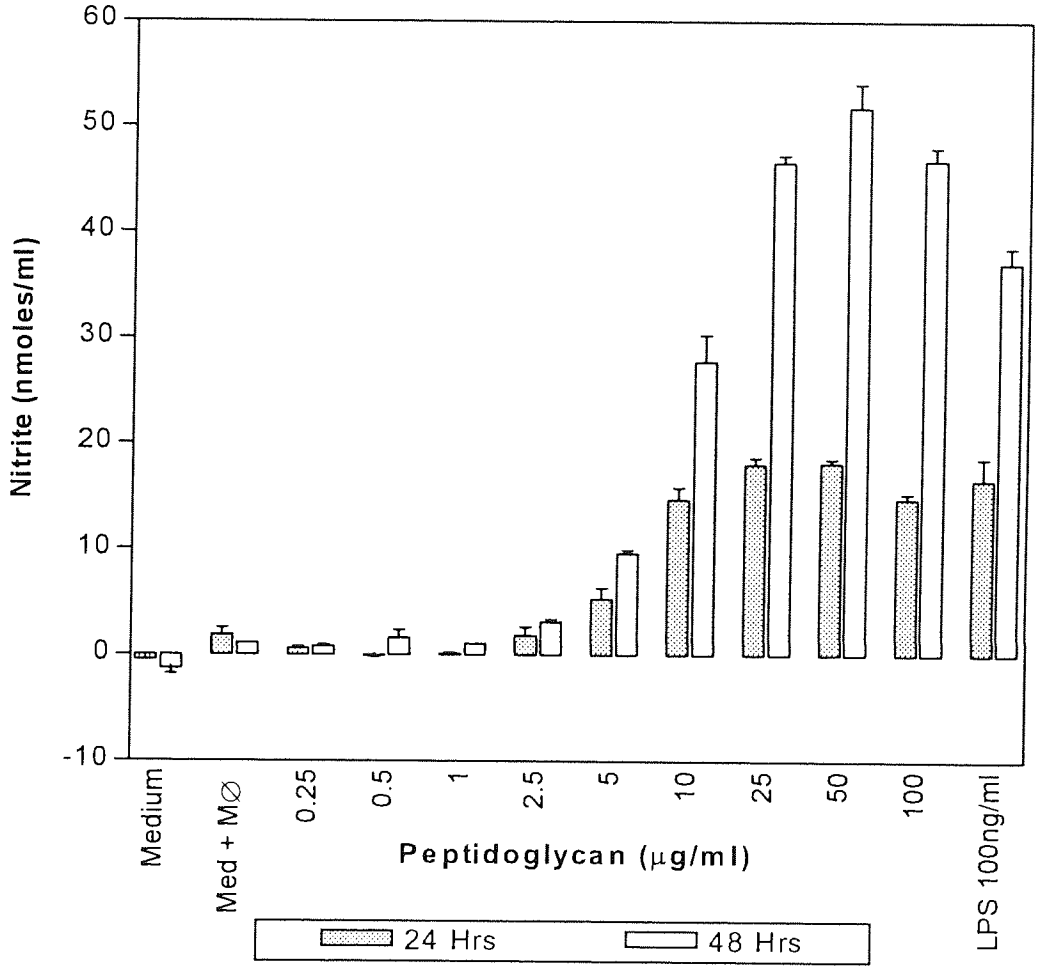


Figure 4.11: Effect of PG from *S. epidermidis* on nitrite production by RAW 264.7 cells (1×10^6/ml).

After PG application (1 - 50µg/ml), nitrite release was progressively enhanced. Concentrations above 5µg/ml at 48 hours and above 10µg/ml after 24 hours were significantly different compared to the macrophage control (p = <0.05). Some inhibition was seen at 100µg/ml and, in most instances, levels of nitrite after 48 hours were double or more those observed after 24 hours. Nitrite production with doses of PG 10µg/ml and higher were all significantly greater at 48 hours compared to a 24 hour incubation (p = <0.05).

It has been suggested that muramyl dipeptide (MDP), the minimal component of PG is responsible for the biological activity of PG (Weidemann *et al.*, 1997) and it can synergise with LPS or LTA to induce the release of cytokines (Yang *et al.*, 2001). This component and LTA from *S. aureus* were tested in the same manner as previous nitrite experiments.

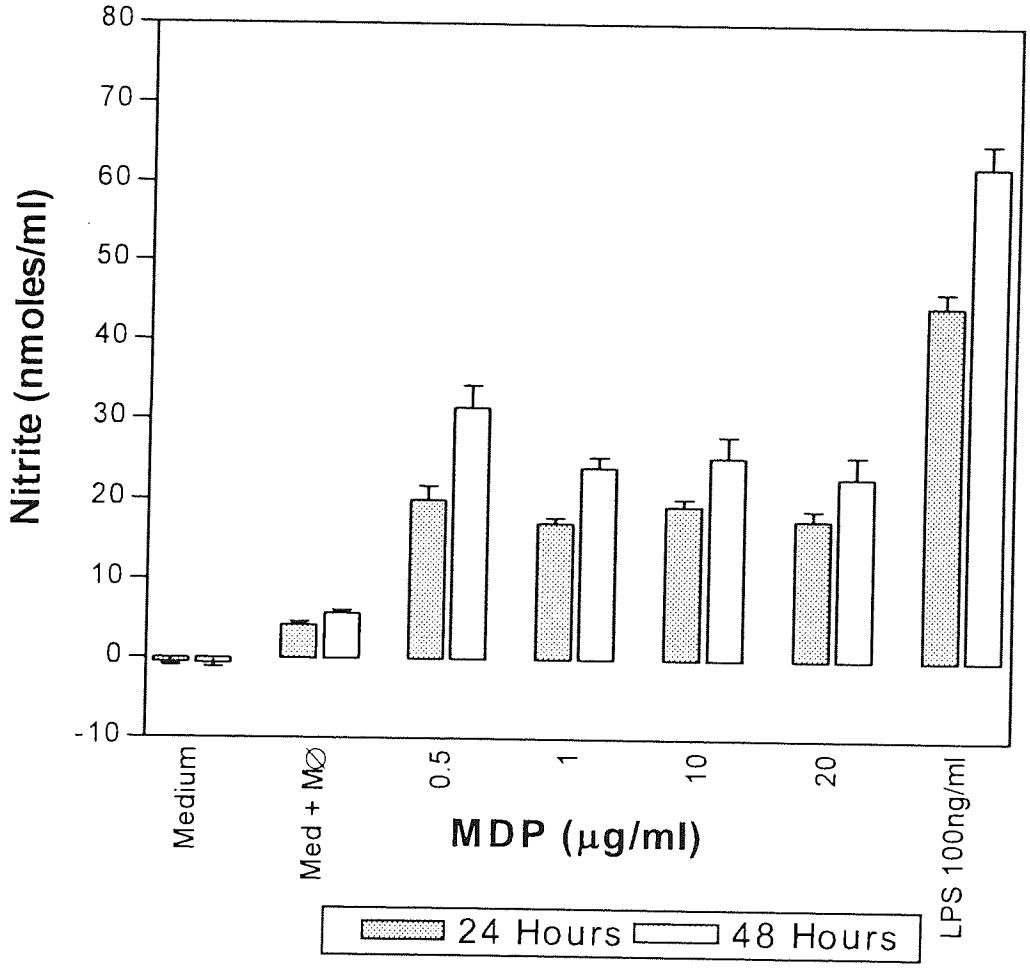


Figure 4.12: Effect of MDP from *S. aureus* on nitrite production by RAW 264.7 cells (1×10^6/ml).

Over the range of concentrations of MDP 20 – 0.5µg/ml little variation in nitrite formation was observed, however significant increases compared to the macrophage

control were observed for all doses (p = <0.05). Further investigations would have to be made to investigate any dose response for MDP at lower concentrations.

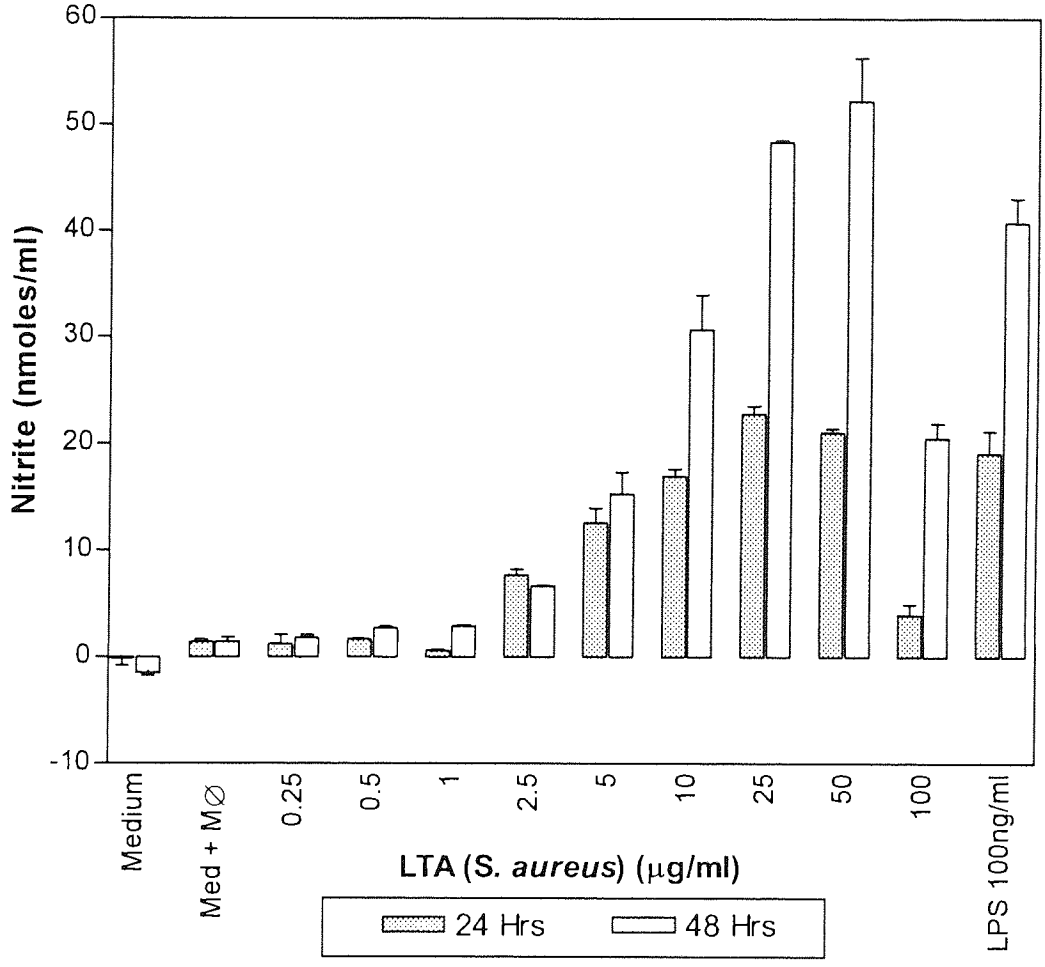


Figure 4.13: Effect of LTA from *S. aureus* on nitrite production by RAW 264.7 cells (1×10^6/ml).

Nitrite levels increased as the dose of LTA increased from 1 to 50µg/ml, a high concentration of 100µg/ml inhibited the release of nitrite. Significant increases in nitrite production were observed for doses 2.5µg/ml and above (p = <0.05) after 48 hours and 2.5µg/ml to 50µg/ml (p = <0.05) after 24 hours compared to the macrophage control.

With the exception of WTA, all bacterial wall products from both *S. epidermidis* and *S. aureus* induced the macrophage cell line RAW 264.7 to produce nitrites in a dose dependent manner (Figs 4.9 – 4.13). LTA from *S. aureus* like that from *S. epidermidis* stimulated NO production and seemed perhaps more effective, although doses are expressed as μg/ml and not in molar terms. Furthermore the comparisons were not made on the same cells within a single experiment (compared Fig 4.9 and 4.13). Lipid S from *S. epidermidis* showed similar activity to LTA from the same organism (Fig 4.9 and 4.10).

When macrophages of the J774.2 cell line were challenged with bacterial components a similar dose dependent pattern emerged (results not shown), although levels of nitrite produced were lower than those seen with RAW 264 cell line, an example is shown using lipid S (Fig 4.14).

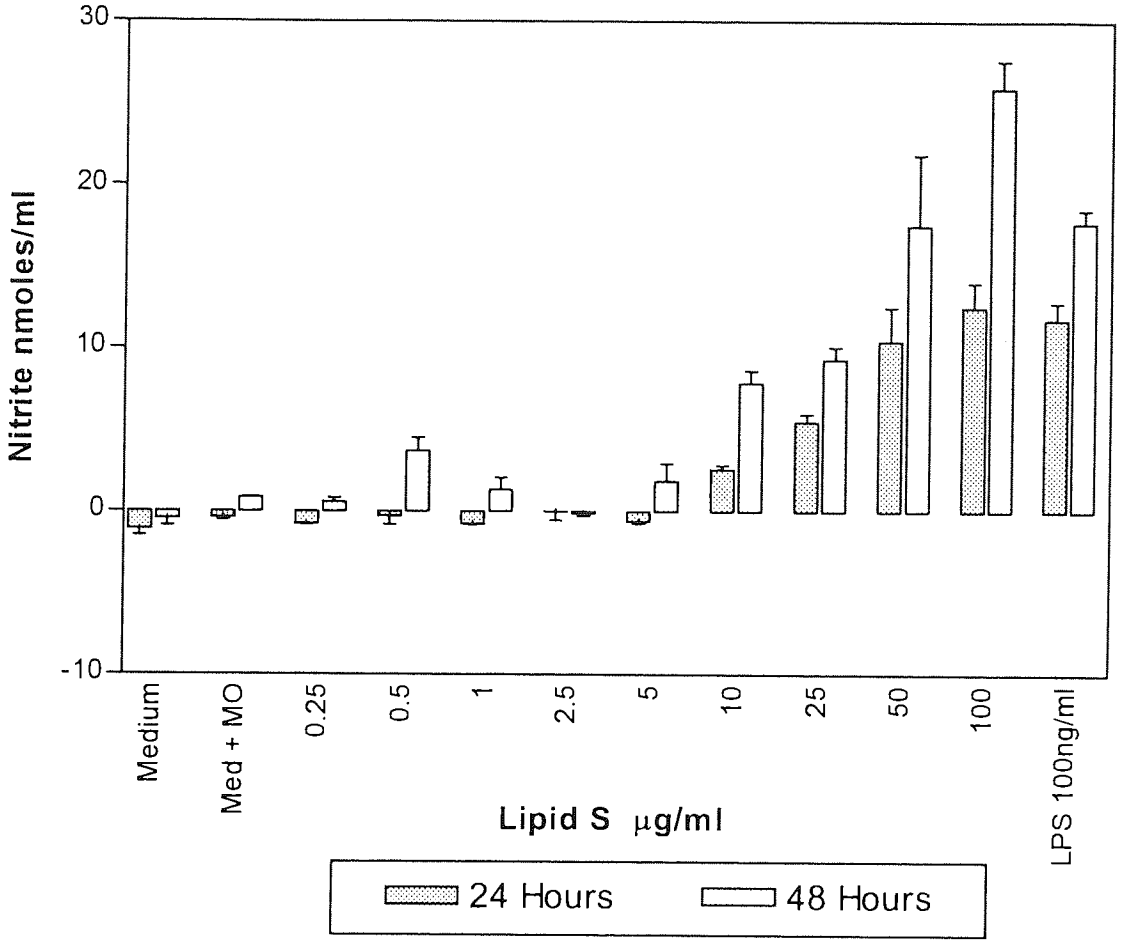


Figure 4.14: Effect of Lipid S from *S. epidermidis* on nitrite production by J774.2 cells (1×10^6/ml).

J774.2 cells (1×10^6) cells in 1 ml of supplemented medium were incubated with antigens at 37°C in 95% air, 5% CO_2 for 24 and 48 hours. LPS 100ng/ml was used as a positive control, medium alone and with macrophages served as negative controls. Bars are standard error of the mean of three samples.

Concentrations ranging from 10 – 100 µg/ml were able to elicit a dose-dependent production of nitrite and all doses in this range were significant compared to controls ($p = <0.05$).

4.3.3: Effects of a combination of bacterial cell components on nitrite production

The results from the dose response assays above indicated that the amount of nitrite formed was dependent on three factors, the nature of the bacterial component, the concentration used and the duration of treatment. However during a Gram-positive bacterial infection a number of these components would be present together. It has previously been shown that LTA and PG from *S. aureus* work in synergy to stimulate nitric oxide production (Kengatharan *et al.*, 1996b). This combination was investigated using LTA and PG from *S. epidermidis* (Fig 4.15 and 4.16) and the combined effects of lipid S and PG was also examined (Fig 4.17 and 4.18)

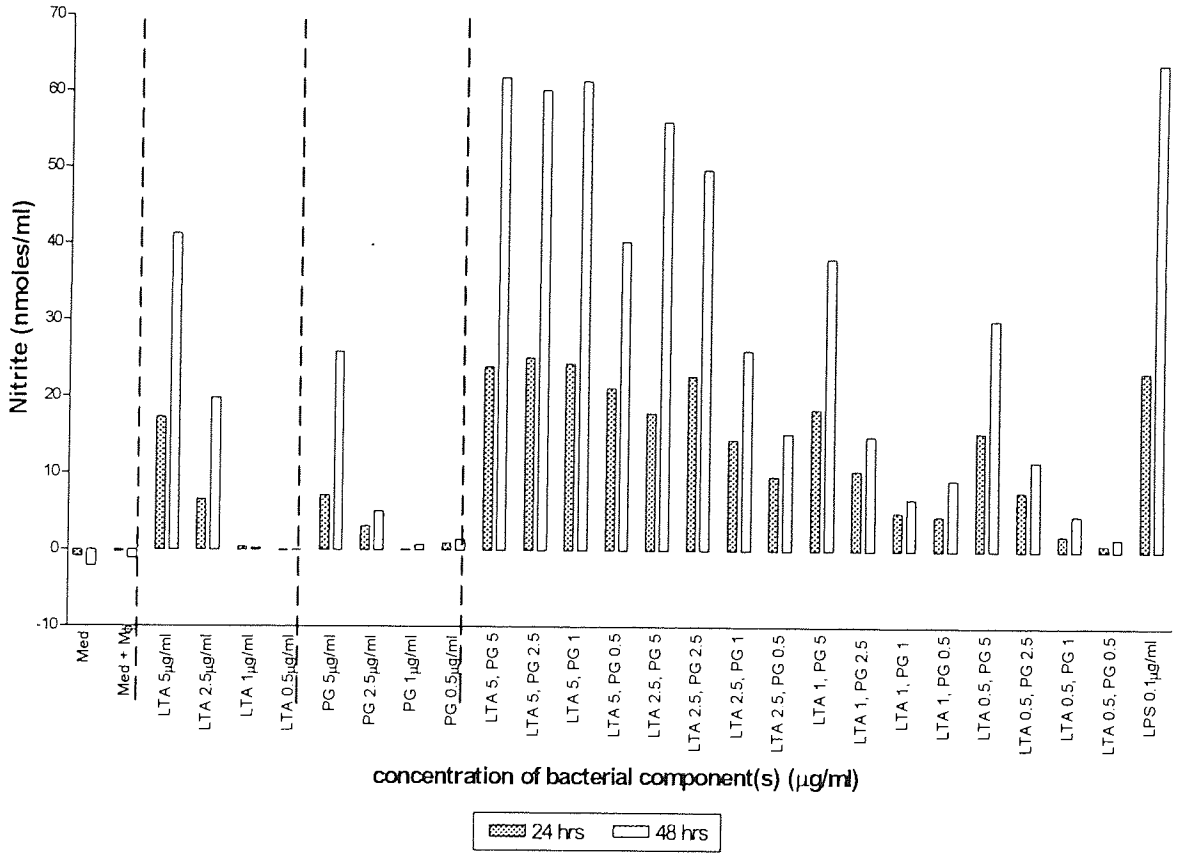


Figure 4.15: The effects of combinations of LTA and PG isolated from *S. epidermidis* on nitrite produced from RAW 264.7 cells.

Columns represent bacterial components alone or in combination after a 24 and 48 hour incubation. Results are the average of duplicate samples from a single experiment.

Experiments employing LTA and PG (*S. epidermidis*) in combination to provoke NO production are shown in Fig 4.15 and the combinations showing enhanced release of NO are shown in Fig 4.16.

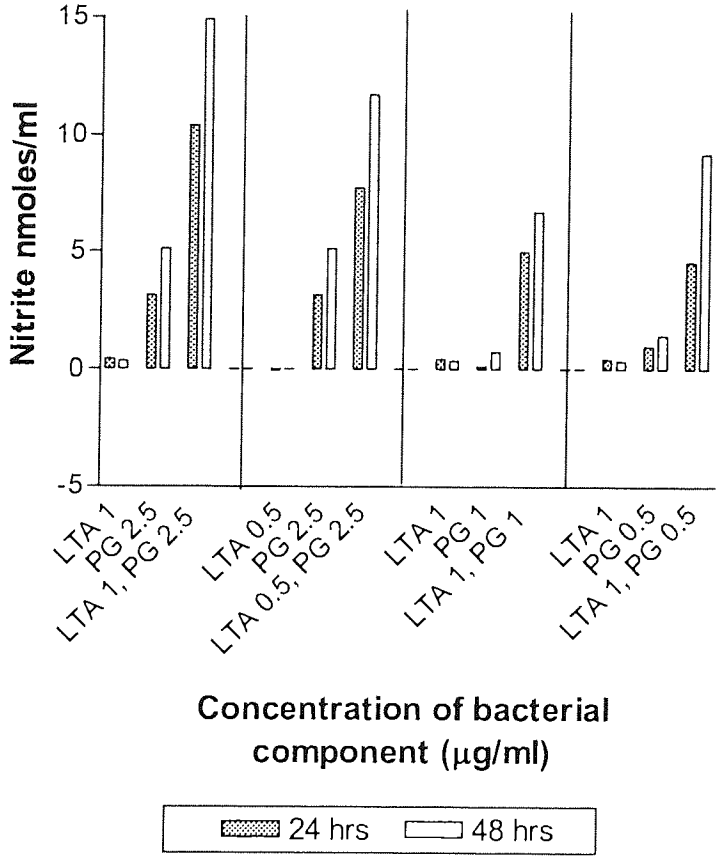


Figure 4.16: The effect of a combination of LTA and PG isolated from *S. epidermidis* on nitrite produced from RAW264.7 cells (1×10^6/ml).

Generally high concentrations of both materials were additive in their effect (Fig 4.15). Figure 4.16 shows clear evidence of synergy between LTA and PG at lower concentrations (especially 1µg/ml each).

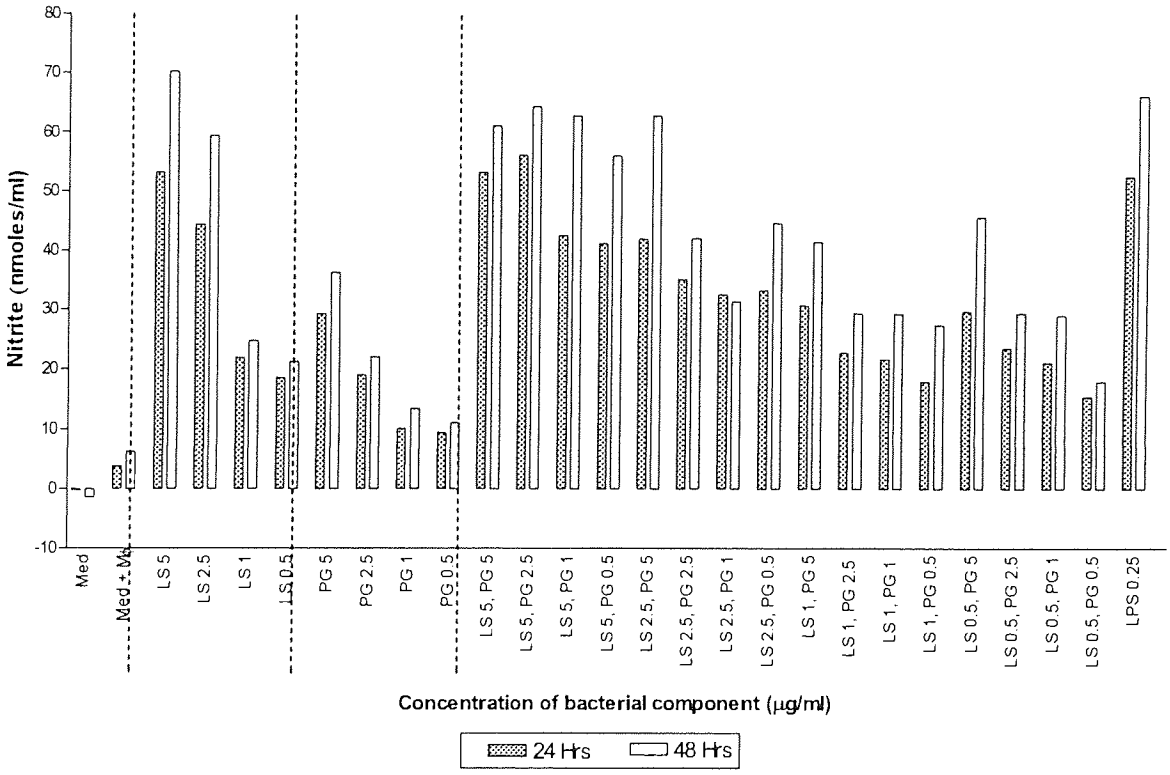


Figure 4.17: The effect of a combination of Lipid S and PG isolated from *S. epidermidis* on nitrite produced from RAW 264.7 cells (1×10^6/ml).

Data represents results from the mean of two samples.

Experiments employing lipid S in combination with PG to elicit NO production are shown in Fig 4.17. High concentrations of both compounds did not increase nitrite production, in fact PG (0.5 – 5 μg/ml), in combination with lipid S (2.5 – 5μg/ml) had an inhibitory effect. However low concentrations of lipid S (0.5μg/ml) and PG (1 - 2.5μg/ml) did increase nitrite above levels produced by either component, which suggests an additive rather than synergistic effect (Fig 4.18).

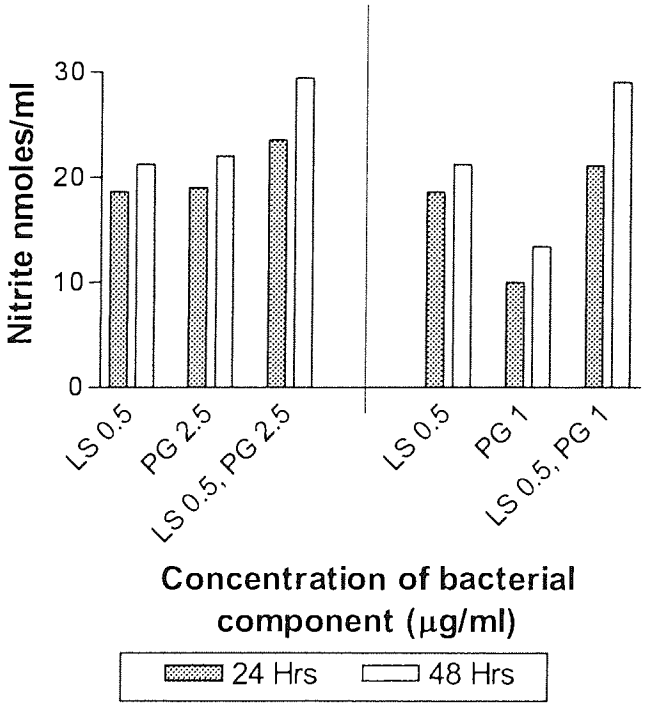


Figure 4.18: Evidence of a combination of lipid S and PG solution from *S. epidermidis* on nitrite produced by RAW264.7 cells (1×10^6/ml).

Data represents results from the mean of two samples.

4.3.4: Effects of doses of lipid S extracted from different Gram-positive bacteria on nitrite production

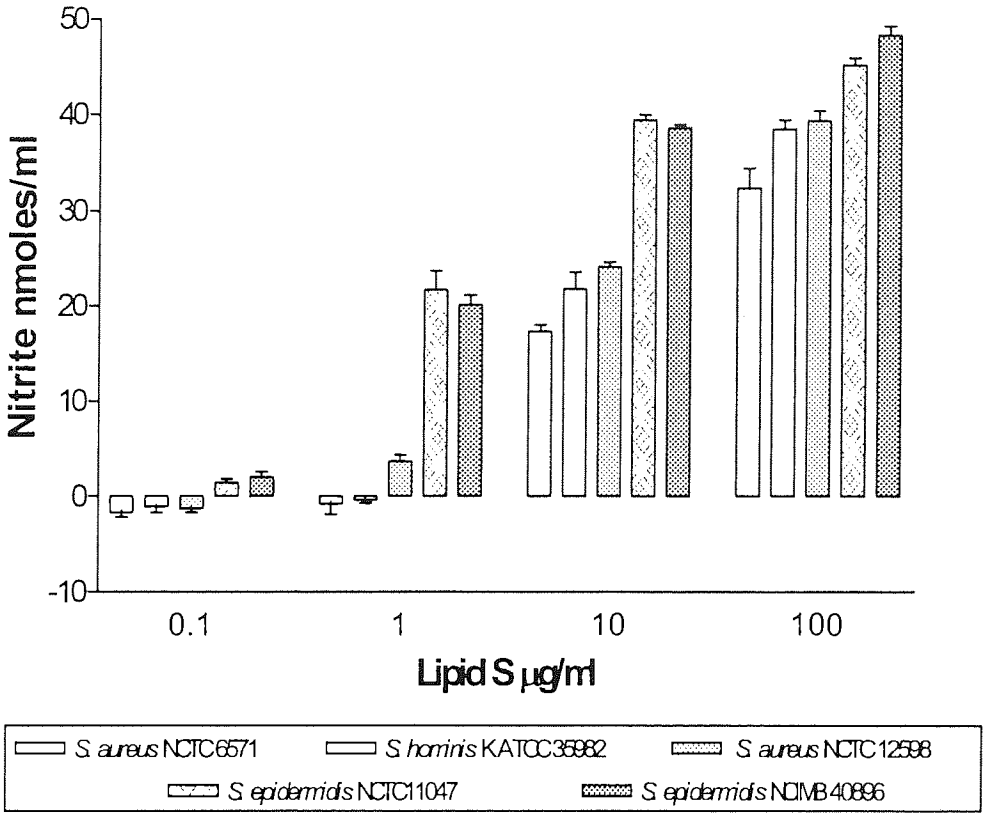


Figure 4.19: Effect of lipid S extracts from various Gram-positive staphylococci species upon nitrite production from RAW264.7 cells (1×10^6/ml) after 48h incubation

RAW264.7 cells (1×10^6) cells in 1 ml of supplemented medium were incubated with doses of lipid S from 5 Gram-positive organisms (grown in HHW medium) at 37°C in 95% air, 5% CO_2 for 48 hours. Bars are standard error of the mean of three samples.

When comparing the means of the different lipid S extracts to each other, very significant values were observed with the two *S. epidermidis* extracts compared to all other extracts at concentrations of 1µg/ml and above ($p = <0.001$). Insignificant values were obtained when the mean nitrite production from all doses of *S.aureus*

NCTC6571, *S.aureus* NCTC12598 and *S. hominis* were compared to each other ($p = >0.05$).

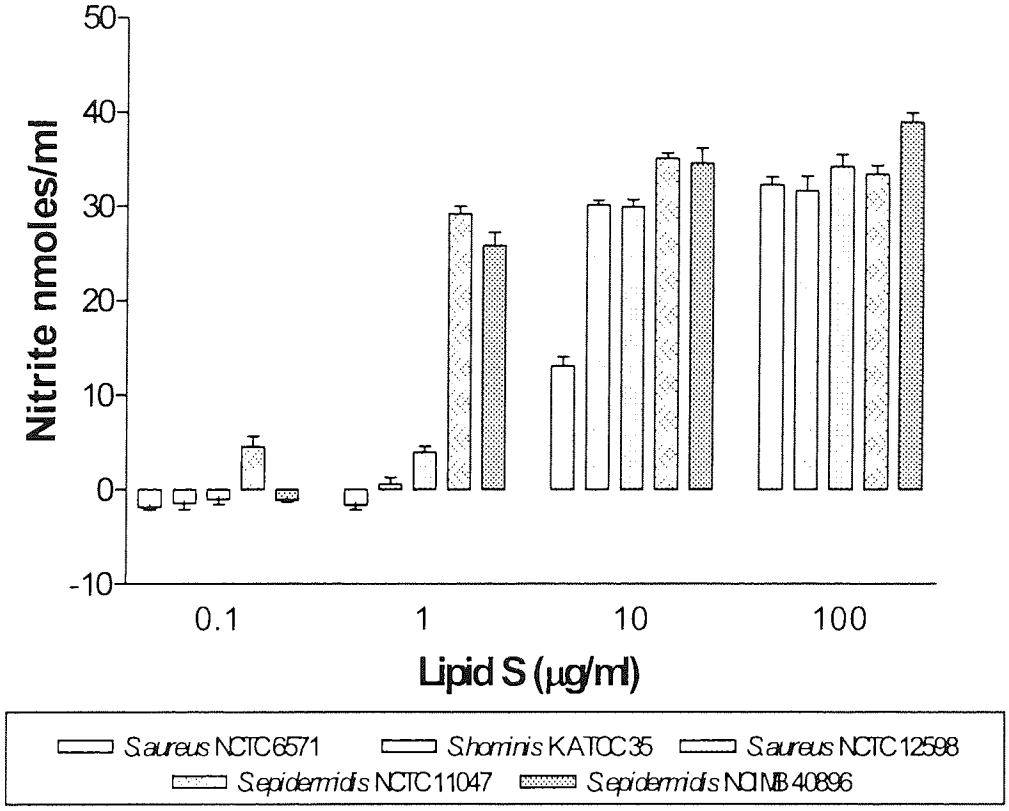


Figure 4.20: Effect of lipid S extracts from various Gram-positive staphylococci species upon nitrite production from J774.2 cells (1×10^6/ml) after 48h incubation

J774.2 cells (1×10^6) cells in 1 ml of supplemented medium were incubated with doses of lipid S from 5 Gram-positive organisms (all grown in HHW medium) at 37°C in 95% air, 5% CO_2 for 48 hours. Bars are standard error of the mean of triplicate samples.

Experiments comparing nitrite release following application of lipid S extracts from different staphylococcal species using both cell lines showed similar results. As expected NO production depended on the dose of lipid S employed and the RAW264.7 cell line responded by producing higher concentrations of nitrite

compared to J774.2 cells. These results also demonstrated that some lipid S extracts were more reactive in their ability to induce nitrite production and therefore potentially more antigenic than others. Both *S. epidermidis* species were able to significantly induce nitrite at low concentrations of 1μg/ml ($p = <0.001$), whilst others had no effect at that concentration ($p = >0.05$).

4.3.5: Measurement of total NO and the quantities of nitrite and nitrate

NO undergoes a number of reactions under biological conditions. These reactions tend to involve the formation of both nitrite and nitrate ions. It has been suggested that the majority of NO is converted into nitrite rather than nitrate and consequently nitrite is a good indicator of total nitric oxide (Jungi, 1999). However in some circumstances, it might be beneficial to determine both nitrite and nitrate concentrations, therefore further experiments were performed to measure nitrate produced in response to components from Gram-negative and Gram-positive bacteria.

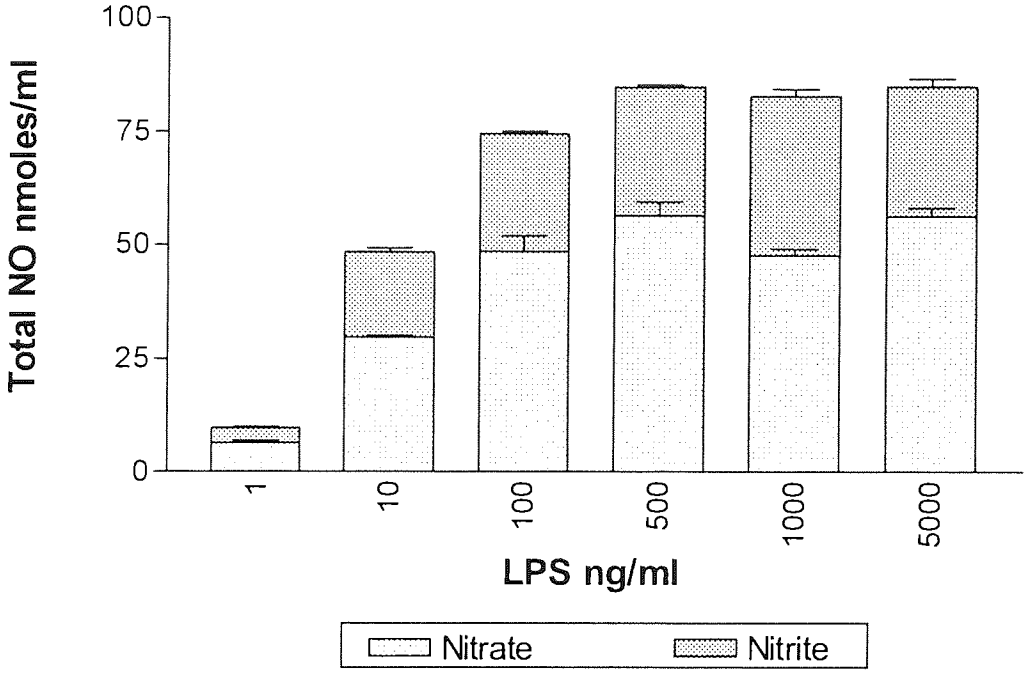


Figure 4.21: Effect of LPS from *E. coli* on nitrate and nitrite produced from RAW264.7 cells (1×10^6)

RAW264.7 cells (1×10^6) cells in 1 ml of supplemented medium were incubated with doses of LPS 37°C in 95% air, 5% CO_2 for 48 hours. Nitrate was measured by subtracting nitrite determinates from total NO (section 4.2.4) Bars are standard error of the mean of three samples.

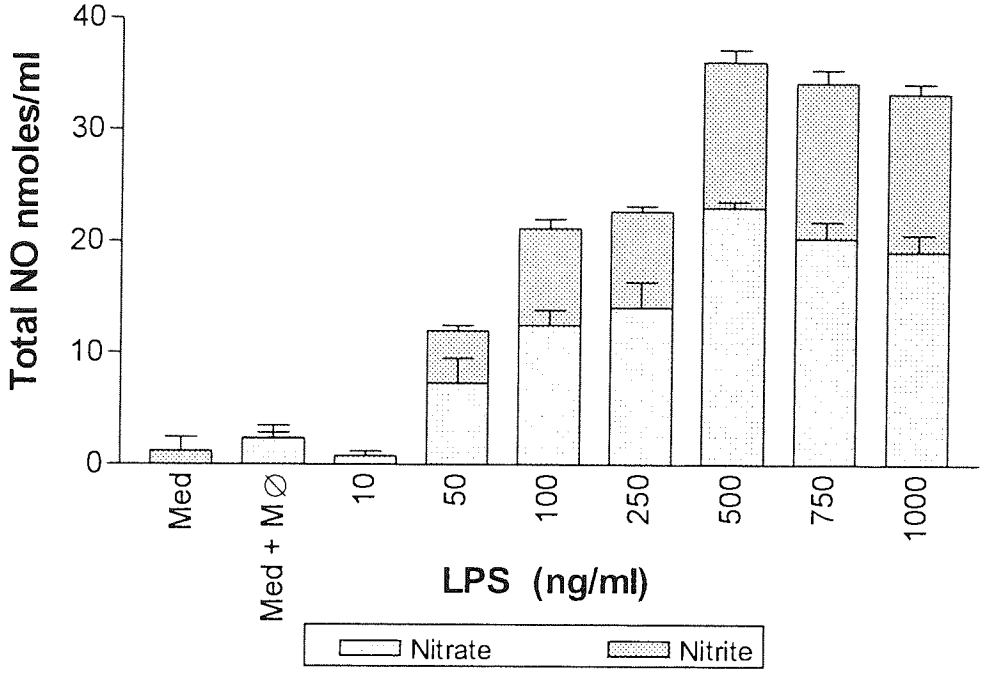


Figure 4.22: Effect of LPS from *E. coli* on nitrate and nitrite produced from J774.2 cells (1×10^6)

J774.2 cells (1×10^6) cells in 1 ml of supplemented medium were incubated with doses of LPS 37°C in 95% air, 5% CO_2 for 48 hours. Nitrate was measured by subtracting nitrite determinates from concentrations of total nitric oxide. Bars are standard error of the mean of three samples.

Both cell lines produced significant quantities of NO in response to LPS, significantly more NO was converted to nitrate (approximately 60- 65%) than to nitrite (35- 40%) $p = <0.05$. Further experiments were then performed to determine nitrite and nitrate produced in response to components from Gram-positive bacteria.

For figures 4.23 – 4.25 assays were performed and observed under the same conditions; RAW264.7 (1×10^6) cells or J774.2 (1×10^6) cells in 1 ml of supplemented medium were incubated with antigens at 37°C in 95% air, 5% CO_2 for 48 hours. Medium alone and with macrophages served as negative controls (results negligible, not shown). Bars are standard error of the mean of three samples.

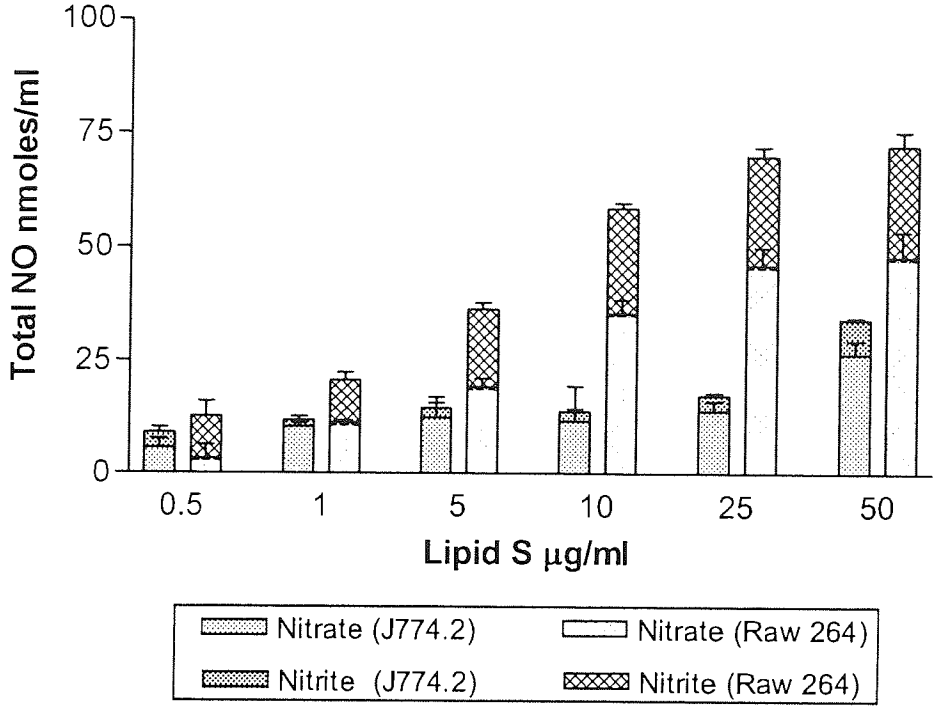


Figure 4.23: Effect of lipid S from *S. epidermidis* on nitrite and nitrate released from RAW 264.7 (1×10^6/ml) and J774.2 cells (1×10^6/ml)

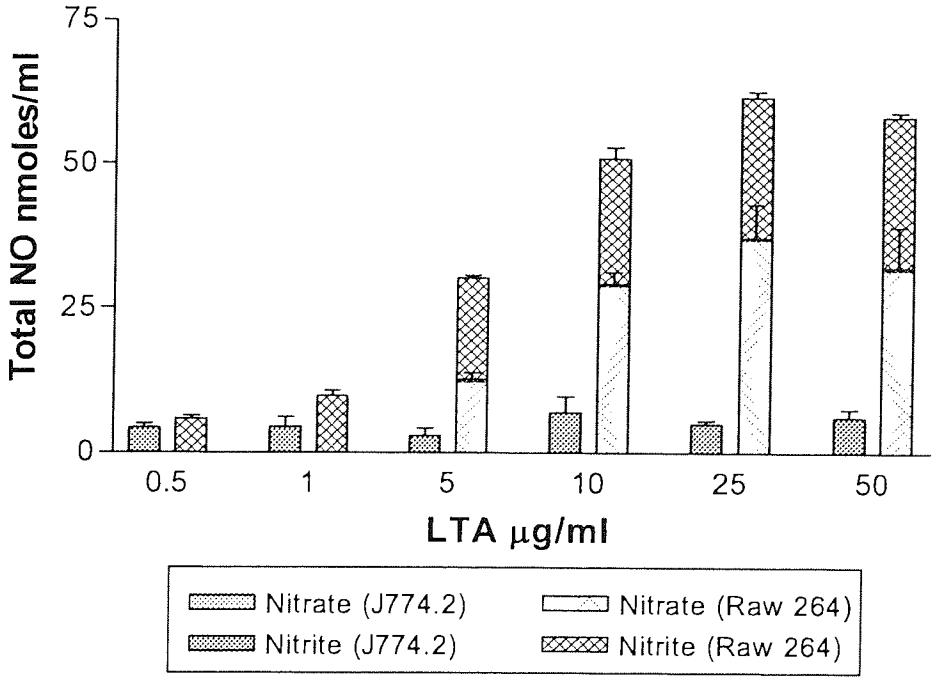


Figure 4.24: Effect of LTA from *S. epidermidis* on nitrite and nitrate released from RAW 264.7 (1×10^6/ml) and J774.2 cells (1×10^6/ml)

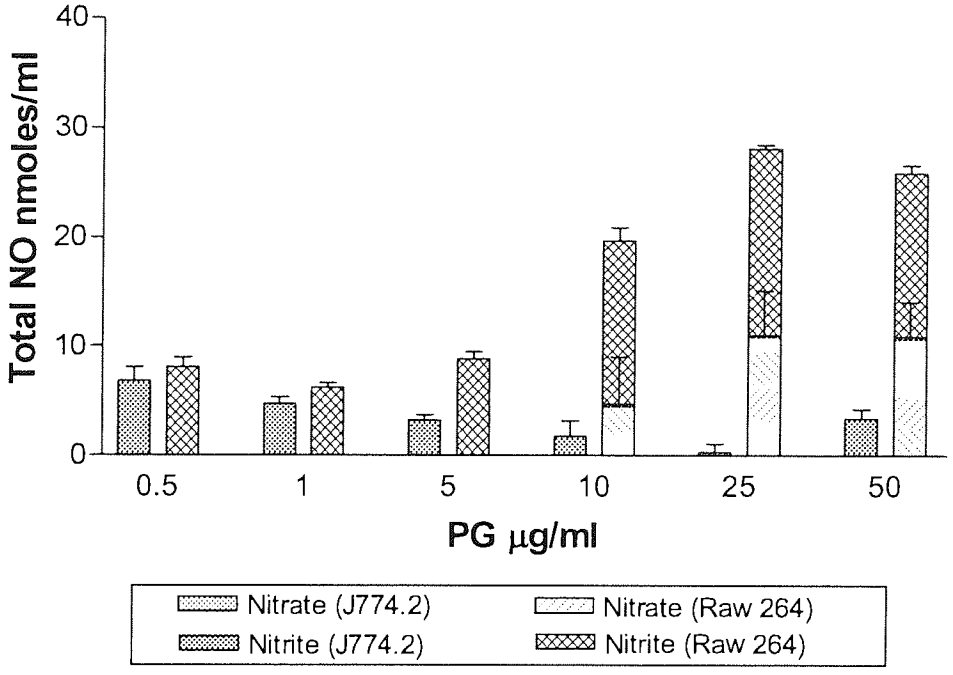


Figure 4.25: Effect of PG on nitrite and nitrate released from RAW264.7 (1×10^6/ml) and J774.2 cells (1×10^6/ml)

The J774.2 cell line did not respond as well as the RAW264.7 cell line in all nitrate experiments (Fig 4.23 -4.25). Generally levels of nitrate produced in response to LTA and PG by J774.2 cells were very low, only marginally above controls ($p = >0.05$) and the majority, 90-100% was in the form of nitrite ($p = <0.01$). In comparison the J774.2 cells response to lipid S differed greatly, in that significant amounts of NO were produced ($p = <0.05$), the majority being converted to nitrate (approximately 80% nitrate and 20% nitrite).

Overall the RAW264.7 cell line was more responsive to all bacterial components; LTA at low concentrations (0.5 -5µg/ml) induced mostly nitrite but at higher concentrations (10 - 25 µg/ml) the major NO product was nitrate but the differences were insignificant, $p = >0.05$ (Fig 4.24). In comparison PG at all concentrations

induced the formation of more nitrite than nitrate, at low concentrations there was no evidence of any nitrate production (Fig 4.25).

For Lipid S at high concentrations (50 and 25μg/ml) the majority of NO produced (approximately 66%) was nitrate ($p = <0.05$). Concentrations of lipid S between 1 and 5μg/ml resulted in equal volumes of nitrite and nitrate ($p = >0.05$) and lower concentrations 0.5μg/ml produced more nitrite 71:29 nitrite to nitrate ratio (Fig 4.23) although this prioved to be insignificant ($p = >0.05$), most likely due to high SEM.

4.3.6: Respiratory burst activity in peritoneal macrophages

A series of experiments were conducted to measure superoxide production from peritoneal macrophages in response to Gram-positive bacterial components LTA, PG and lipid S from *S. epidermidis* in conjunction with opsonised zymosan. No response was observed when these bacterial components were incubated in the absence of opsonised zymosan, also neither RAW 264.7 nor J774.2 cell lines produced significant levels of superoxide (results not shown).

For the following experiments (Figures 4.26 – 4.28) cells were treated in the same manner, see section 4.2.6.2 for contents of wells. Peritoneal macrophages (5×10^5 cells /ml pooled from four MF1 mice), supplemented medium, lucigenin and bacterial cell wall components were dispensed into wells on a microtitre plate and left to incubate at 37°C until they had reached stable levels. At time 0 the basal luminescence level was recorded and opsonised zymosan was added to all wells except the blank (which contained only medium) and the oxidative burst was recorded (1240 Victor, Wallac) at time intervals over a period of 55 minutes, by which time oxidative burst activity had declined. Control cells were incubated in the absence of bacterial components.

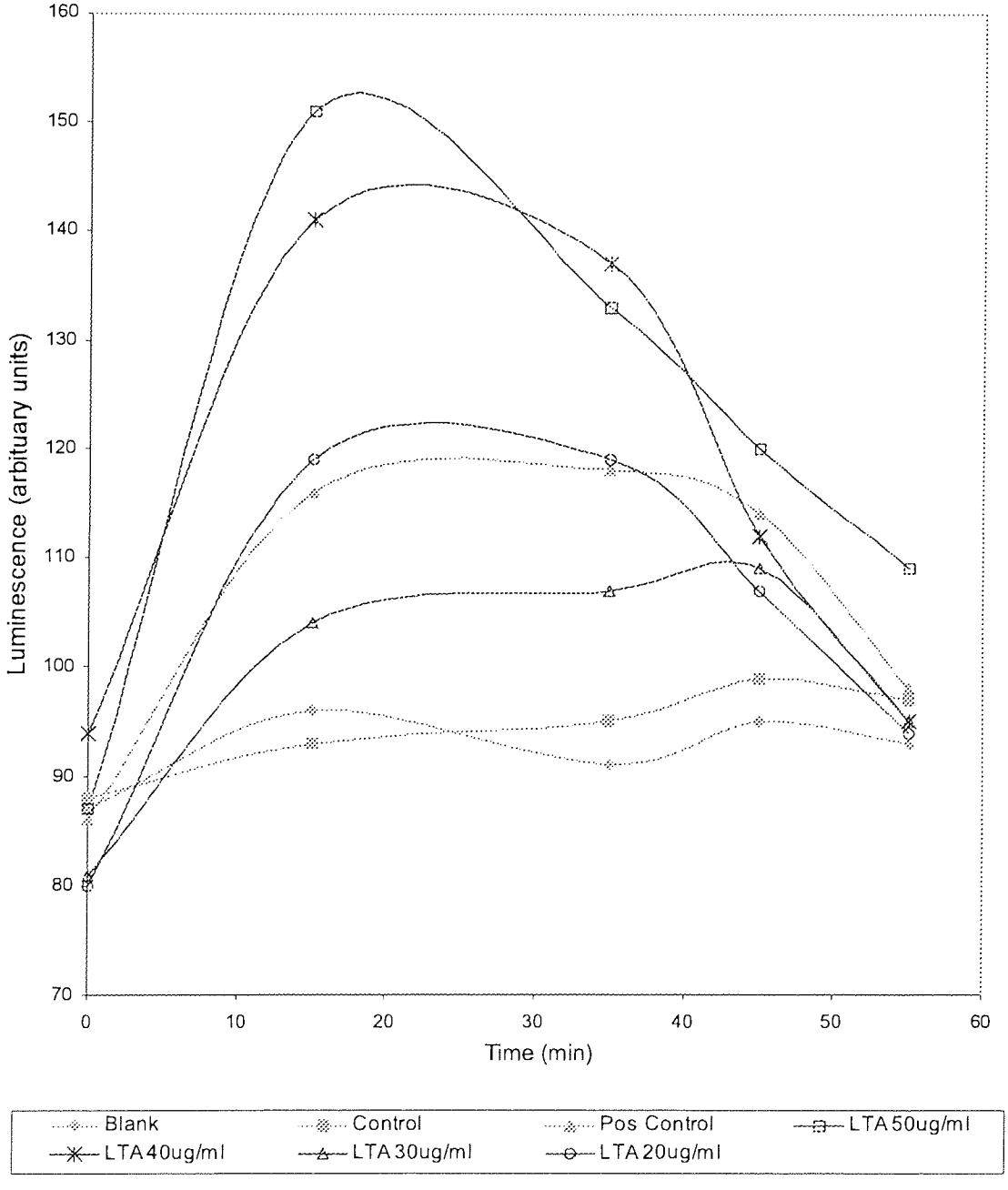


Figure 4.26: Oxidative burst elicited by peritoneal macrophages (5×10^5 cells/ml) in the presence of LTA from *S. epidermidis* and opsonised zymosan (positive control)

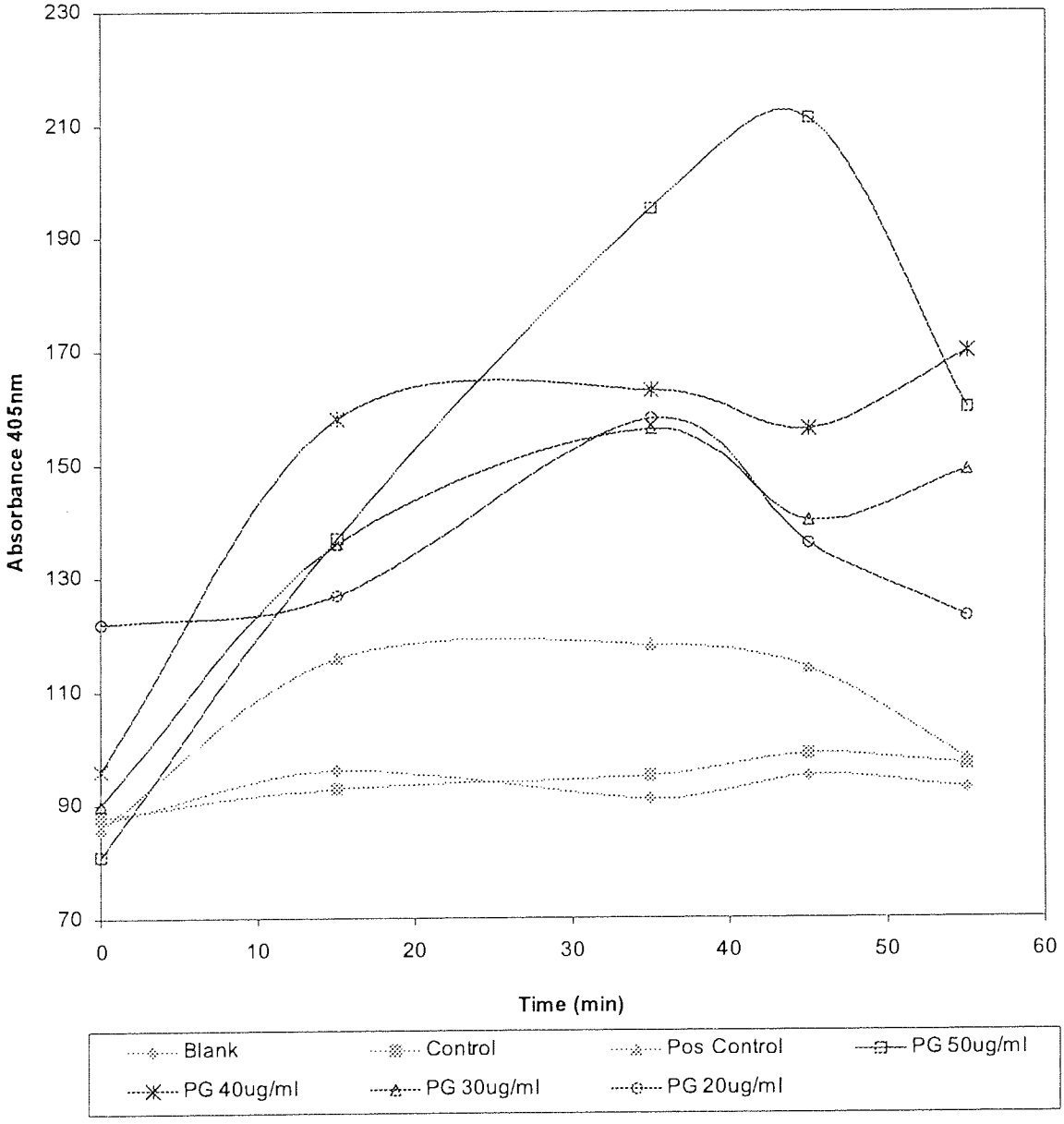


Figure 4.27: Oxidative burst elicited by peritoneal macrophages (5×10^5 cells/ml) in the presence of PG from *S. epidermidis* and opsonised zymosan (positive control)

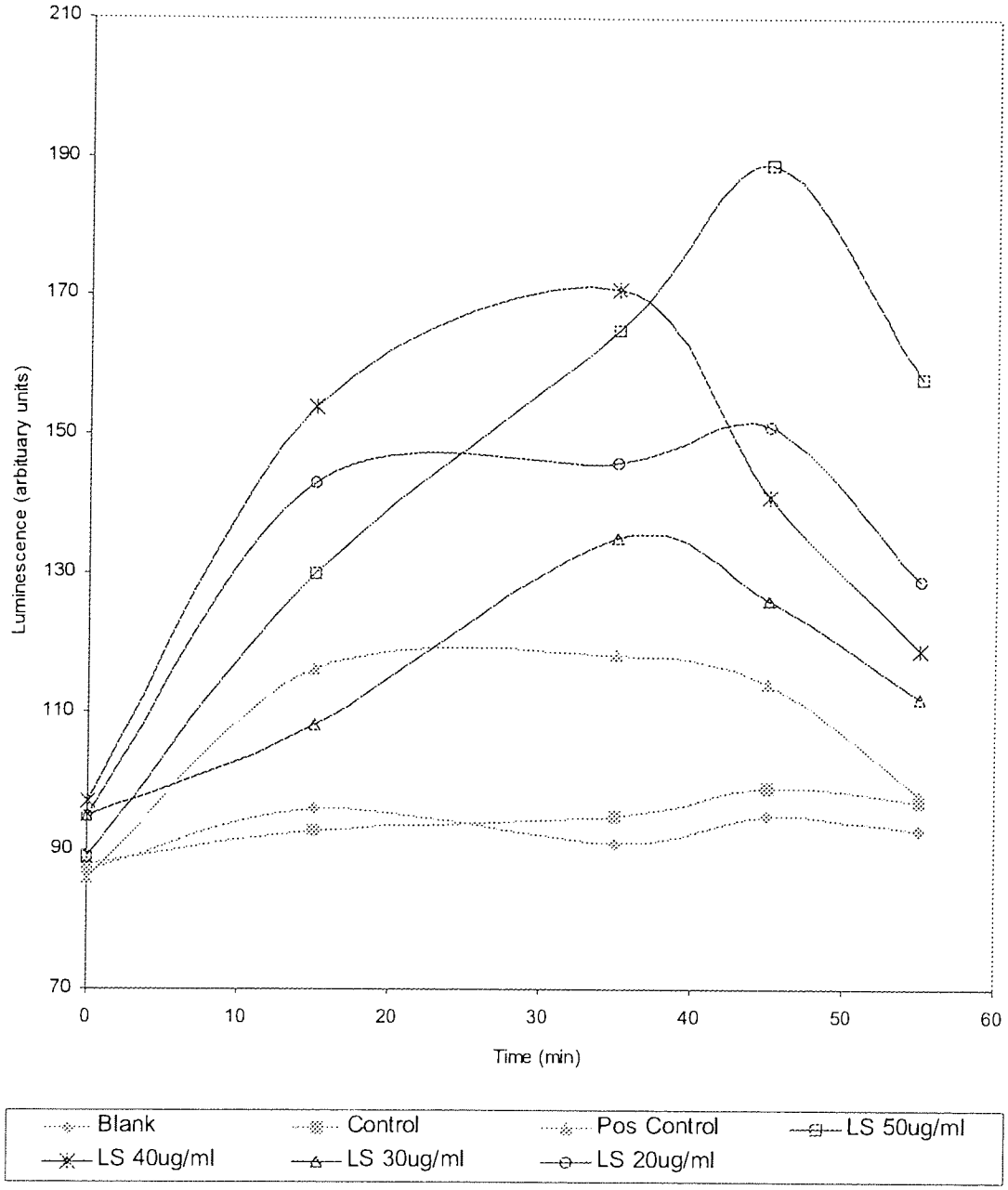


Figure 4.28: Oxidative burst elicited by peritoneal macrophages (5×10^5 cells/ml) in the presence of Lipid S from *S. epidermidis* and opsonised zymosan (positive control)

Control samples of medium alone (blank) and medium with macrophages but no stimulants showed no significant activity. Positive control samples (cells with opsonised zymosan) elicited an oxidative burst significantly above control levels, which peaked at 15 minutes, remained constant until 35 minutes and thereafter

declined. For the following analysis peak oxidative burst activity from the positive control is expressed as 100 percent. In most instances the bacterial components elicited a burst higher than that observed from the positive controls.

Lipid S significantly potentiated the oxidative burst with levels 60% more than the positive control at a concentration of 50μg/ml, 44% higher at 40μg/ml, smaller, modest increases were observed for 20μg/ml and 30μg/ml, which resulted in increases of 14% and 27% respectively. Peak oxidative burst levels were seen after 45 minutes incubation with a concentration of 50μg/ml and 35 minutes for all other concentrations (Fig 4.28).

Similarly PG significantly enhanced the oxidative burst above the positive controls levels, potentiation in burst resulted in a 78% 38% 32% and 33% with 50, 40, 30, and 20μg/ml respectively. Burst activity occurred after 45 min with the highest concentration and after 35 min for all other concentrations (Fig 4.27).

In comparison LTA had a lesser effect, concentrations of 50μg/ml and 40μg/ml modestly increased the oxidative burst above controls at 27% and 19% respectively, whereas the lower concentration of 30μg/ml inhibited the oxidative burst, reaching only 88% of control levels. Oxidative burst activity induced by LTA at 20μg/ml only marginally differed from levels detected in the positive control cells. At all concentrations of LTA the oxidative burst peaked earlier than lipid S and PG samples, after only 15 min incubation (Fig 4.26).

4.4 DISCUSSION

It has been demonstrated that NO plays a significant role in host defences as a vasodilator and a diffusible toxic mediator that can attack invading organisms and high levels of NO can be produced over a sustained period. The toxicity of NO is useful in host defence; however, it also represents a potential danger to the host if prolonged release is uncontrolled. Murine peritoneal macrophages have the ability to produce NO and subsequently nitrite. LPS is known to be the bacterial component primarily responsible for Gram-negative septic shock (Opal and Cohen, 1999), its ability to stimulate release of nitrites even at low concentrations of 100ng/ml is evident in results obtained here (Fig 4.4). Stuehr and Marletta (1985) also reported that LPS induces NOS and NO formation in activated macrophages. PG and WTA did not enhance the release of nitrites above those found in controls, whereas at a concentration of 100μg/ml, both LTA and lipid S elicited the formation of nitrites. This assay was only the result of a single experiment and was therefore only used to demonstrate that peritoneal macrophages have the ability to produce nitrite under *in-vitro* conditions. Later experiments were performed on 4 mice which supported these results (Fig 4.5), a range of doses were used to measure the accumulation of nitrite production over a 48h period. In all instances the amount of nitrite produced was dependent upon the concentration of microbial component.

The effect of LPS concentrations on nitrite production was similar in both murine macrophage cell lines (Fig 4.7 – 4.8) and in primary cultures of murine peritoneal macrophages (Fig 4.5). It seems probable, therefore, that these cell lines can be used reliably to simulate effects of cell wall products on macrophage NO production.

Cunha *et al.* (1993) had previously demonstrated that murine peritoneal macrophages express high levels of NO when stimulated with LTA and De Kimpe *et al.* (1995b) reported LTA induced hypotension and circulatory failure in an anaesthetised rat, which was prevented by the use of L-NMMA, an inhibitor of NO. Hattor *et al.* (1997) also suggested that Gram-positive sepsis was due to more than one component of the bacteria and demonstrated that PG could cause shock. In this study macrophages

stimulated with LTA from both *S. epidermidis* and *S. aureus* was investigated along with MDP, PG and lipid S. They all induced macrophages to produce nitrite, though at varying levels (Figs 4.9 – 4.14). Most increased the production of nitrite in a dose-dependent manner and levels of nitrite produced after 48 hours were more than those measured after 24 hours, indicating that the activity of macrophages is substained over this period. However, high concentrations inhibited the formation of nitrite; this could be due to a number of factors. Microbial components may bind together and hence block binding to macrophage receptors. Alternatively receptors may be saturated by the abundance of microbial component or high doses of these materials could be toxic to macrophages. Recent studies indicate that macrophages may undergo apoptosis during septic infection, and it appears to be mediated by the release of reactive oxygen/nitrogen species and cysteine protease activity (Williams *et al.,* 1997), this may account for the low levels of NO produced in response to high concentrations of microbial components. Another possible explanation is that high concentrations induce the formation of NO inhibitors as a negative feedback mechanism that prevents NO synthesis and therefore inhibiting the production of NO (Marcinkiewicz *et al.*, 1994).

The co-administration of LTA and PG from *S. epidermidis* increased the production of nitrite (Fig 4.14), in some instances this enhancement was two-fold greater than that produced when each component was tested alone (Fig 4.15), indicating that these components may act in synergy. Similar results were found when LTA and PG from *S. aureus* were used in conjunction (Kengatharan *et al.,* 1996b). By contrast lipid S in conjunction with PG (Fig 4.17) did not potentiate an increase of NO to the levels observed with the combination of LTA and PG, although there was an additive effect at some concentrations (Fig 4.18). These results suggest that lipid S and PG may be linked to same second messenger system as very little increase was observed when the two were used in combination. Interestingly lipid S alone stimulated greater levels of nitrite than those produced by LTA and PG together, indicating that it may be a major factor as a mediator of NO formation.

The above results have indicated that lipid S from *S. epidermidis* (strain NCIMB 40896) is potentially an important antigenic molecule and is more effective in inducing higher levels of nitrite than other Gram-positive cell wall products, including

LTA and PG. Following these results other strains, which had previously been shown to release lipid S into culture medium (see section 3.9) were tested to assess the ability of their lipid S preparations to induce nitrite formation in the two cell lines, RAW264.7 and J774.2. Results showed similar patterns in both cell lines, in all strains the effects of lipid S were dose-dependent. Also lipid S from most samples (with the exception of *S. aureus* NCTC 6571) at high concentrations (10 – 100μg/ml) were effective and comparable to each other. However at a low concentration of 1μg/ml lipid S from both the *S. epidermidis* strains were the most effective, significant levels of nitrite were produced in response to these organisms ($p = <0.01$), whereas lipid S from other strains failed to stimulate nitrite levels above those of controls (Fig 4.19 – 4.20). This indicates that lipid S released from all the strains tested has the ability to induce the production of nitrite, though some were more effective than others. Therefore antigenic effects of lipid S samples are dependent on the strain it was released from.

Up to this point, because it was assumed that nitrite is a good indicator of total nitric oxide and because of the simplicity of the assay only nitrite had been measured. For a more complete picture it was necessary to measure the other stable end product of NO, nitrate. It is not known under what conditions or what circumstances nitrate or nitrite is formed from NO, although it has been suggested that when NO encounters superoxides then the conversion is to nitrate (Lincoln *et al.*, 1997).

Both cell lines were employed to measure total NO from bacterial components, and hence the production of nitrite and nitrate (Fig 4.21 – 4.25). LPS, LTA, lipid S and PG all induced RAW264.7 cells to produce nitrate. J774.2 cells were not so responsive and in some instances only nitrite was formed and this was marginally above control levels and therefore insignificant ($p = >0.05$). Possibly the J774.2 cell line was compromised due to passage number 25 at this stage, possibly losing their ability to respond. However the J774.2 cells did respond well to LPS and lipid S so it is unlikely that they were fully compromised.

Whereas RAW264.7 cells did respond well to all bacterial components, the general pattern emerging was that at high concentrations the majority of nitric oxide formed was nitrate and at lower concentration the formation of nitrite was more prominent.

Macrophages are professional phagocytic cells; they are proficient at internalising and destroying foreign particles, cellular debris and senescent cells. They recognise sugar moieties on the zymosan particles via their mannosyl-fucosyl receptors (Adams, 1989). Incubation of zymosan with guinea pig complement results in the coating of zymosan particles with the complement components C3b or C3bi. Macrophages, in addition to binding glucan molecules, are now able specifically to recognise and bind to these components via their complement receptors, CR1and CR3 (Roitt, 1997), thus increasing target recognition and uptake of the particle, a process known as opsonisation (Janeway *et al.*, 2001). As a result of this opsonised zymosan particles are phagocytosed with greater specificity.

Gram-positive components, lipid S, LTA and PG, were unable themselves to elicit an oxidative burst in either cell line or murine peritoneal macrophages. However, the addition of opsonised zymosan in conjunction with these products did result in a potentiation of the oxidative burst. This suggests that murine peritoneal macrophages may possess receptors for these compounds which do not trigger a burst themselves, but act by enhancing the burst initiated by subsequent stimuli, e.g. opsonised zymosan. This increase in burst may be due to increased affinity of the oxidase complex for NADPH.

Peritoneal macrophage activation in terms of oxidative burst activity was investigated for Gram-positive components, lipid S, LTA and PG in the presence of opsonised zymosan (Fig 4.26 – 4.28). All components were shown to sensitise the peritoneal macrophages to produce an oxidative burst. LTA is known to bind to the macrophage scavenger receptor (Greenberg *et al.*, 1996) and therefore macrophages may have a greater responsiveness towards LTA. However no significant potentiation in burst over control levels was seen after incubation with LTA at low concentrations of 20 and 30μg/ml. In comparison, higher concentrations of LTA (40 and 50μg/ml) did modestly enhance burst activity (Fig 4.26).

Cells incubated with PG resulted in significant oxidative bursts above control levels, the high concentration of 50μg/ml resulted in a potentiation of 78% above positive

controls levels, a modest increase was observed at lower concentrations (40 - 20μg/ml), where enhancement was between 32 -38% above controls (Fig 4.27).

Lipid S also enhanced the oxidative burst; concentrations were dose-dependent and all induced the macrophage to oxidative activity above control levels. Potentiation was greatest at the highest concentration (50μg/ml), this resulted in a oxidative burst of 60% more than controls, and lower concentrations also had moderate effects.

NO reacts directly with superoxide anions to produce nitrate and other species such as $ONOO^-$ (Archer, 1993), the combined effects of NO, ROI and other RNI's released by macrophages will induce massive vasodilatation, and tissue damage, characteristics of septic shock. These experiments have shown that lipid S is a potent antigen and is potentially the principal mediator in the onset of an immune response in a Gram-positive infection. Lipid S is a small diffusible molecule that is released during growth of a Gram-positive organism and would therefore have systemic effects, whereas cell wall products remain within the bacterial cell structure and primarily will not have a direct effect, although some would be released during the process of cell division. Other Gram-positive components such as LTA and PG have also been shown to play a role in an immune response, and evidence here suggests that they may act in synergy.

CHAPTER 5: CYTOKINES

5.1: INTRODUCTION

Cytokines are small proteins (~25 kDa) that are released by various cells in the body, usually in response to an activating stimulus, and induce responses through binding to specific receptors.

In response to the initial host-bacterial interaction there is widespread activation of the innate immune system. Macrophages are key components of this defence system and one of their actions is to up-regulate the production and release of numerous cytokines including the pro-inflammatory cytokines IL-1, IL-6 and TNF-α (Janeway *et al.*, 2001). These cytokines have a wide spectrum of biological activities that help to coordinate the body's responses to infection. They are all endogenous pyrogens, raising body temperature, which is believed to help eliminate infections. A major effect of these cytokines is to act on the hypothalamus, altering the body's temperature regulation, and on muscle and fat cells, altering energy mobilization to increase the body temperature (Henderson *et al.*, 1998). At elevated temperatures, bacterial and viral replication are decreased, while the adaptive immune response operates more efficiently, however it has been demonstrated that persistent increases in TNF-α are associated with mortality and multiple organ failure in patients diagnosed with sepsis (Wang and Tracey, 1999).

LPS induces the production of TNF-α from macrophages and is a contributory factor to Gram-negative septic shock (Cerami and Beutler, 1988). Recent reports claim that both TNF-α and IL-1 are the inflammatory cytokines involved in a response to LPS (Cohen, 2002). Other reports suggest that Gram-positive organisms can also prompt the release of inflammatory mediators that may be associated with sepsis; high levels of TNF-α, IL-1 and IL-6 were found in patients with Gram-positive sepsis (Bone, 1994). Bhakdi *et al,* (1991) demonstrated that LTA from several Gram-positive species induced the release of TNF-α, IL-1 and IL-6 from cultivated macrophages at

levels similar to those elicited by LPS from Gram-negative organisms. In addition these cytokines can stimulate release of their own production and that of other cytokines which serves to enhance their inflammatory effects, for example TNF-α acts on macrophages in a autocrine fashion to increase its own production (Blasi *et al.*, 1994). TNF-α and IL-1 can also induce the production of each other and amplify the production of IL-6 (Wang and Tracey, 1999). Furthermore TNF-α has been reported to enhance the production of NO and contributes to the circulatory failure and organ failure seen in septic shock (Thiemermann, 1997).

After establishing that some cell wall components had strong antigenic properties and could induce the production of nitric oxide, experiments to determine their capacity to elicit cytokine production in murine macrophage cell lines and murine peritoneal and splenic macrophages were carried out.

5.2 MATERIALS AND METHODS

5.2.1: Measurement of Cytokines

TNF-α, IL-1 and IL-6 produced by macrophages were measured by enzyme-linked immunosorbent assay (ELISA) using the appropriate mouse TNF-α (lot # 0012034), IL-1β (lot # 9939075) or IL-6 (lot # 0010234) Duoset ELISA development system kits (R and D Systems).

5.2.2: Buffers and Reagents

5.2.2.1: Phosphate buffered Saline (PBS)

1 litre of distilled water
10 tablets of PBS (Dulbecco A, Oxoid)
This was then autoclaved and adjusted to pH 7.2

5.2.2.2: Block Buffer

1% Bovine serum albumin (BSA) (Sigma)
5% Sucrose (Fisher Chemicals)
0.05% Sodium azide (NaN_3, Difco Laboratories)
dissolved in PBS

5.2.2.3: Wash Buffer

0.05% Polyoxyethylene sorbitan monolaurate (Tween 20)
dissolved in PBS
adjusted to pH 7.2

5.2.2.4: Reagent Diluent

1% BSA (Sigma)
dissolved in PBS, sterile filtered using sterile filter unit (Fisher Scientific) and 0.2μm sterile membrane micropore (Whatman) and adjusted to pH 7.2

All the above buffers and reagents were stored at 4°C, prior to use they were all warmed to room temperature.

5.2.2.5: Substrate Solution

10mg 3,3',5,5'-tetramethybenzidine was dissolved in 1ml of dimethyl sulphoxide.
This solution was diluted in 100mls sodium acetate/citrate buffer 0.1M pH 6 containing 20μl H_2O_2 immediately prior to use.

5.2.2.6: Stop solution

1M Sulphuric acid (H_2SO_4) (Fisher Chemicals)

The following substances were provided in the ELISA kits (R and D Systems).

5.2.2.7: Capture antibody

Capture antibody was diluted in PBS to give stock concentration of 8μg/ml goat anti-mouse (TNF-α), 40μg/ml (IL-1β) and 20μg/ml (IL-6).
1ml samples were stored at -20°C
Immediately prior to use the samples were defrosted and diluted in PBS to working concentration of 0.8μg/ml (TNF-α), 4μg/ml (IL-1β) and 2μg/ml (IL-6)

5.2.2.8: Detection antibody

Detection antibody was reconstituted in reagent diluent (5.2.2.4) to give stock concentrations of 3μg/ml biotinylated goat anti-mouse (TNF-α), 1μg/ml (IL-1β) and 2μg/ml (IL-6)

1ml samples were stored at -20°C

Immediately prior to use samples were defrosted and diluted in reagent diluent to working concentrations of 300ng/ml (TNF-α), 100ng/ml (IL-1β) and 200ng/ml (IL-6)

5.2.2.9: Standards

Standards were reconstituted with reagent diluent to give stock concentrations of 4000pg/ml (TNF-α), 1000pg/ml (IL-1β) and (IL-6)

1ml samples were stored at -70°C

5.2.2.10: Streptavidin-horseradish peroxidase

Conjugate was stored at 4°C, immediately prior to use it was diluted with reagent diluent to a working concentration of 5μl/ml

5.2.3: ELISA protocol

5.2.3.1: Preparation of ELISA plates

- 100μl capture antibody (working concentration) was added to each well of a 96 well microtitre plate (Immulon 2HB, Dynex Technologies Inc)
- Plates were sealed using a plate sealer film (Appleton Woods) and left overnight at room temperature.
- Wells were aspirated and washed 4 times (4×) in wash buffer
- Plates were inverted and blotted dry against clean paper towels
- 300μl block buffer was added to all wells
- Plates were resealed and left at room temperature for 2 hours
- Plates were re-washed 4x in wash buffer and dried against paper towels

- Plates were then dried under vacuum, using a vacuum drier (Jencons, Hemel Hempstead) and high vacuum pump (Edwards); resealed and stored at 4°C for up to 6 months.

5.2.3.2: Assay procedure

- 100µl standard was added to 2 wells and using reagent diluent a linear dilution was prepared in duplicate, using a total of 16 wells. To all other wells 100µl of treated culture supernatants and control samples were added in duplicate or triplicate. The plate was then covered and left at room temperature for 2 hours.
- Unbound plate contents were then discarded and the plate was washed 4× in wash buffer and blotted dry on clean paper towels.
- 100µl detection antibody was added to all wells; the plate was then covered and left to incubate at room temperature for a further 2 hours.
- The wash process was then repeated 4× and the plate was blotted dry on clean paper towels.
- 100µl of streptavadin-HRP conjugate was added to all wells, the plate was protected from light and left to incubate at room temperature for 20 minutes.
- The wash process was repeated again 4× and the plate was blotted dry on clean paper towels.
- 100µl substrate solution was then added to all wells, the plate was then left to incubate at room temperature protected from light for 20 minutes. A clear blue colour in the wells indicated a positive reaction.
- The reaction was then stopped by the addition of 100µl of stop solution to all wells. The plate contents were gently tapped to ensure thorough mixing. A yellow colour within the wells indicated the presence of cytokines.
- The optical density of each well was determined using a microplate reader (Anthos 2001, Labtech Instruments) set to 450nm; optical imperfections were corrected by using a correction wavelength of 570nm
- For each plate a standard curve was plotted from the results of wells containing the standard solutions of cytokines and unknown quantities of

samples were read off the standard curve and were expressed as picograms/ml (pg/ml).

5.3 RESULTS

5.3.1 Cytokine standard curves

Enzyme-linked immunosorbent assays (ELISA) were used to detect and measure the concentration of each cytokine produced from both primary macrophages and the cell lines RAW264 and J774.2. Figures 5.1 – 5.3 represent typical standard curves for each cytokine tested, the results were derived by linear dilution of a recombinant mouse cytokine standard solution. The standard curve was used to determine cytokine production in macrophage supernatant samples. A standard curve was generated for each 96 well microtitre plate assayed.

Data in Fig 5.1 – 5.3 is representative of typical data generated when using the Duoset kits. The standard curves were derived by linear dilution of the standards and results were plotted using 4-PL curve fit (Graphpad prism 3). Standards are in duplicate; hence only mean values are shown

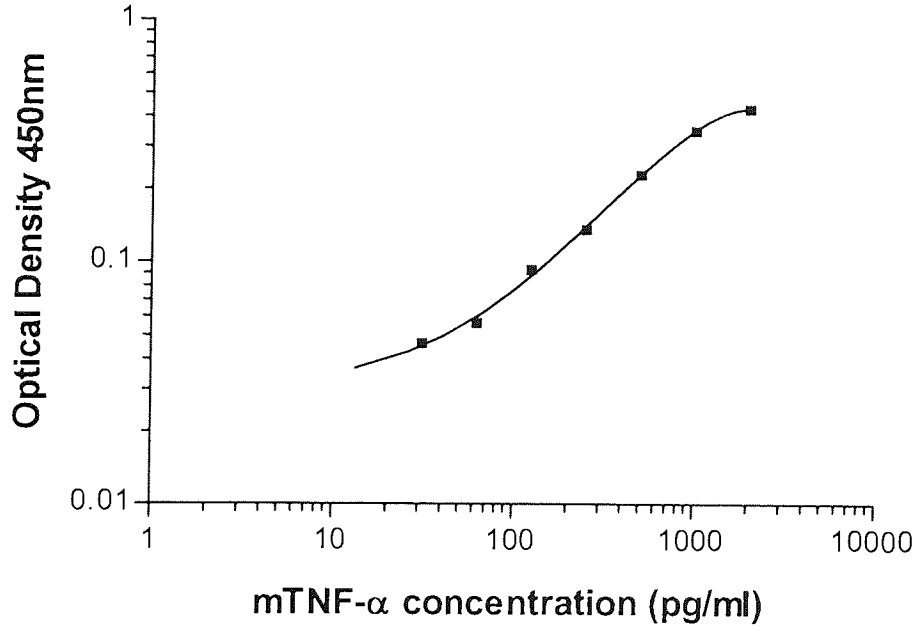


Figure 5.1: Standard Curve for mouse TNF-α

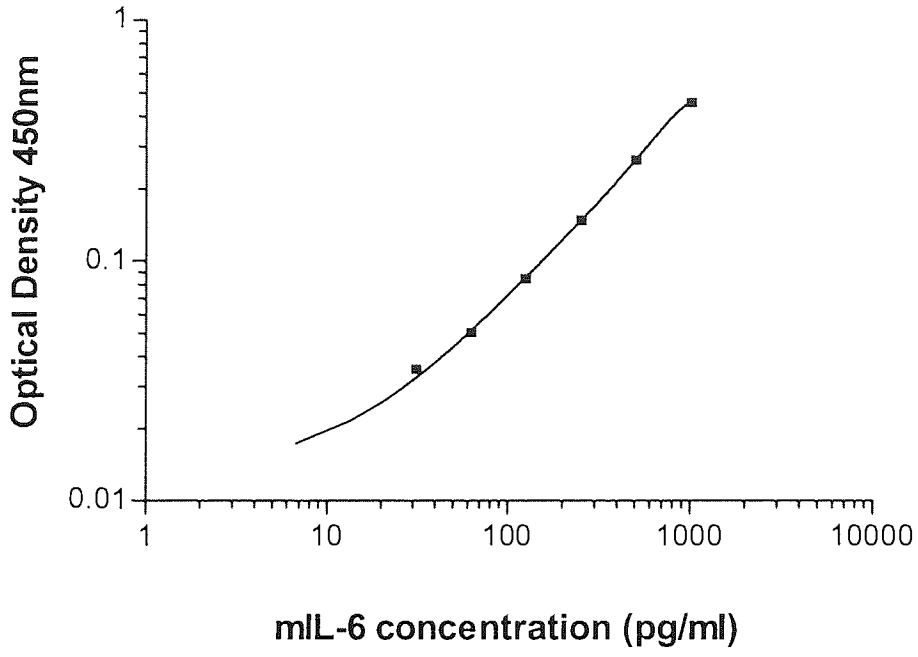


Figure 5.2: Standard Curve for mouse IL-6

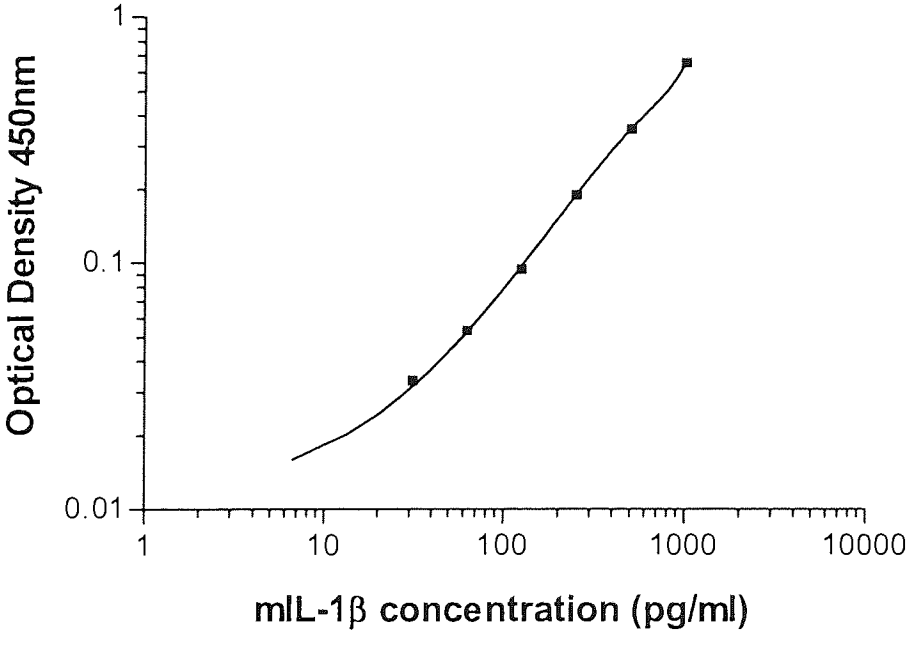


Figure 5.3: Standard Curve for mouse IL-1β in

5.3.2 Effects of bacterial components on the production of TNF-α

To determine the optimal cell concentration a cell density assay was performed in the following manner. Cells were incubated at 37°C in 95% air, 5% CO_2 in 1ml of supplemented medium with the addition of LPS (100ng/ml). Medium and macrophage controls were used as negative controls. Supernatant was collected 24 and 48 hours after incubation and assayed using the mouse TNF-α ELISA kit. Data represents the mean of two samples.

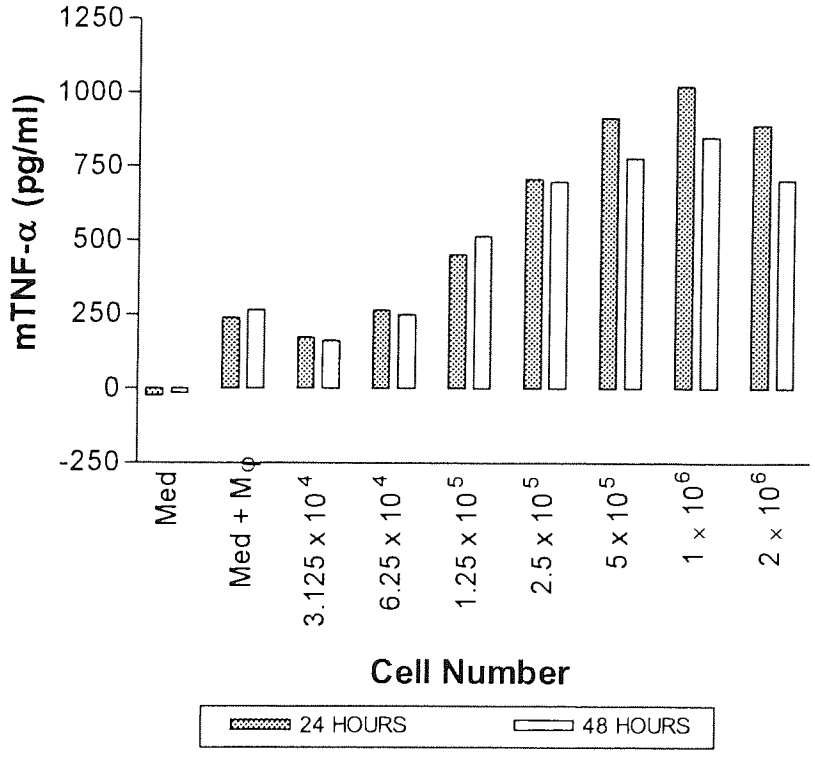


Figure 5.4: Effect of cell number by RAW264.7 cells after 24 and 48h incubation with LPS from *E. coli* (100ng/ml)

Figure 5.4 demonstrates that LPS (100ng/ml) elicited TNF-α production by RAW 264.7 cells. With increasing cell number output at 24 or 48 hours increased proportionately and in most instances there was no more release of TNF-α at 48 hours than 24 hours. Slight inhibition was observed at 2×10^6, possibly due to contact inhibition of cells or some extra-cellular degradation process was taking place. Using

1×10^6 cells/ml, high levels of cytokine could be detected after 24 hours of culture so this density was employed in all subsequent experiments. A similar pattern was obtained when J774.2 cells were incubated with LPS (results not shown).

To determine the optimal time of TNF-α production RAW264.7 cells were incubated with LPS (100ng/ml) and cells (1×10^6 cells/ml) were assayed over a range of time intervals. Controls of cells and medium alone were assayed as negative controls after 48 h. Data represents the mean ± SEM of triplicate samples.

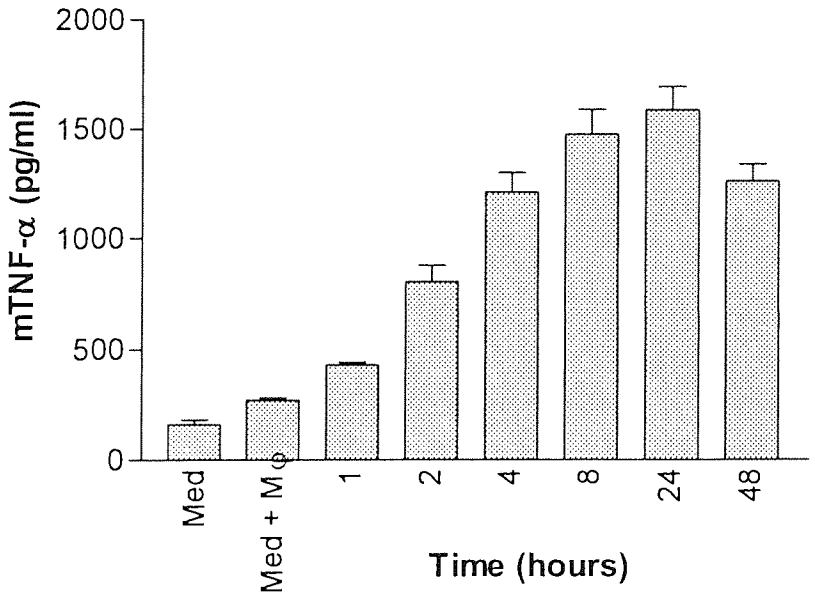


Figure 5.5: Effect of time on TNF-α production by RAW264.7 cells (1×10^6/ml) following stimulation with LPS from *E. coli* (100ng/ml)

Figure 5.5 shows that a moderate increase in TNF-α production was seen after only 1 hour, and thereafter an increase of TNF-α released into the supernatant with time was observed up to the peak production at 24 hours compared to the macrophage control ($p = 0.0002$). After 48 hour incubation the TNF-α release had diminished slightly. A 24 hour incubation period was therefore used in all subsequent experiments.

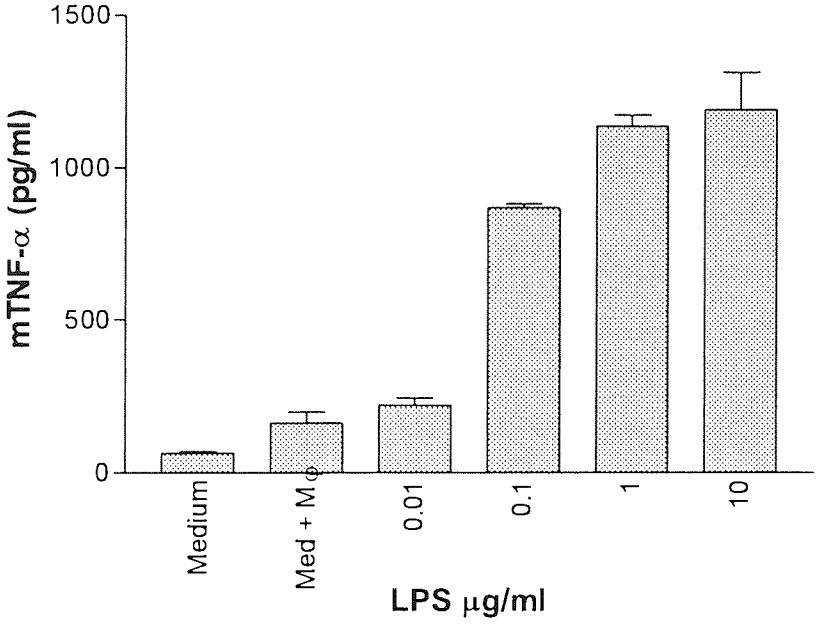


Figure 5.6: Effect of LPS from *E. coli* on TNF-α production from RAW 264.7 cells (1×10^6/ml).

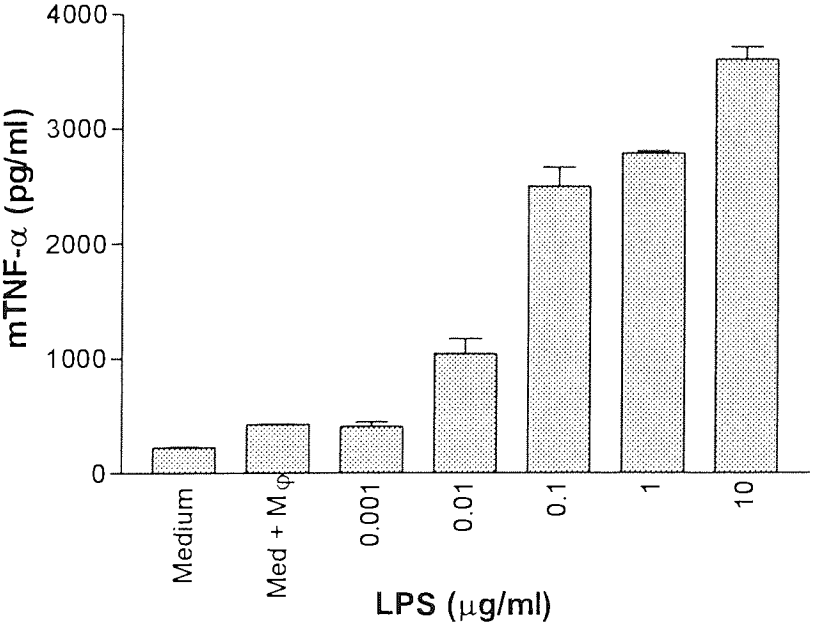


Figure 5.7: Effect of LPS from *E. coli* on TNF-α production from J774.2 cells (1×10^6/ml).

For figures 5.6 and 5.7; TNF-α was measured 24 hours after incubation with various concentrations of LPS. Data represents the mean ± SEM of triplicate samples.

Results clearly show that there is a linear relationship between the concentration of LPS and TNF-α produced from RAW 264.7 cells. A similar pattern was produced using both adherent and non-adherent cells of the J774.2 cell line, although concentrations of TNF-α released in response to LPS were more than two-fold greater than those produced by RAW 264.7 cells for the same concentration of LPS (Figure 5.6 and 5.7). Peak concentrations in both cell lines were observed at 10 μg/ml, this was the highest dose used. However, significant levels of TNF-α were seen at the lower concentration of LPS 0.1 μg/ml, a seven-fold and a six-fold difference compared to control levels of cells alone was observed for the RAW264.7 and J774.2 cell lines respectively ($p = <0.01$). In previous experiments a dose of LPS at 0.1 μg/ml was used for positive controls and, because results of TNF-α production were also significant for this concentration ($p = <0.001$), it was therefore used for the following experiments.

After establishing the experimental procedures with LPS from *E. coli*, products of Gram-positive bacteria were investigated for their capacity to enhance macrophage activity and consequently the release of cytokines. The following experiments (Fig 5.8 – 5.11) were performed in the same manner and observed under the same conditions; RAW 264.7 (1×10^6) cells in 1 ml of supplemented medium were incubated with bacterial components at 37°C in 95% air, 5% CO_2 for 24 hours. LPS 100ng/ml was used as a positive control, medium alone and with macrophages served as negative controls. Different concentrations of lipid S, PG and LTA derived from *S. epidermidis,* and LTA from *S. aureus* were all investigated for their effects on TNF-α release. Values shown are the mean of two samples. WTA did not induce cytokine production at any concentration (results not shown).

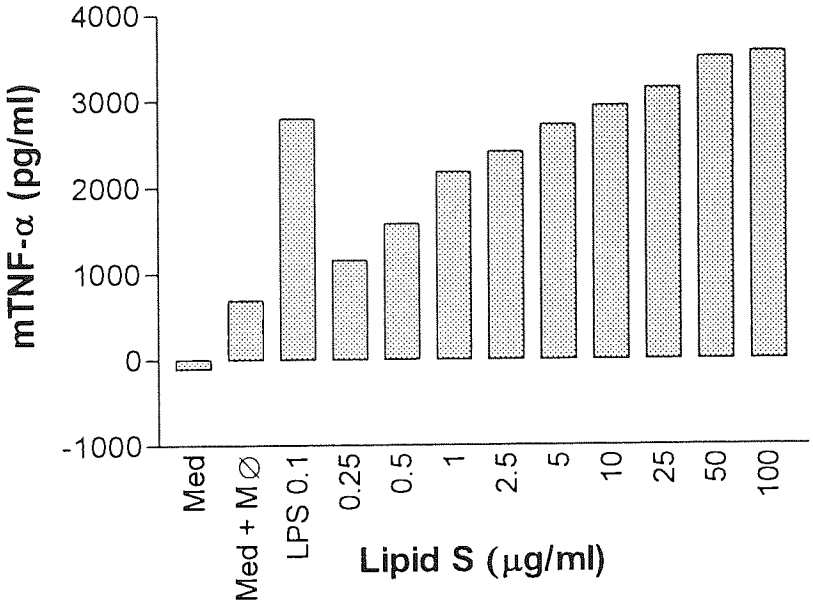


Figure 5.8: Effect of lipid S from *S. epidermidis* on TNF-α production from RAW 264.7 cells (1×10^6/ml) after 24h. Data represents the mean of two samples

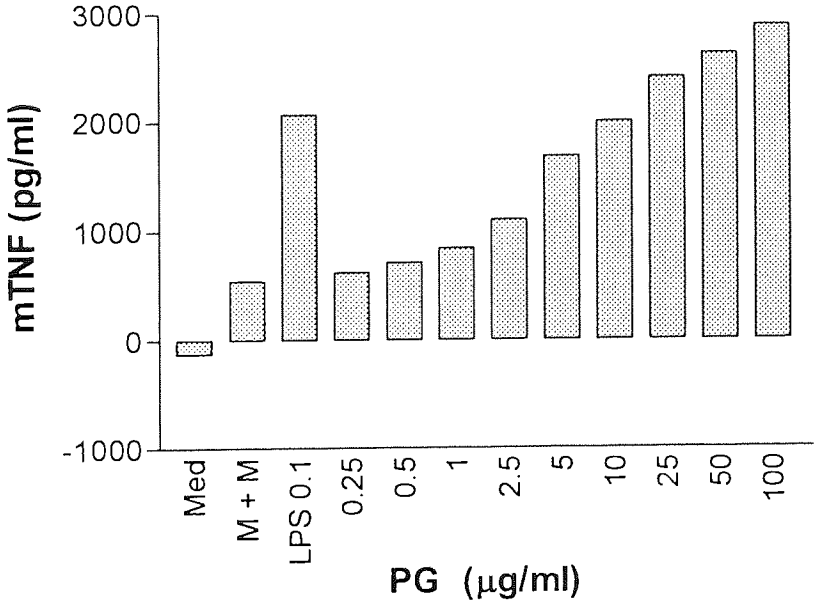


Figure 5.9: Effect of PG from *S. epidermidis* on TNF-α production from RAW 264.7 cells (1×10^6/ml) after 24h. Data represents the mean of two samples

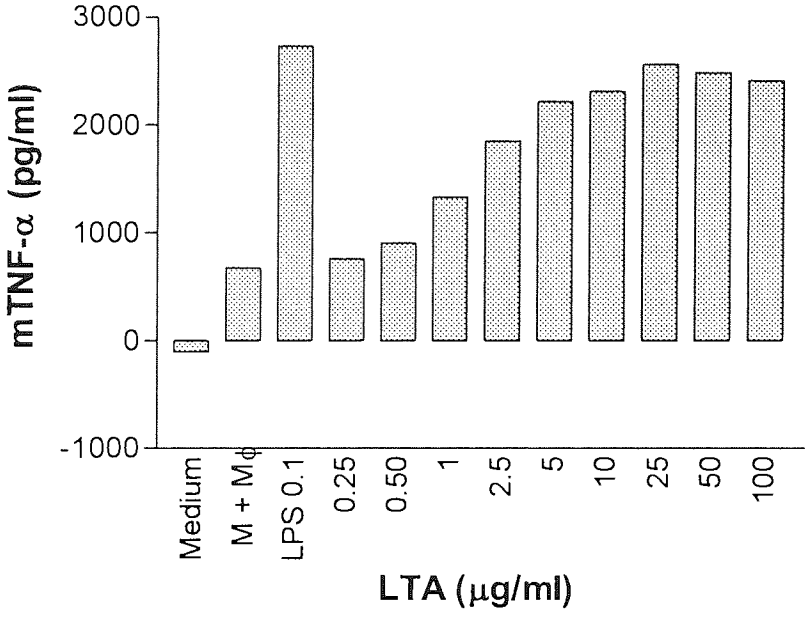


Figure 5.10: Effect of LTA from *S. epidermidis* on TNF-α production from RAW 264.7 cells (1×10^6/ml) after 24h. Data represents the mean of two samples

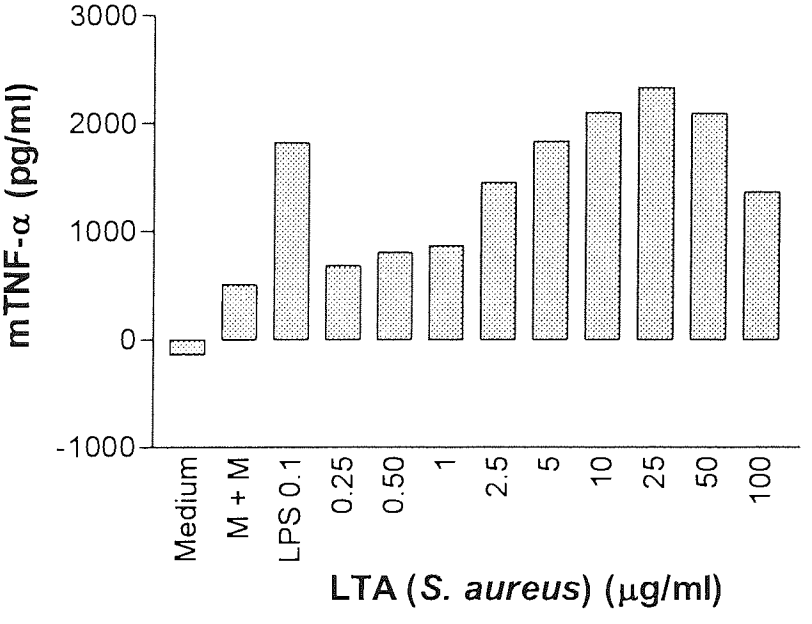


Figure 5.11: Effect of LTA from *S. aureus* on TNF-α production from RAW 264.7 cells (1×10^6/ml) after 24h. Data represents the mean of two samples

Different concentrations of lipid S (Fig 5.8), PG (Fig 5.9) and LTA (Fig 5.10) all derived from *S. epidermidis*, and LTA derived from *S. aureus* (Fig 5.11) were all investigated for their effects on TNF-α production. Lipid S and PG both displayed a dose-dependent effect; with peak production of TNF-α occurring at the highest concentration of 100 μg/ml. A five-fold increase above control samples was observed for both bacterial components; slightly lower increases were observed at concentration between 1 - 50 μg/ml for lipid S and 5 – 50 μg/ml for PG. Lower concentrations of 0.25 – 2.5μg/ml from PG were less effective. LTA from both species of bacteria showed a similar pattern to each other, both were dose-dependent with an optimal production of TNF-α at 25 μg/ml; higher concentrations diminished the response.

It was observed in the above experiments investigating the effects of bacterial components to induce TNF-α production that the levels of TNF-α varied greatly in positive control samples containing RAW264.7 cells and LPS (100ng/ml). For example the LPS control in figures 5.6 was considerably different from the LPS control in figure 5.9; the difference being nearly 1000 pg/ml. This was most likely due environmental factors or to the passage number of the cell line. Consequently to limit variation and for results to be more comparable the subsequent experiments were performed using cells of the same passage number.

To maximise cell usage the following experiments (Fig 5.12 – 5.15) were performed in the same manner and observed under the same conditions. A 250μl aliquot of 1×10^6 cells/ml (from the same cell line and passage number) were added to individual wells of a 96 well tissue culture plate, bacterial components were added and the suspension was incubated at 37°C in an atmosphere of 95% air and 5% CO_2 for 24 hours. Medium alone and with macrophages served as negative controls. Different concentrations of lipid S, PG and LTA derived from *S. epidermidis* were all investigated for their effects on cytokine release. After 24 hours, supernatants were collected and cytokines were measured by ELISA.

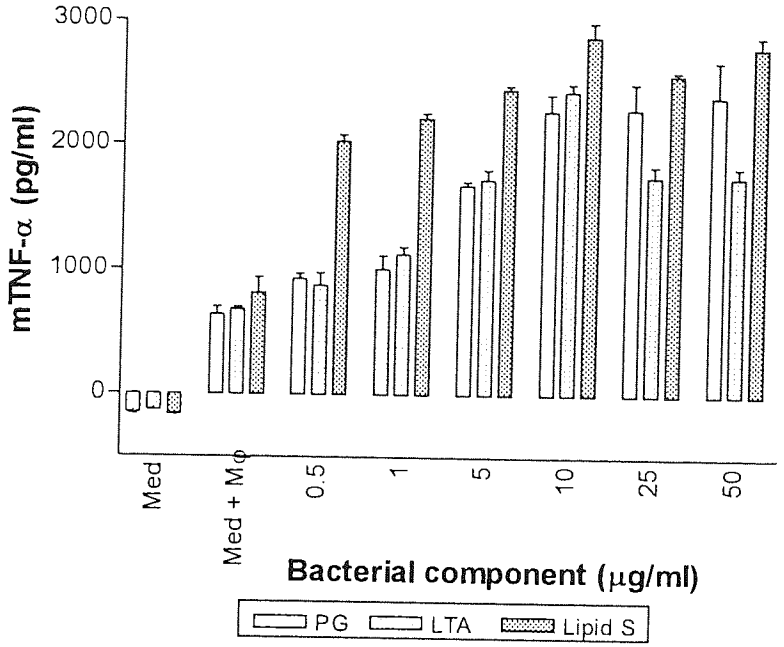


Figure 5.12: Effects of different concentrations of Lipid S, LTA and PG from *S. epidermidis* on TNF-α production from RAW 264 cells (1×10^6) after 24 hours Data represents the mean □ SEM of triplicate samples

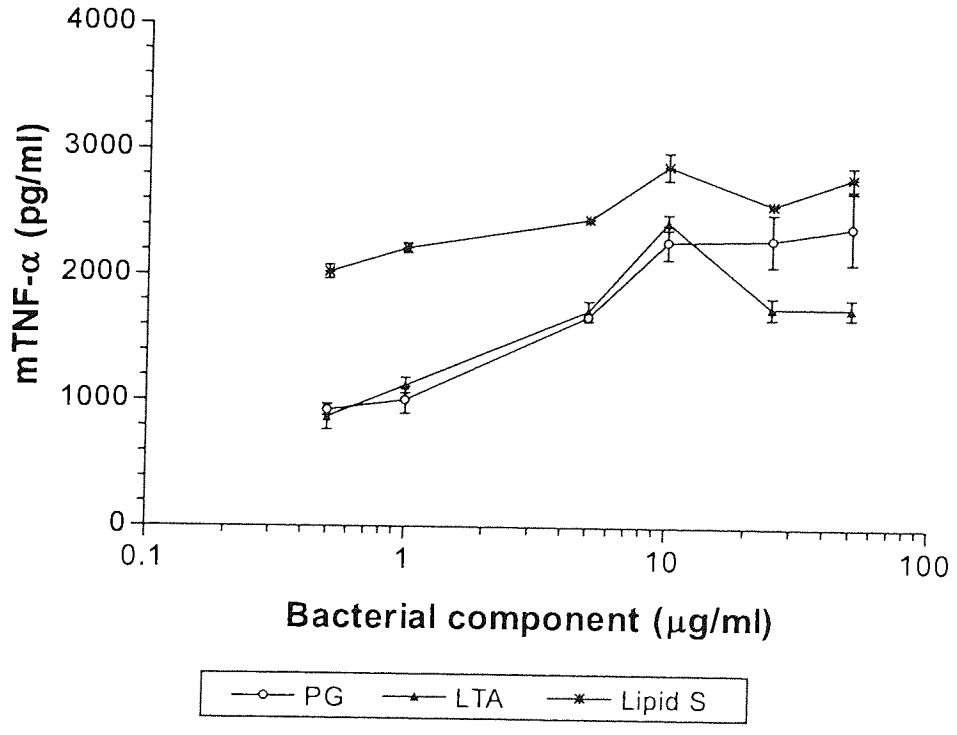


Figure 5.13: Log dose response curves demonstrating effects of Lipid S, LTA and PG from *S. epidermidis* on TNF-α production from RAW 264 cells (1×10^6) after 24 hours. Data represents the mean ± SEM of triplicate samples

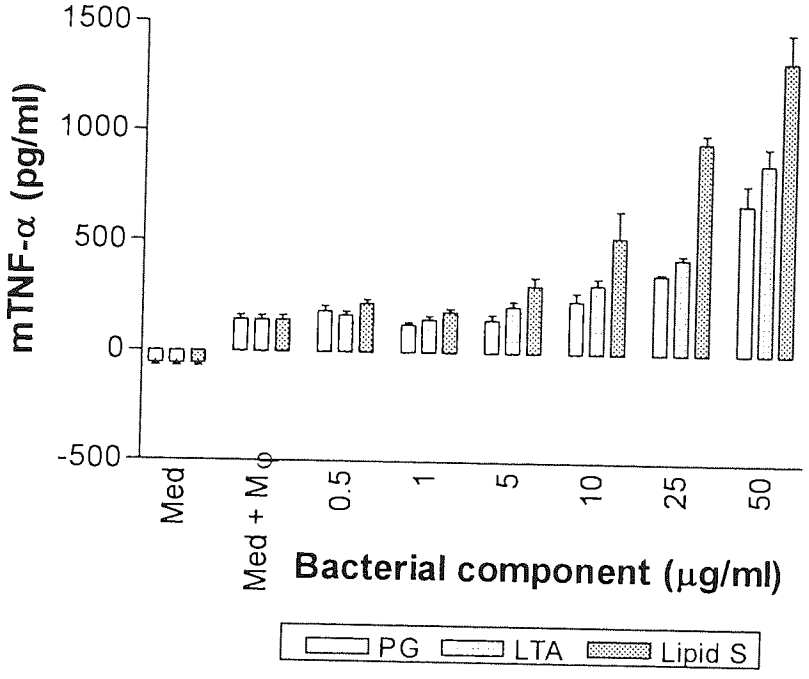


Figure 5.14: Effects of different concentrations of Lipid S, LTA and PG from *S. epidermidis* on TNF-α production from J774.2 cells (1×10^6) after 24 hours. Data represents the mean ± SEM of triplicate samples

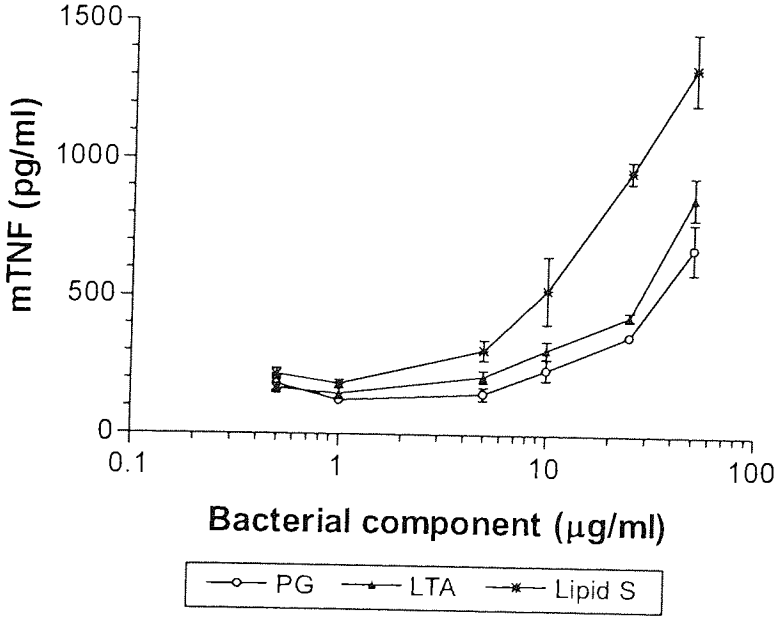


Figure 5.15: Log dose response curves demonstrating effects of Lipid S, LTA and PG from *S. epidermidis* on TNF-α production from J774.2 cells (1×10^6) after 24 hours. Data represents the mean ± SEM of triplicate samples

Experiments comparing TNF-α production on different concentrations of lipid S, LTA and PG using both cell lines, RAW264.7 (Figs 5.12 and 5.13) and J774.2 (Fig 5.14 and 5.15) showed similar results. As expected all were dose-dependent and the RAW264.7 cell line responded by producing higher concentrations of TNF-α compared to J774.2 cells, however negative controls of cells alone were much higher in RAW264.7 than J774.2 cells.

These results demonstrate that all the Gram-positive products tested except WTA are able to stimulate significant release of pro-inflammatory TNF-α release in macrophage cell lines, and on a weight basis lipid S was most active in this respect. The TNF-α release from RAW264.7 cells in response to lipid S showed significant increases in concentrations as low as 0.5 μg/ml; where a two-fold increase was observed compared to control samples ($p = 0.0019$), thereafter increases in TNF-α production occurred with each higher dose; with optimal dose occurring at 10 μg/ml, demonstrating a 3.5-fold increase compared to control cells ($p = 0.0012$); higher doses of lipid S slightly inhibited the release of TNF-α. PG and LTA were similar in their effects on RAW 264.7 cells; the peak concentration of 10 μg/ml occurred for both products.

The J774.2 cell line released less TNF-α than RAW 264.7 cells in respect to all Gram-positive samples. The results shown in figure 5.14 are more clearly shown in log dose response curves in figure 5.15, demonstrating the differences between samples. All samples tested showed a dose-dependent pattern. Low doses; between 0.5 - 5 μg/ml were insignificant for all products tested ($p = >0.05$). Lipid S again showed a significant difference to the other compounds, doses of 10, 25 and 50 μg/ml when compared with control cells elicited 3.6 ($p = <0.05$), 6.6 ($p = <0.001$), and 9-fold ($p = <0.001$) increases in TNF-α respectively. Whereas doses of LTA and PG were not significant until the highest concentration of 50 μg/ml was used ($p = <0.001$), where a 6 and 4.7-fold increase in TNF-α production was observed.

After establishing that cell lines had the ability to up-regulate the production of TNF-α both splenic and peritoneal macrophages were utilised to compare effects of LPS from *E. coli* and lipid S and LTA from *S. epidermidis*

To maximise the use of mouse macrophages and to limit any variations between batches of mouse cells they were pooled together prior to the addition of bacterial components and samples were tested in duplicate (Figures 5.16 and 5.17). For preparation of cells see section 2.4.1.

Mouse macrophages were pooled and a 250µl aliquot of 5×10^5 cells/ml were added to individual wells of a 96 well tissue culture plate and allowed 24 hours to adhere. Following adherence bacterial components were added and the suspension was incubated at 37°C in an atmosphere of 95% air and 5% CO_2 for a further 24 hours. Medium alone and with macrophages served as negative controls. After 24 hours, supernatants were collected and TNF-α was measured by ELISA.

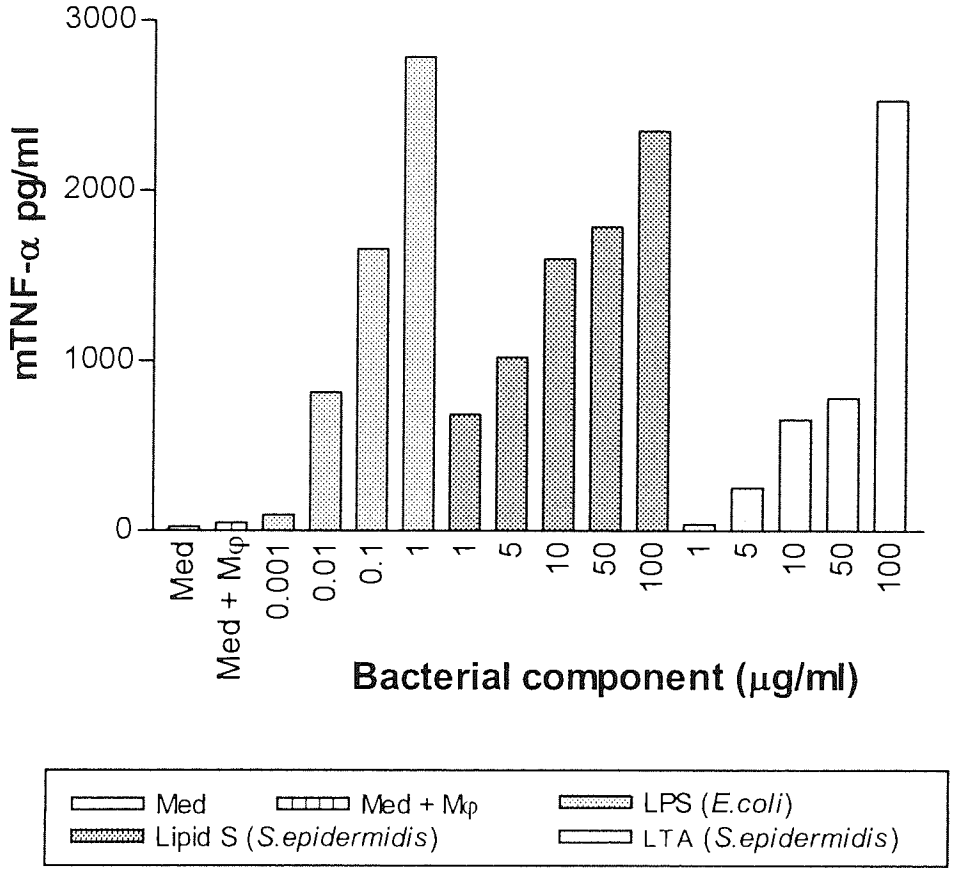


Figure 5.16: Effects of different concentrations of Lipid S and LTA from *S. epidermidis* and LPS from *E. coli* on TNF-α production from mouse splenic macrophages (5×10^5 cells/ml) after 24 hours

Results are expressed as the mean of duplicate samples from a single experiment.

All bacterial components stimulated splenic macrophages to elicit a dose-dependent production of TNF-α, increases over basal levels were observed for low concentrations of LPS at 0.01 μg/ml (16-fold), lipid S at 1 μg/ml (14-fold) and LTA at 10μg/ml (13-fold). LTA at lower concentrations was less effective than lipid S but at the higher concentration of 100 μg/ml it elicited a greater response than lipid S. TNF-α levels produced by lipid S at 10 μg/ml were comparable to those produced by LPS at 0.1 μg/ml, suggesting that on a weight for weight basis that LPS is 100-fold more active than lipid S.

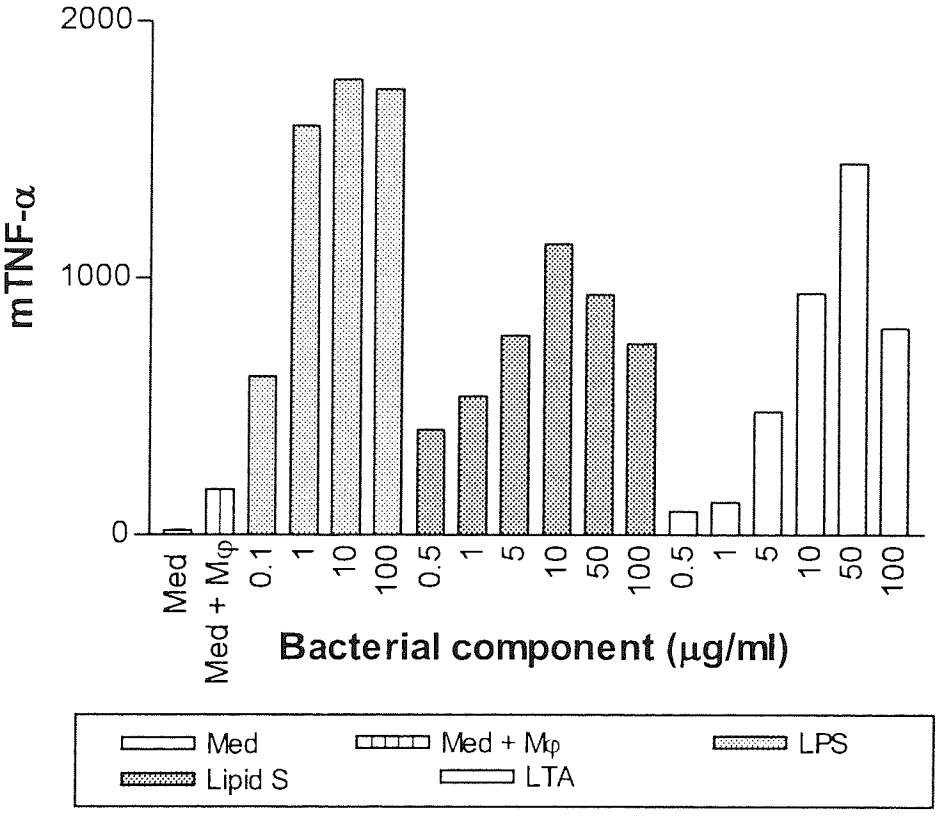


Figure 5.17: Effects of different concentrations of Lipid S and LTA from *S. epidermidis* and LPS from *E. coli* on TNF-α production from mouse peritoneal macrophages (5×10^5) after 24 hours

Results are expressed as the mean of duplicate samples from a single experiment.

All of the bacterial components tested caused mouse peritoneal macrophages to produce TNF-α. Peak production of TNF-α was observed for LPS and lipids S at 10μg/ml and LTA at 50μg/ml, thereafter higher concentrations were inhibitory.

5.3.3 Effects of bacterial components on the production of IL-6

Following the TNF-α experiment, assays were then conducted to measure IL-6 produced by the RAW264.7 and J774.2 murine cell lines in response to bacterial components from *S. epidermidis*. Experiments were performed in the same manner and observed under the same conditions as for TNF-α (Figures 5.18 – 5.21). 250µl aliquots of 1 × 10^6 cells/ml (from the same cell line and passage number) were added to individual wells of a 96 well tissue culture plate, bacterial components were added and the suspensions were incubated at 37°C in an atmosphere of 95% air and 5% CO_2 for 24 hours. Medium alone and with macrophages served as negative controls. Different concentrations of lipid S, PG and LTA derived from *S. epidermidis* were investigated for their effects on cytokine release. After 24 hours, supernatants were collected and IL-6 levels were measured by ELISA.

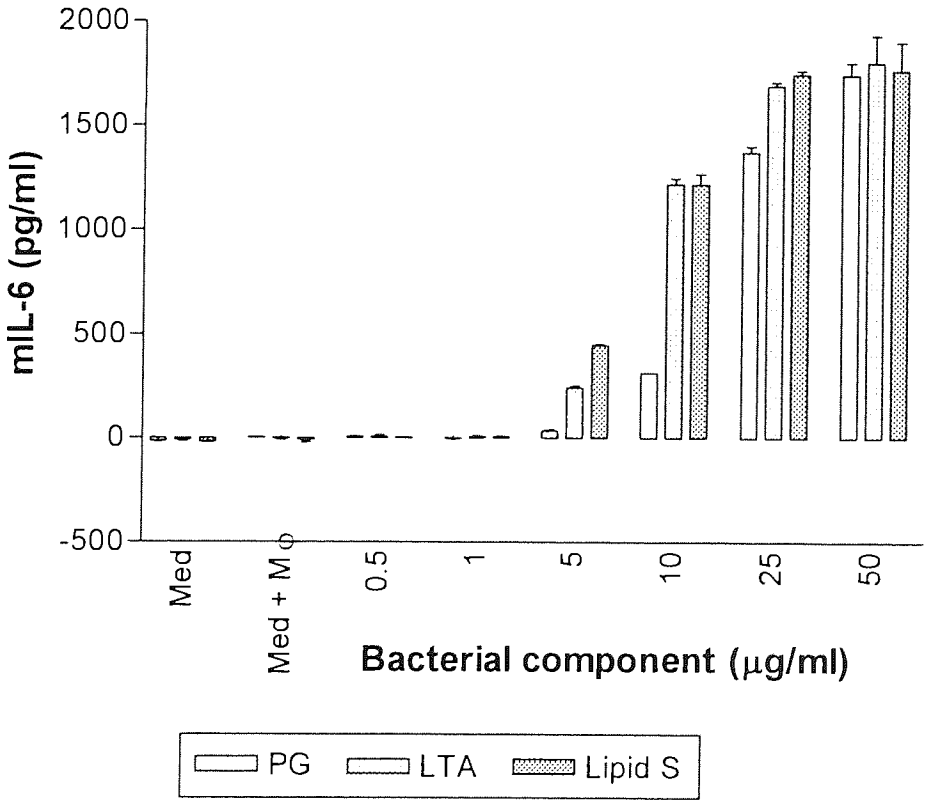


Figure 5.18: Effects of different concentrations of Lipid S, LTA and PG from *S. epidermidis* on the production of IL-6 from RAW 264.7 cells (1 ×10^6) after 24 hour incubation. Data represents the mean ± SEM of triplicate samples

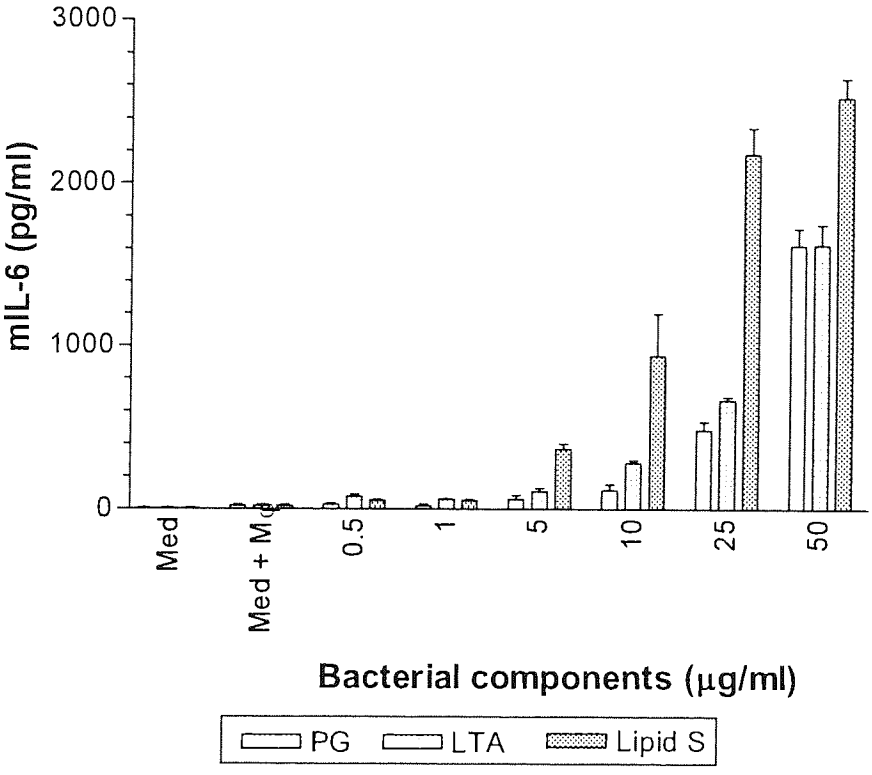


Figure 5.19: Effects of different concentrations of Lipid S, LTA and PG from *S. epidermidis* on the production of IL-6 from J774.2 cells (1×10^6) after 24 hour incubation. Data represents the mean ± SEM of triplicate samples

As expected all bacterial components exhibited a dose-dependent induction of IL-6 in the J774.2 cell line. LTA and PG elicited similar effects for all doses used and both peaked at the highest concentration of 50 µg/ml; lower concentrations gave insignificant induction. Lipid S was more active in its ability to induce IL-6 release; significantly greater effects were seen at concentrations 10, 25 and 50 µg/ml, resulting in increases of 40, 90 and 105-fold respectively when compared to control cells $p = <0.001$). To emphasise the dose response the results were also plotted on a log concentration axis in figure 5.20.

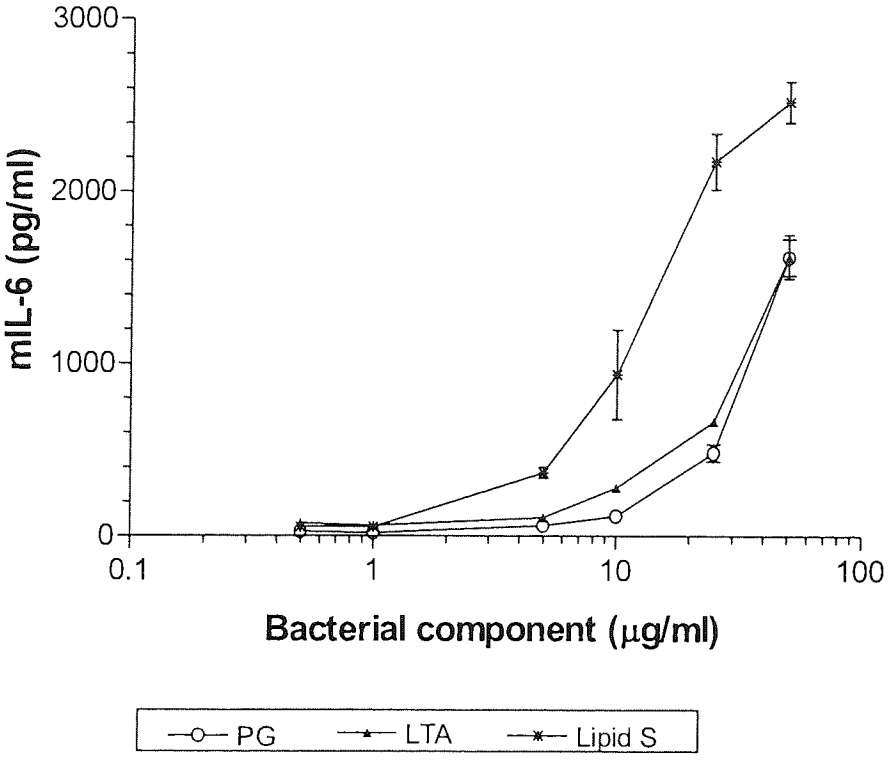


Figure 5.20: Effects of different concentrations of Lipid S, LTA and PG from *S. epidermidis* on the production of IL-6 from J774.2 cells (1×10^6) after 24 hour incubation. Data represents the mean ± SEM of triplicate samples.

5.3.4: Effects of bacterial components on the production of IL-1β

Following the above experiments the two cell lines were then investigated for their release of IL-1β in response to the different components from *S. epidermidis*.

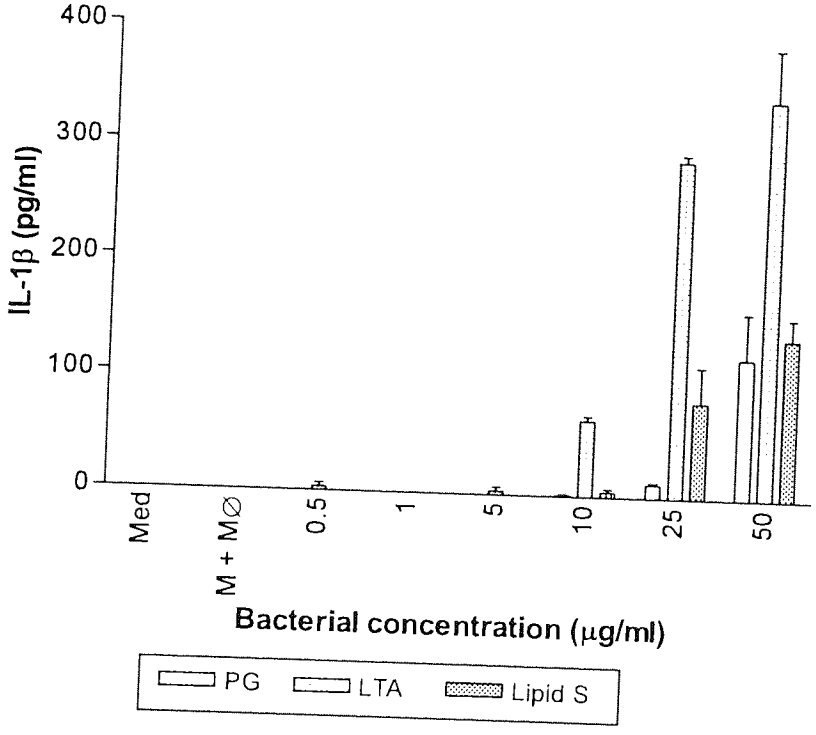


Figure 5.21: Effects of different concentrations of Lipid S, LTA and PG from *S. epidermidis* on the production of IL-1β from RAW 264.7 cells (1×10^6) after 24 hour incubation. Data represents the mean ± SEM of triplicate samples.

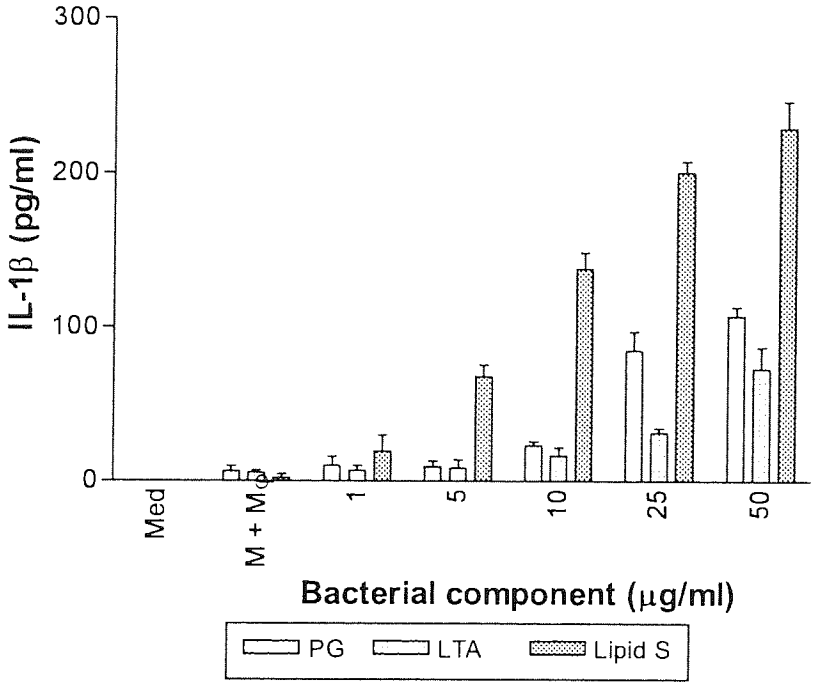


Figure 5.22: Effects of different concentrations of Lipid S, LTA and PG from *S. epidermidis* on the production of IL-1β from J774.2 cells (1×10^6) after 24 hour incubation. Data represents the mean ± SEM of triplicate samples.

The two cell lines showed different responses for IL-1β production. Figure 5.21 which represents the effects of bacterial components on the RAW264.7 cell line shows that LTA was the most active compound from 10 – 50μg/ml ($p = <0.05$). Lipid S and PG were ineffective below concentrations of 25 and 50μg/ml respectively. In contrast the J774.2 cells (Fig 5.22) were much less responsive to LTA and more sensitive to lipid S, with concentrations between 5 – 50μg/ml being the most significantly effective ($p = <0.001$). Significant levels of IL-1β were only observed at the higher concentrations of LTA (50μg/ml) and PG (25μg/ml), $p = <0.05$).

5.3.5 Effects of a combination of bacterial cell components on cytokine production

Following the earlier investigations into the additive and synergistic effects of combinations of bacterial components on nitric oxide production, similar studies on cytokines production from the RAW264.7 cell line were made.

The effect of combinations of LTA and PG from *S. epidermidis* upon TNF-α (Fig 5.23) and IL-6 (Fig 5.24) and the combined effects of lipid S and PG on TNF-α production (Fig 5.25) were examined.

The experiments (Fig 5.22 – 5.26) were performed as follows, a 250μl aliquot of 1×10^6 cells/ml (from the same passage number) were added to individual wells of a 96 well tissue culture plate, bacterial components, which had been pre-incubated for 30 minutes were added and the suspension was incubated at 37°C in an atmosphere of 95% air and 5% CO_2 for 24 hours. Medium alone and with macrophages served as negative controls and LPS (0.1μg/ml) was used as a positive control. Data represent results from the means of two samples.

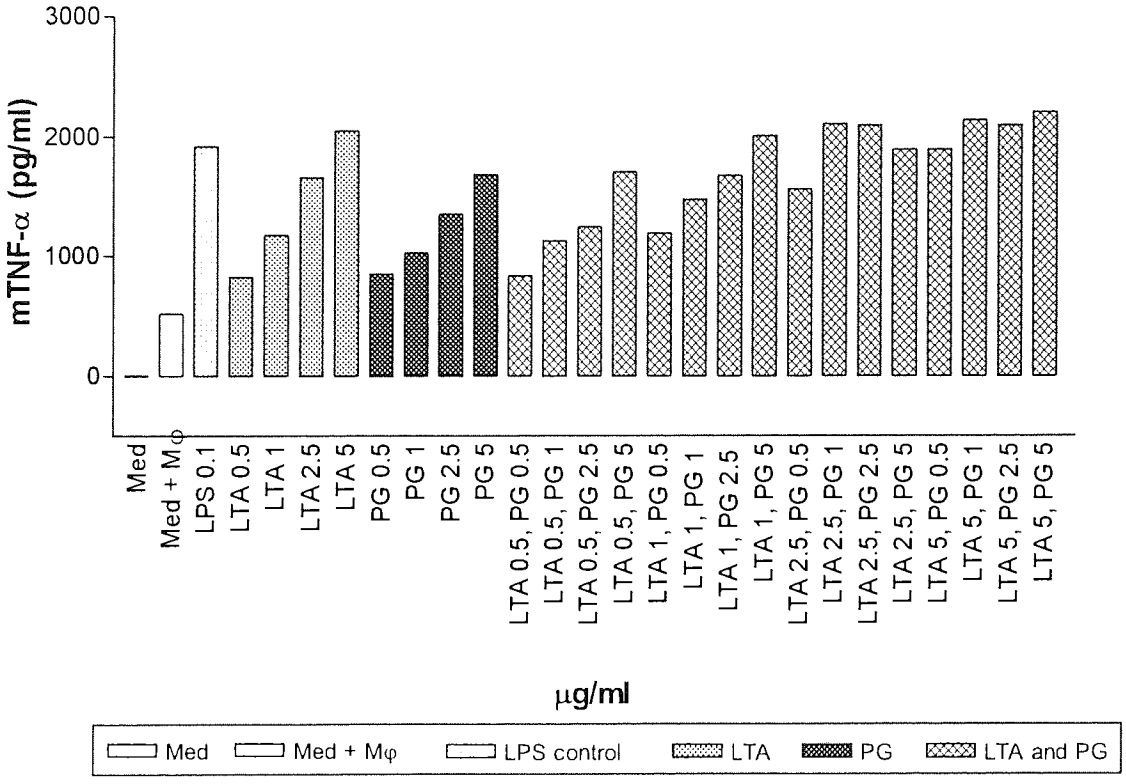


Figure 5.23: The effect of LTA and PG from *S. epidermidis* alone and in combination on TNF-α produced from RAW264.7 cells (1×10^6/ml). Bars represent the mean of two samples.

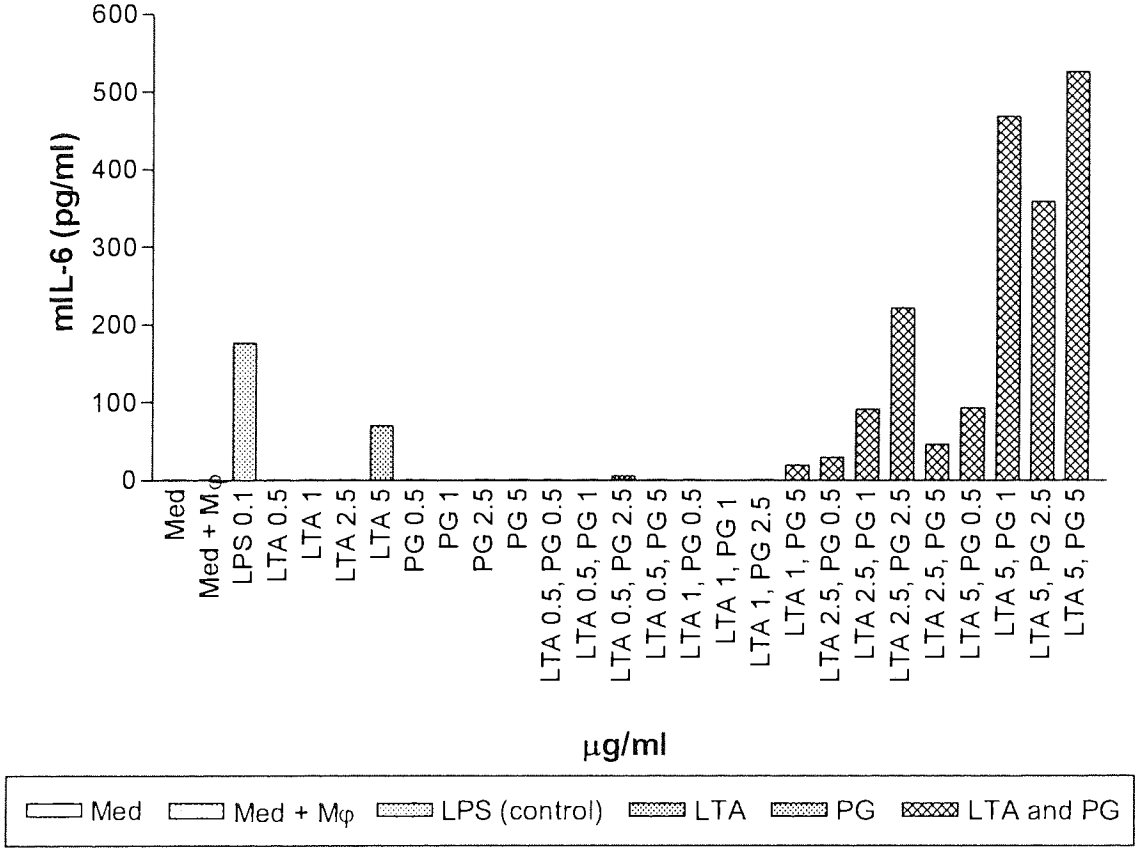


Figure 5.24: The effect of LTA and PG from *S. epidermidis* alone and in combination on IL-6 produced from RAW264.7 cells (1×10^6/ml). Bars represent the mean of two samples.

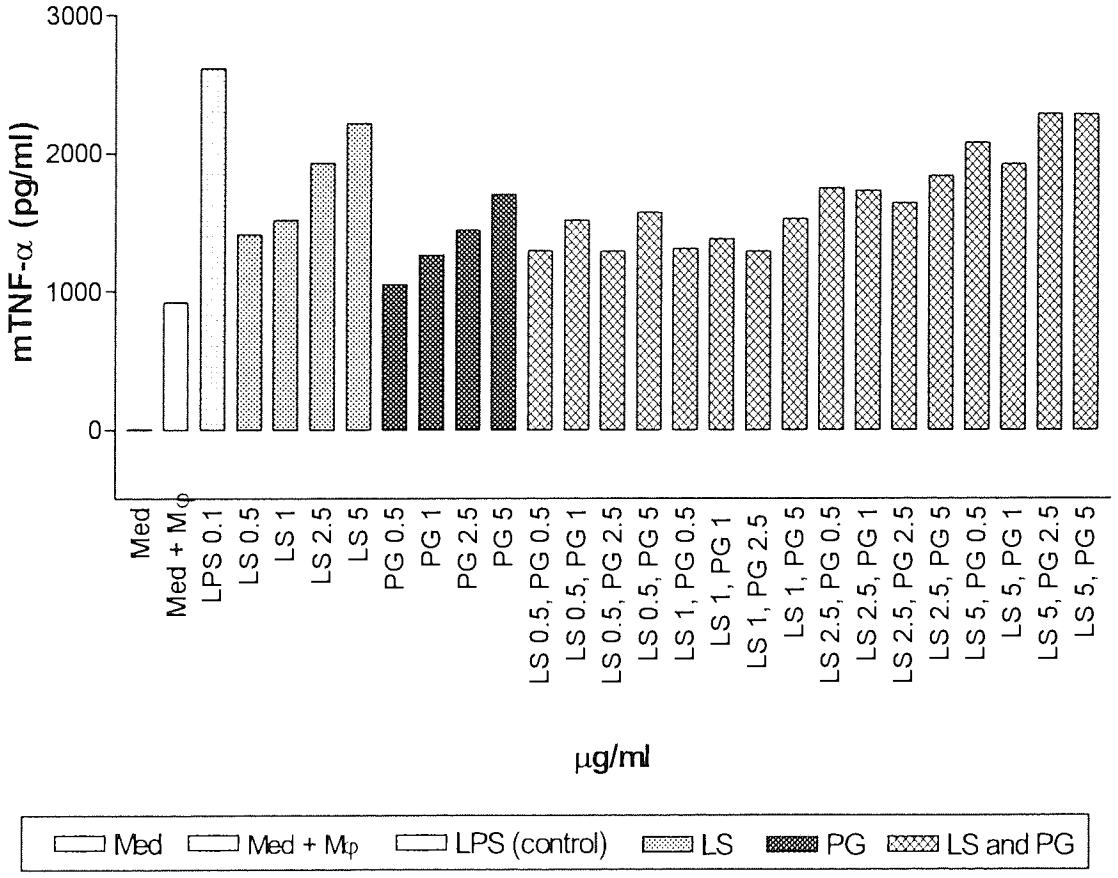


Figure 5.25: The effect of lipid S and PG from *S. epidermidis* alone and in combination on TNF-α produced from RAW264.7 cells (1×10^6/ml). Bars represent the mean of two samples.

No synergy or an additive enhancement was observed for the release of TNF-α in response to the combinations of LTA and PG (Fig 5.23) or lipid S and PG (Fig 5.25) in RAW264.7 cells. All combinations were produced similar or lower cytokine levels than the bacterial components alone.

The measurement of IL-6 produced by RAW264.7 cells in response to the combination of LTA and PG revealed a different picture (Fig 5.24). All concentrations (with the exception of LTA at 5 μg/ml) had no effect on the release of IL-6, whereas combinations of the two products stimulated IL-6 production by

RAW264.7 cells. Considerably higher levels of IL-6 were observed with LTA at 5μg/ml and PG at 1, 2.5 and 5 μg/ml (6.7, 5.1 and 7.5-fold enhancement respectively). A slightly lower concentration of LTA (2.5μg/ml) and PG (2.5μg/ml) resulted in a 3.1-fold increase in IL-6 compared to control cells of LTA and PG alone.

5.3.6 Effects of doses of lipid S extracted from different Gram positive bacteria on TNF-α production

Following the earlier investigations into the effects of lipids extracts from different Gram-positive bacteria on nitric oxide production, similar studies on TNF-α production from the RAW264.7 cell line were made.

RAW264.7 cells (1×10^6) cells in 1 ml of supplemented medium were incubated with doses of lipid S from 5 Gram-positive organisms (all grown in HHW medium) at 37°C in 95% air, 5% CO_2 for 24 hours. Bars are standard error of the mean of triplicate samples.

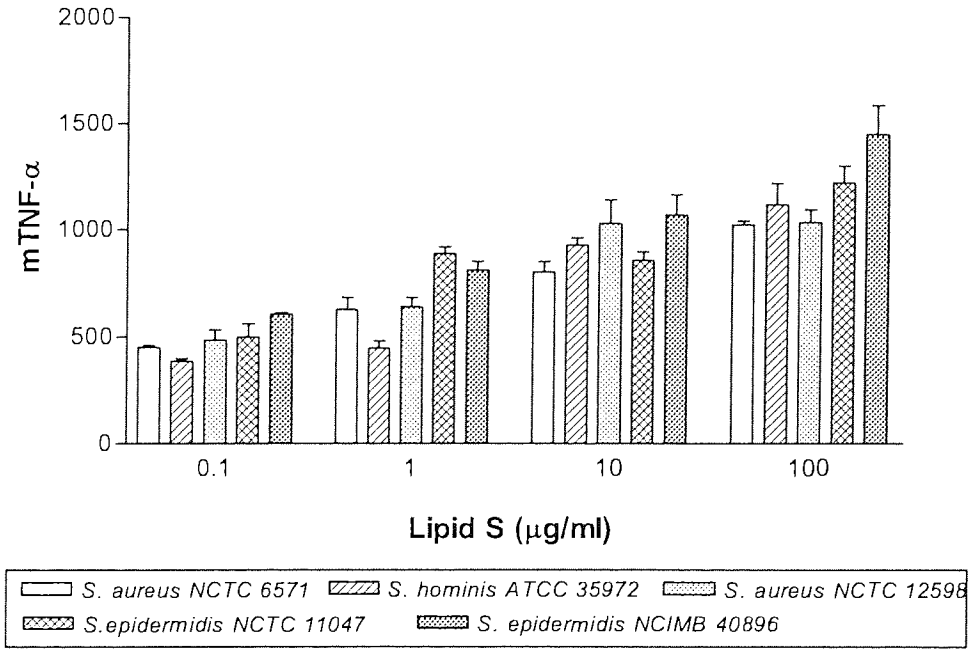


Figure 5.26: Effect of lipid S extracts from various Gram-positive staphylococci species upon TNF-α production from RAW264.7 cells (1×10^6/ml) after 24h incubation

The results demonstrate that all the lipid S extracts tested here are all dose-dependent and all are potent stimulators of TNF-α, even at low concentrations. Insignificant values were obtained when comparing the means within the groups.

5.4 DISCUSSION

Results in this chapter show that lipid S, LTA and PG from *S. epidermidis,* LTA from *S. aureus*, and LPS from *E. coli* stimulate the release of cytokines from macrophage-like cell lines, and from primary mouse macrophage cells isolated from the spleen and peritoneum. The actions of cytokines are normally beneficial to the hosts' response to infection; however they can also be detrimental if they are over-produced. High levels have been reported in patients with sepsis (Waage and Aasen, 1992).

Preliminary experiments established the optimal cell density to obtain measurable levels of TNF-α from RAW264.7 cells. LPS is the major component of Gram-negative bacteria which induces the release of TNF-α and was utilised here to measure optimal time for assays to run and optimal cell density. A linear increase in TNF-α production occurred with increasing RAW264.7 cell numbers (Fig 5.4), peaking at 1×10^6, hence this density was employed for all subsequent assays involving cell lines. A higher cell density of 2×10^6 gave lower TNF-α levels, suggesting that cell contact inhibition was occurring through over-crowding of cells or possibly some toxicity to cells was taking place. This experiment also revealed that levels of cytokines were slightly lower after 48 hours compared to the 24 hour incubation period. After 24 hours there would be a reduction of available nutrients in the culture medium which would result in diminished metabolic activities, lower levels of protein synthesis and possibly a proportion of the cell population would be dying.

A further experiment was conducted to determine the optimal time for testing samples for TNF-α production. RAW264.7 cells were incubated with LPS and samples were taken at intervals of 2, 4, 8, 24 and 48 hours (Fig 5.5). A linear increase was seen with time up to a maximal level of TNF-α production after a 24 hour incubation period. After 48 hour incubation a lower level was observed, confirming the results from the previous experiment. It would be expected that the production of TNF-α would be at least the same at 24 and 48 hours because it was released into the culture cell supernatant and would therefore be confined in culture supernatant. The lower level

could be due to instability of TNF-α or the action of proteases in the medium. Alternatively high levels in the supernatant could have inhibited further synthesis and release of TNF-α, resulting in feedback inhibition. Consequently a 24 hour incubation period was used as the time to conduct all subsequent experiments. However the fact that peak TNF-α was produced within 24 hours could have major implications for treatment, Michie *et al.* (1989) suggest that any attempts to block the effects of TNF-α require rapid diagnosis and would have to be administered very early in the course of the disease and not when clinical manifestations of septic shock are maximal.

The effects of a range of concentrations of bacterial components on both RAW264.7 and J774.2 cells were investigated. Both cell lines demonstrated similar effects in their response to LPS (Fig 5.6 and 5.7), TNF-α was produced in a dose-dependent manner in both cell lines, although levels of TNF-α differed greatly with RAW264 .7 cells producing three-times more TNF-α than J774.2 cells for the same concentration of LPS. This suggests that RAW264.7 cells have a higher number of cell receptors that can respond to LPS than J774.2 cells.

The incidence of Gram-positive sepsis has increased in the last two decades with significant mortality (Cui *et al.*, 2000). The initiation of sepsis does not require the presence of live bacteria; it can also be initiated from the components of dead or lysed bacteria (Bone, 1994). LTA and PG have been previously reported to provoke a marked stimulation of TNF-α (Greenberg *et al.*, 1996). These results confirm previous findings that LTA induces inflammation but is much less potent than LPS (Henderson *et al.*, 1996). PG has also previously been implicated in playing a partial role in the initiation of septic shock (Mehlin *et al.*, 1999). Greenberg *et al.* (1996) also reported that PG and LTA are required at concentrations 2 to 3 orders of magnitude higher than for LPS. However lipid S which is released from the bacterial cell wall has not been investigated for its effects on cytokine production before and could well be an important factor in clinical infections particularly if it goes systemic when released by multiplying *S. epidermidis* on biofilm. Lipid S, PG and LTA from *S. epidermidis* and LTA from *S. aureus* all induced production of TNF-α from RAW264.7 cells, though at varying levels (Fig 5.8 – 5.11). Lipid S and PG both induced the release of TNF-α in a dose-dependent manner, with peak production occurring at their highest concentration used (100μg/ml). Concentrations of 10μg/ml of lipid S and PG gave

similar results of TNF-α produced by LPS at 0.1μg/ml. In contrast LTA from both *S. epidermidis* and *S. aureus* were dose-dependent up to an optimal concentration of 25μg/ml; some inhibition was seen at higher concentrations of 50 and 100μg/ml. This is contrary to other reports, which showed that LTA from *S. aureus* does not induce cytokine release from cultured monocytes (Bhakdi *et al.*, 1991; Heumann *et al.*, 1994). However it was noted that the effects of the standard positive control samples of LPS differed greatly in the levels of TNF-α between different experiments. This was most likely due to the passage number of the cell lines rather than experimental variation. Consequently to narrow the margin of error and to make more comparable judgements subsequent experiments were designed to measure the effects of all bacterial components on the same microtitre plates using cells from the same passage number.

Results from the same passage number cells in response to bacterial components lipid S, LTA and PG from *S. epidermidis* were all dose dependent (Fig 5.12 - 5.15). Lipid S proved to be the most active compound in this respect with higher levels of TNF-α released into culture supernatants compared to LTA and PG. Peak levels of TNF-α were seen with the addition of 10 μg/ml of lipid S in RAW264.7 cells and 50μg/ml in J774.2 cells, these were 3.5 and 5-fold increases respectively compared to control cells. High concentrations of lipid S were slightly inhibitory towards RAW264.7 cells. This was also evident with the addition of high concentrations of LTA incubated with RAW264.7 cells, where the response decreased by a third in samples containing 25 and 50 μg/ml compared to the response observed after addition of 10μg/ml. LPS and LTA are amphiphilic molecules which form micelles in solution (Keller *et al.*, 1992a; Deininger *et al.*, 2003). Lipid S is structurally related to LTA (Lambert *et al.*, 2000) and it is presumed that this molecule also forms micelles. Micelle formation could explain the lower levels of stimulation at higher concentrations, possibly by blocking receptor binding. Critical micelle concentrations for LTA are in the range 1 -10μM (Wicken *et al.*, 1986)

It is known that macrophages isolated from different anatomical sites display a diversity of phenotypes and capabilities. Because macrophage function is dependent in part on signals received from the immediate microenvironment, it is suggested that macrophage heterogeneity may arise from unique conditions within specific tissues.

The spleen and peritoneum macrophages exist in a sterile, anaerobic environment and will impart different constraints on resident macrophages than does the aerobic environment of the alveolar macrophage. These cells were employed to measure TNF-α in response to products from both Gram-positive products and Gram-negative LPS. A range of doses was used to measure the accumulation of TNF-α over a 24-hour period under *in-vitro* conditions. LPS, LTA and lipid S induced a dose-dependent release of TNF-α from splenic macrophages, with the highest concentration initiating the greatest activity. Concentrations of LTA and lipid S at 100μg/ml were comparable to each other; however lower concentrations of 1, 5, 10 and 50μg/ml showed that lipid S was twice as active as the LTA molecule. Concentrations of lipid S at 10μg/ml were comparable with LPS at 0.1 μg/ml confirming earlier predictions that LPS is 100-times more potent than lipid S on a weight to weight ratio (Fig 5.16).

In contrast, results from peritoneal cells (Fig 5.17) displayed a different pattern; lower concentrations were more effective and higher concentrations of LPS, LTA and lipid S inhibited the response, though at varying levels. LPS was the most active compound and peak levels of TNF-α was seen at 10μg/ml, little difference was seen at the higher concentration of 100μg/ml. Lipid S gave the greatest response at 10μg/ml; LTA at 50μg/ml; lipid S at lower concentrations of 0.5 and 1μg/ml was 5-times more potent than LTA.

The differences in response observed by the murine splenic and peritoneum macrophage cells are probably due to their heterogeneity. The spleen acts a filter against foreign organisms that infect the bloodstream. Splenic macrophages have an important filtering and phagocytic role in removing bacteria from the circulation .and therefore it seems likely that splenic macrophages are more sensitive to bacterial products than peritoneal cells, possibly having more receptors that enable a higher degree of binding.

Bacterial cell wall products, LTA, PG and lipid S were investigated for their ability to induce the production of IL-6 and IL-1; both have been suggested to play a role in severe infections (Waage *et al.*, 1989). When IL-6 was measured from RAW264.7

(Fig 5.18) and J774.2 cells (Fig 5.19), all components gave dose–dependent responses and in all instances low concentrations (0.5 -5μg/ml) of the compounds tested were ineffective. Results from the RAW264.7 cell line showed that lipid S and LTA were equivalent on a weight-to-weight ratio in their ability to induce the secretion of IL-6, but PG was less effective. In contrast, the J774.2 cells were far more responsive to lipid S and results observed for PG and LTA were comparable, this is demonstrated clearly in figure 5.20.

When IL-1 was measured in response to the same Gram-positive products a different pattern emerged from the RAW264.7 cells (Fig 5.21). RAW264.7 cells had previously been more responsive to lipid S than to the other two products, LTA and PG. However, in this assay LTA proved to be more potent and displayed a three-fold increase in the amount of IL-1 secreted compared to lipid S and PG, which gave similar results. Low levels of all components had negligible effects on IL-1 release. In comparison, the results showed IL-1 released from the J774.2 cell line was greater in the presence of lipid S and PG than LTA (Fig 5.22). Lipid S was more than three-times more potent than LTA ($p = <0.001$) and twice as potent as PG at 50μg/ml ($p = <0.01$), lower concentrations of lipid S (5 and 10μg/ml) were also significantly more active than LTA ($p = <0.001$) and PG ($p = <0.001$). Therefore it seems likely that some experimental error in the RAW264.2 cell line assay had occurred. However, unfortunately these results were conducted as a single experiment and should be viewed with care.

WTA had previously been reported to be a weak stimulator of TNF-α production (Henderson *et al.*, 1996), however WTA derived from *S. epidermidis* had no effect on either of the cell lines on TNF-α, IL-6 or IL-1 release (results not shown).

The co-administration of some bacterial components which had previously been shown to act in synergy or to exert an additive effect on the release of NO was investigated to observe the effects on TNF-α and IL-6 production from RAW 264.7 cells. *S. aureus*-derived PG and LTA have previously been shown to cause an increase in release of TNF-α in anaesthetised rats which was not observed following activation by PG and LTA when used alone (De Kimpe *et al.*, 1995a). When LTA and PG used

in conjunction (Fig 5.23) there was no evidence of a synergistic or an additive response, levels of TNF-α from the combination of these bacterial components were no higher than those seen with the two products alone. This was also seen in results from the co-incubation of lipid S and PG (Fig 5.25). It has been suggested that LTA and PG are recognised by membrane-bound CD14 on macrophages (Yang *et al.*, 2001) which implies that the absence of a synergistic or additive effect on TNF-α levels may be due to receptor saturation.

In contrast, the amount of IL-6 resulting from the co-administration of LTA and PG showed a dramatic difference. Low concentrations of both components alone showed no activity in the production of IL-6, however low doses of the two combined components induced an additive effect. This suggests that receptors for IL-6 are more receptive when a combination of LTA and PG are present together than when they are present alone. Alternatively the additional production of IL-6 is the consequence of LTA and PG binding to TNF-α and IL-1 receptors as it has previously been reported that TNF-α and IL-1 can induce production of each other and act together to amplify the production of IL-6, further heightening the inflammatory response (Sundy *et al.*, 1999; Wang and Tracy, 1999).

Lipid S extracts from different Gram-positive bacteria were all found to stimulate the RAW264.7 macrophage cell line to up-regulate the production of TNF-α (Fig 5.26). These results showed a dose dependent response and significant levels of TNF-α were observed even at low concentrations; however there was very little variation in peak responses of TNF-α between each species at each concentration used suggesting they all have similar potency.

These results have demonstrated that in addition to LPS, the Gram-positive components lipid S, LTA and PG all exhibit a significant macrophage activation potentiation which resulted in the up-regulation and release of TNF-α, IL-1 and IL-6, though at varying levels. From the Gram-positive products tested, lipid S has been shown in numerous experiments to be the most potent in this respect on a weight basis. On a molar basis lipid S (MW ~2400) has a similar activity to LTA (MW

~18000) (Lambert *et al.*, 2000). Recently Deininger *et al.* (2003) reported to have discovered the active agent within LTA that causes the release of inflammatory mediators, they synthesised a molecule of LTA which had 6 glycerophoshate subunits which has the same structure as the lipid S model extracted in this study. This molecule also proved to stimulate the release of cytokines to the same effect as native LTA and they suggested that the key structural requirements for immune activation are two fatty acids and a glycerophosphate backbone with D-alanine substituents. How lipid S and other Gram-positive components interact with macrophages and other cells is largely unknown, but they undoubtedly induce pathways common to Gram-negative bacteria.

In a clinical situation it is the sum of all these agents that determines the outcome in a patient with a Gram-negative or Gram-positive infection by their direct and indirect effects. This has serious implications for induction of the inflammatory cascades that can lead to septic shock.

CHAPTER 6: INVESTIGATION OF POSSIBLE CONTAMINATION BY ENDOTOXIN AND TOXICITY OF BACTERIAL COMPONENTS TO CELLS

6.1 INTRODUCTION

6.1.1: Endotoxin contamination

There have been many reports that LTA is the main component of the Gram-positive cell wall that stimulates an immune reaction and may be responsible for septic shock induced by these bacteria. However, it has been suggested that some commercial preparations of LTA are contaminated with endotoxin and that the effects seen with LTA are, in fact, due to the contamination of the material with LPS (Morath *et al.*, 2002). Indeed polymyxin B, which is a specific inhibitor of LPS, suppresses the production of NO stimulated by LTA from some bacteria. Furthermore, purifying commercial LTA resulted in loss of activity of the LTA molecule to produce NO (Gao *et al.*, 2001). This suggests that the extraction methods are unsuitable and the purity of some commercial LTA has to be questioned. Endotoxin is ubiquitous in the environment and it is possible that during the purification process some endotoxin could have entered samples.

To ensure the several effects of LTA, lipid S and PG outlined in previous chapters was not due to contamination with LPS, the bacterial components isolated from *S. epidermidis* were subjected to endotoxin contamination analysis. This chapter also examines effects of phenol extraction to further purify commercial LTA to establish whether there is any loss in activity after purification.

It is possible that high concentrations of bacterial products could be toxic and kill macrophages and release further damaging materials. To determine if the bacterial products had an effect on cells that would induce apoptosis or necrosis and ultimately cell death their viability was assessed by flow cytometry.

6.1.2: Apoptosis and necrosis

Cell death can occur by two different mechanisms, apoptosis and necrosis. Apoptosis is a controlled form of cell death; it is pre-programmed into the cell as a method of controlling numbers. There is no pre-set programme for necrosis and this form of cell death is considered to be accidental. It usually occurs following a severe and sudden physiological or chemical trauma and leads to dysfunction of the mitochondria. In early necrosis the shape and function of the mitochondria changes and the cell is unable to maintain homeostasis, also due to damage to the cell membranes the cell cannot control osmotic pressure. In the latter stages of necrosis the cell swells and the membrane ruptures, cell contents are released into the local surrounding tissues, which can result in inflammation (Cohen, 1993).

6.2 MATERIALS AND METHODS

6.2.1: Detecting Endotoxin contamination

Two different methods of detecting contamination of endotoxin in samples of bacterial components were used.

6.2.1.1: Endotoxin contamination analysis using the antibiotic polymyxin B

One means to investigate if samples are contaminated with endotoxin is to use the antibiotic polymyxin B, which forms complexes with LPS and neutralises its ability to bind to macrophages (Kengatharan *et al.*, 1996a).

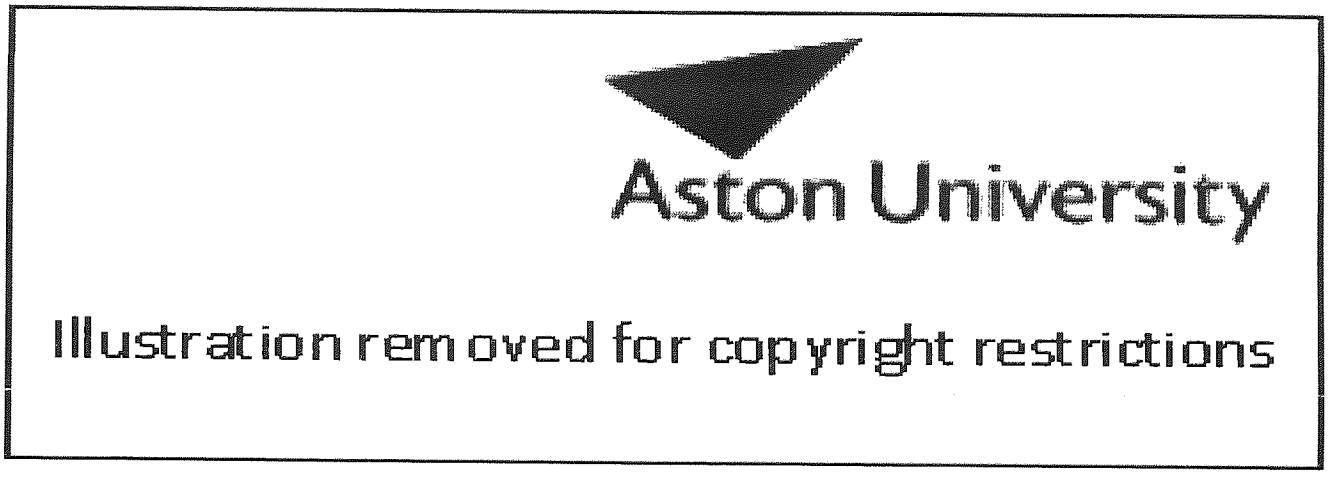


Figure 6.1: Structure of Polymyxin B

PMB is a decapeptide characterised by a heptapeptide ring containing four 2,4-diaminobutyric acids. An additional peptide chain covalently bound to the γ-amino group carries an aliphatic chain attached to the peptide through an amide bond. The molecule carries five positively charged residues of diaminobutyric acid (Storm *et al.*, 1977)

RAW264.7 cells were prepared as in section 2.4.6; polymyxin B sulphate (Sigma) was diluted to 5μg/ml in culture media and pre-incubated with cells 1 hour prior to the addition of cell wall components.

6.2.1.2: Limulus amoebocyte lysate assay (LAL)

A more sensitive test called the Limulus amoebocyte lysate assay (LAL) was employed (LAL kit QCL-1000, BioWhittaker Inc., MD). The test kit is a fast, quantitative, endpoint assay for the detection of Gram-negative bacterial endotoxin. It is based on the fact that endotoxin in the range of 0.01 to 0.1ng/ml activates a proteolytic enzyme in LAL that cleaves a chromogenic substrate proportional to the concentration of enzyme-activating endotoxin. Analysis was done according to the manufacturer's instructions. Samples were mixed with the LAL reagent and chromogenic substrate reagent 16 min at 20°C and the absorbance measured at 405nm. The endotoxin levels were calculated from the standards provided by the kit manufacturer. Pyrogen-free water was used as a control and to dilute samples and all plastic ware was endotoxin-free (Falcon).

6.2.2: Purifying commercial LTA

As previously discussed commercial LTA has been reported to contain LPS endotoxin that can be removed by further purification (Gao *et al.*, 2001). Commercial LTA (*S. aureus*, Sigma) was subjected to the same phenol extraction process as *S. epidermidis* LTA (section 3.2.3).

6.2.3: Viability tests using flow cytometry analysis

Flow cytometry is a quick way of performing quantitative sensitive measurements on each individual cell within a large population (King, 2000). Propidium iodide (PI) is the dye used most often to detect dead cells by flow cytometry; it carries two positive charges which prevent it from entering intact cells (Shapiro, 1995). In apoptotic cells or necrotic cells PI is able to enter through the membrane in the process of degradation and bind to DNA and RNA. When binding to DNA, PI has no sequence preference and intercalates between bases, this binding to nucleic acids causes the

fluorescence of cells to increase 20 – 30 fold. Cells that take up the PI dye are therefore identified as dead or dying.

To determine if any of the bacterial components were toxic to cells, the viability and DNA cell cycle analysis of macrophage cells was determined by flow cytometry. RAW264.7 cells (1×10^6) were prepared and incubated in the presence and absence of high concentrations of bacterial components (LTA, lipid S, PG and WTA at 100µg/ml and LPS 1µg/ml) for 24 hours as in section 2.4.6.

Flow cytometric analysis was performed on an EPICS® XL-MCL flow cytometer (Beckham-Coulter, Miami, USA).

6.2.3.1: Viability Assay

The propidium iodide used for the viability assay (Sigma, P4170) was first prepared as a stock solution 25mg/ml in PBS with 0.1% BSA (Sigma) and stored at 4°C in the dark. Further dilutions were prepared to obtain a working solution of 25µg/ml in PBS with 0.1% BSA prior to analysis.

Cell suspensions were removed from wells after 24 hours, transferred to 1.5ml microfuge tubes and centrifuged at 2,300rpg for 5 minutes. The supernatant was removed and the remaining cell pellet was resuspended in 1ml of ice-cold PBS. Samples were centrifuged again and the washing process was repeated once more in ice-cold PBS. The resulting cell pellet were resuspended to 1×10^6 in 1ml of PI working solution and incubated at room temperature in the dark for 15 minutes prior to analysis by flow cytometry. The PI fluorescence of individual cells was analysed upon a single parameter histogram of linear fluorescence (FL2, red fluorescence, 560 – 590nm) against event count. For each treatment the percentage of cells that had taken up dye was recorded. To differentiate between normal, apoptotic and early or late necrotic cells a dual parameter histogram of FSC v Log PI fluorescence was used.

6.2.3.2: DNA cell cycle analysis

Flow cytometry with PI staining can also be used to investigate, nuclei from apoptotic cells show a condensed or fragmented morphology.

25mg propidium iodide (Sigma P4170) was dissolved in 1ml of 0.1% sodium citrate in water and stored in the dark at 4°C. This solution was further diluted in a hypotonic fluorochrome solution to a working concentration of 50µg/ml in 0.1% sodium citrate and 0.1% Triton X-100 immediately prior to use.

Cell suspensions were removed from wells after 24 hours, transferred to 1.5ml microfuge tubes and centrifuged at 2,300rpg for 5 minutes. The supernatant was removed and the remaining cell pellet was resuspended in 1ml of PBS. Samples were centrifuged again and the washing process was repeated once more in PBS. The resulting cell pellet was resuspended to 2×10^6 in 1ml of PI working solution and incubated at 4°C in the dark for 24 hours and then analysed by flow cytometry. The PI fluorescence of individual nuclei was measured at a low flow rate and was excited by a 488nm Argon laser. In addition to the measurement of forward and side scatter of the nuclei; the linear red fluorescence (FL3, bandwidth 605 – 635nm) was also measured. Clumps of nuclei were eliminated by appropriate gating.

6.3 RESULTS

6.3.1 Determining endotoxin contamination using polymyxin B

It was first established that polymyxin B (PMB) could actually bind to LPS in the cell culture and suppress the production of nitric oxide.

RAW264.7 cells (1×10^6) in 1 ml of supplemented medium were incubated with different doses of LPS at 37°C in 95% air, 5% CO_2 for 24 hours. A fixed concentration of PMB (5μg/ml) was incubated with cells 1 hour prior to the addition of LPS. Medium alone and with macrophages, with and without PMB served as negative controls.

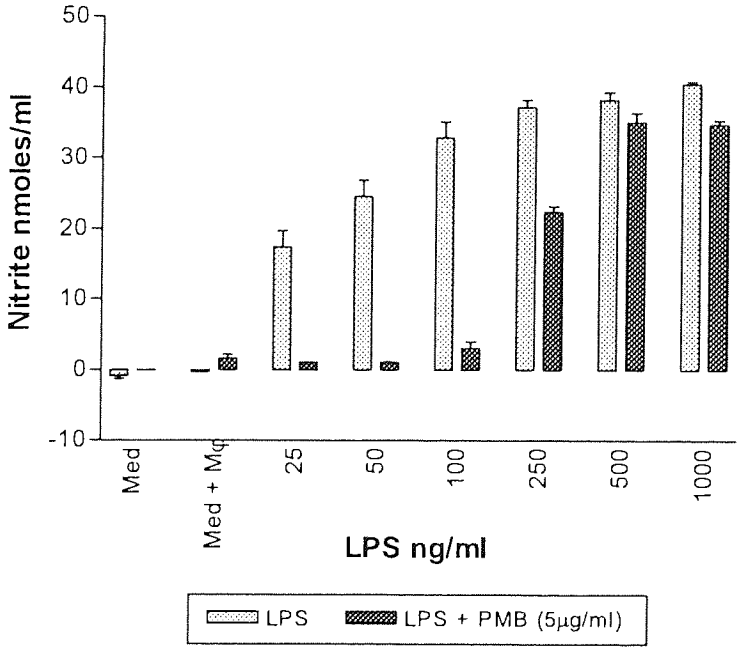


Figure 6.2: Effect of doses of LPS from *E. coli* on nitrite production from RAW264.7 cells (1×10^6) with and without the presence of PMB. Bars are standard error of the mean of three samples.

At concentrations of LPS up to 100ng/ml the preincubation with 5μg/ml PMB almost completely inhibited the LPS-induced NO production ($p = <0.01$). At higher

concentrations of LPS (250 – 1000ng/ml) there was progressively less inhibition of NO by this concentration of PMB. In the absence of bacterial components PMB did not activate macrophages to produce NO. Complete inhibition was not observed at higher concentrations because there was a higher ratio of LPS to PMB, hence all the available PMB was bound to LPS but, because of the abundance of LPS, there was enough unbound LPS still available to bind to receptors and initiate a response.

After confirming that PMB did bind to LPS and did therefore inhibit the production of nitrite two more experiments were set up to investigate if PMB could also bind to Gram-positive cell wall components and inhibit the production of NO (Fig 6.2) and TNF-α (Fig 6.3). Concentrations of bacterial components were chosen that had previously been shown to induce the production of both NO and TNF-α.

For figures 6.2 and 6.3 RAW264.7 cells (1×10^6) in 1 ml of supplemented medium were incubated with bacterial components at 37°C in 95% air, 5% CO_2 for 24 hours A fixed concentration of PMB (5μg/ml) was incubated with cells 1 hour prior to the addition of bacterial products. LPS served as a positive control and medium alone and with macrophages; with and without PMB and served as negative controls.

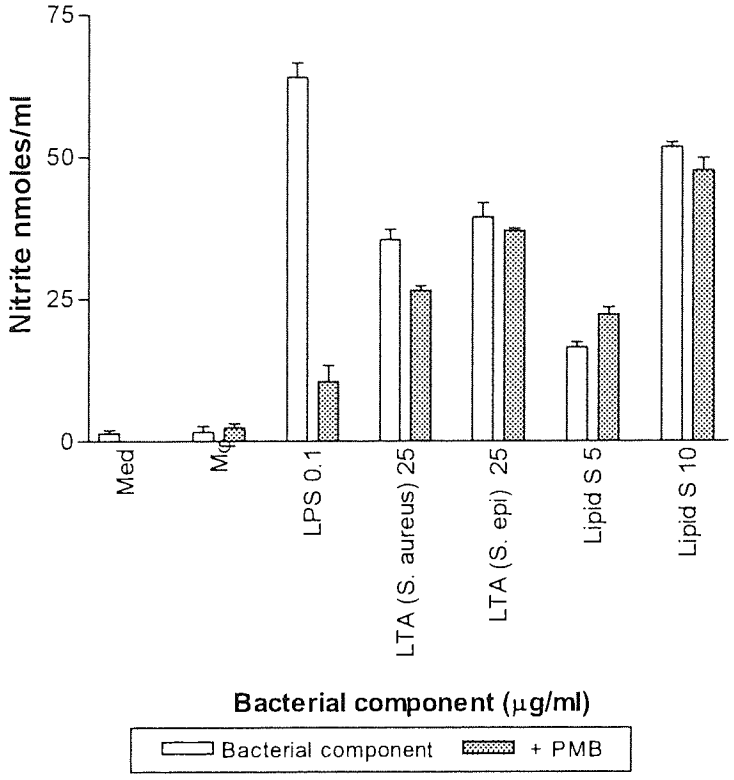


Figure 6.3: Effect of bacterial components on nitrite released from RAW 264.7 (1×10^6/ml) in the presence and absence of PMB (5μg/ml). Bars are standard error of the mean of three samples.

A significant 84% reduction of NO was observed in cells containing LPS with PMB compared to LPS alone ($p = 0.0051$), this confirmed previous results (Fig 6.1). PMB had either no or only a slight effect on the NO stimulation by the Gram-positive components tested. The production of NO elicited by lipid S and LTA, both purified from *S. epidermidis* was marginally reduced by 7% and 6% respectively in the presence of PMB and were proved to be insignificant ($p = >0.05$). However, incubation of lipid S at the lower concentration of 5μg/ml with the addition of PMB caused an increase in production of NO, although insignificant ($p = >0.05$). In contrast, commercial non purified LTA from *S. aureus* with PMB resulted in a significant 24% inhibition of NO ($p = 0.0478$).

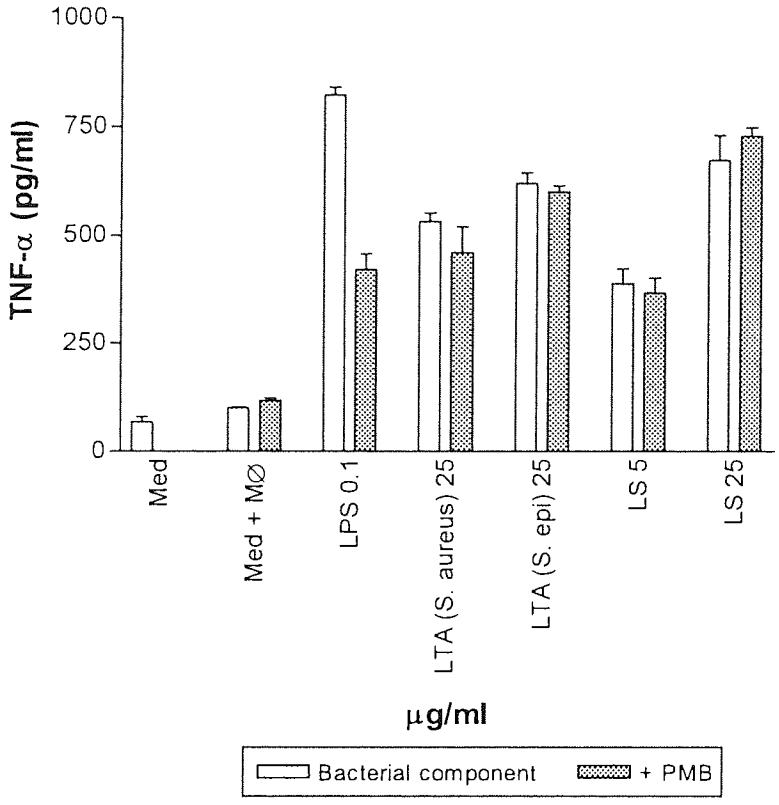


Figure 6.4: Effect of bacterial components on TNF-α released from RAW264.7 (1×10^6/ml) in the presence and absence of PMB (5μg/ml). Bars are standard error of the mean of three samples.

TNF-α production in the presence of LPS (0.1μg/ml) and PMB resulted in an inhibition of 49% ($p = 0.01$). In contrast, Gram-positive components were affected by PMB to a much lesser extent; there were no significant differences with LTA (*S. epidermidis*) or lipid S at either concentration, only minor differences of between 2 and 5 % were observed ($p = >0.05$). However, TNF-α released in response to commercial LTA (*S. aureus*) was suppressed by 14% in the presence of PMB, although this proved to be insignificant ($p = >0.05$) suggesting this preparation might have some endotoxin present as a contaminant.

The results obtained from figures 6.2 – 6.3 suggest that the stimulation of NO and TNF-α by lipid S and LTA from *S. epidermidis* is not due to contaminating LPS. By

contrast, the commercial preparation of LTA (*S. aureus*) may be contaminated with LPS as reported by Morath *et al.* (2002).

6.3.2: Determining endotoxin contamination using the Limulus assay.

The commercial Limulus amoebocyte lysate assay (LAL, Biowhittaker) was employed under sterile conditions using pyrogens free reagents and containers.

A known standard solution of LPS (*E. coli*, 0111:B4) was provided by the manufacturers of the LAL kit. A standard curve was derived by linear dilution of the LPS solution and was used to determine endotoxin contamination in samples of bacterial components.

Table 6.1: Amount of endotoxin present in samples of Gram-positive cell wall products using the Limulus assay

Bacterial component	LPS (ng/ml)
Blank	0.0118
WTA (100μg/ml)	0.0116
PG (*S. epidermidis*) (25μg/ml)	0.0156
LTA (*S. epidermidis*) (25μg/ml)	0.0248
Lipid S (*S. epidermidis*) (10μg/ml)	0.0189
LTA (*S. aureus*) (25μg/ml)	0.0374

These results show that at concentrations of PG, LTA and lipid S which would maximally stimulate NO or TNF-α production there would be less than 0.03ng of LPS present and this would be quite insufficient to stimulate NO or TNF-α output on its own. Similar levels were also found in the pyrogen-free water samples used as a blank.

6.3.3: Purification of commercial LTA by phenol extraction

Following reports that commercial preparations of LTA contain sufficient amounts of LPS purification of commercial LTA by phenol extraction was carried out (section 3.2.3) to investigate any effect on its stimulatory action in the production of NO and TNF-α.

For figures 6.4 and 6.5, RAW264.7 cells (1×10^6) in 1 ml of supplemented medium were incubated at 37°C in 95% air and 5% CO_2. The phenol extracted commercial LTA (*S. aureus*) was tested alongside un-purified commercial LTA (*S. aureus*) and LTA from *S. epidermidis* (also isolated by phenol extraction). Cell wall components were added to wells at time 0. Supernatants were removed after 48 hour incubation and assayed for nitrite and TNF-α production.

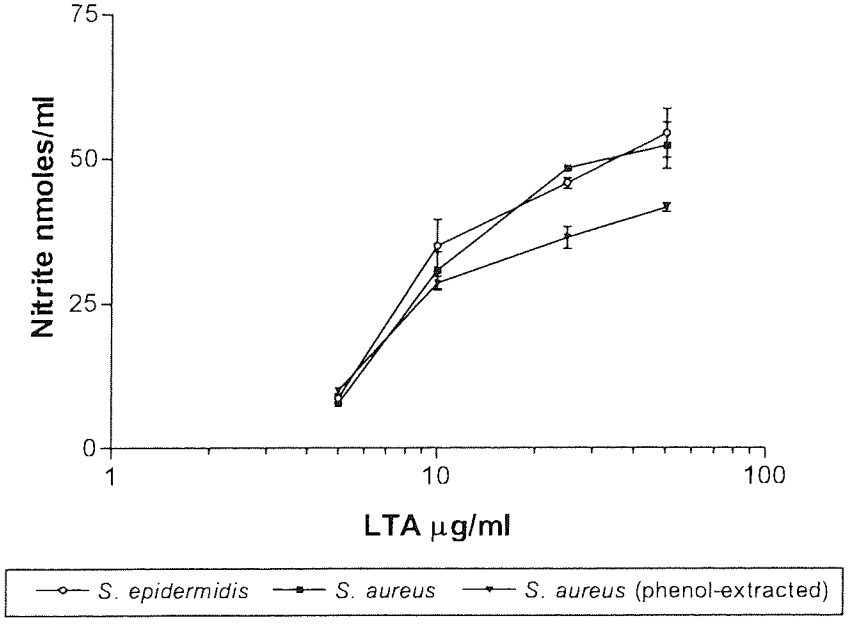


Figure 6.5: Effect of commercial LTA (*S. aureus*), phenol extracted purified commercial LTA (*S. aureus*) and LTA from *S. epidermidis* on nitrite production from RAW 264.7 cells. Bars are standard error of the mean of three samples

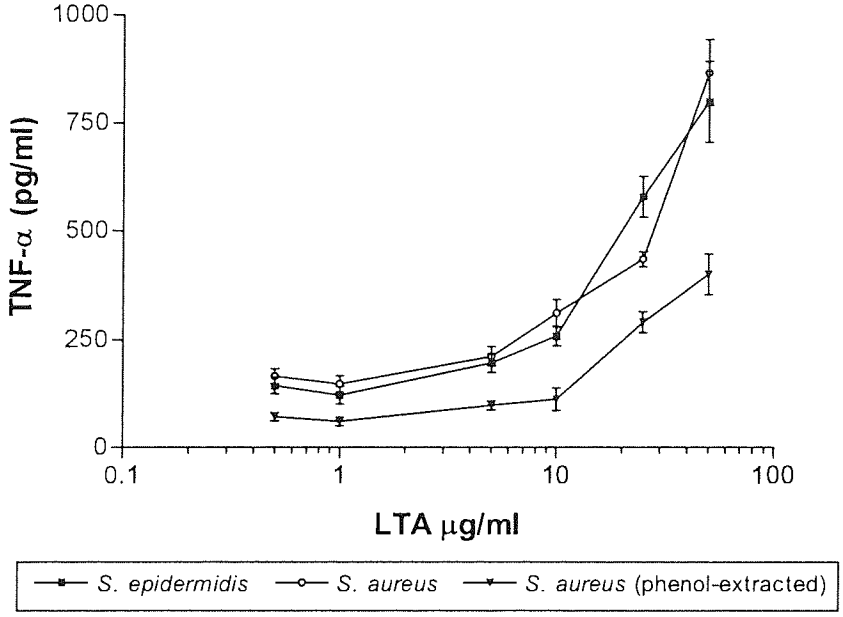


Figure 6.6: Effect of LTA (*S. aureus*), purified commercial LTA (*S. aureus*) and LTA from *S. epidermidis* on TNF-α production from RAW 264.7 cells Bars are standard error of the mean of three samples

LTA isolated from *S. epidermidis* and commercial LTA from *S. aureus* (unpurified) induced similar levels of both NO and TNF-α. In contrast, the LTA from *S. aureus* re-purified by phenol-extraction was a weaker stimulant of both NO and TNF-α. At lower concentrations (>10μg/ml) the differences in NO production were minimal, however effects at high concentrations (25 - 50μg/ml) show that the activity of re-purified commercial LTA was approximately 23% ($p = <0.05$) less than the other LTA preparations (Fig 6.5). The same preparation resulted in a significant reduction of between 34 – 50% in TNF-α production when compared to the LTA unpurified form ($p = <0.05$). These results indicate that the commercial LTA contains some unidentified active agents and that purification by phenol extraction removes some immunostimulatory activity from the commercial LTA (possibly LPS).

6.3.4: Viability studies

To determine if bacterial products could induce apoptosis a cell viability assay was performed. RAW264.7 (1×10^6) cells in 1 ml of supplemented medium in the presence of bacterial components (previously shown to stimulate cells) were incubated at 37°C in 95% air and 5% CO_2 for 24 hours and cell viability was analysed. Results are shown in table 6.2.

Initially data was obtained on a dual parameter histogram of forward scatter (FS) v side scatter (SS) on which of 2 x10^6 cells /ml were analysed and a cell population was gated (Gate A) as shown in figure 6.6. PI uptake was analysed by flow cytometry and assessed on dual parameter histograms of FS versus log PI fluorescence (Fig 6.7). Normal cells fall into quadrant 3, early necrotic cells are in quadrant 1 because they swell and increase in size. Late necrotic cells appear in quadrant 2 because in addition to their increased size their cell membrane is disrupted which allows PI entry into the cell. Late apoptosis cells are in quadrant 4 because this is a more controlled form of cell death, the membrane is disrupted which allows PI uptake but with a reduction in cell size.

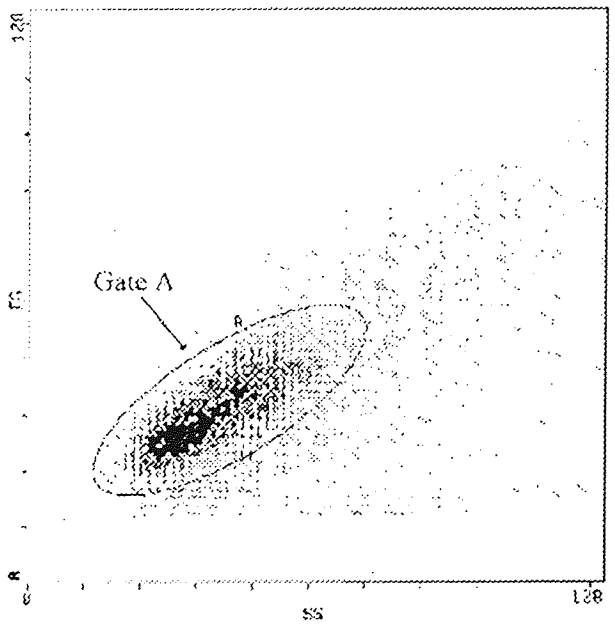


Figure 6.7: Flow cytometric analysis of RAW264.7 cells, FS forward scatter, SS side scatter

A viable population of cells was selected and gated (gate A), from within this gate a count of 20,000 events was analysed to measure the apoptotic and necrotic state of the cells using a single histogram of FL2log versus FS (Fig 6.7).

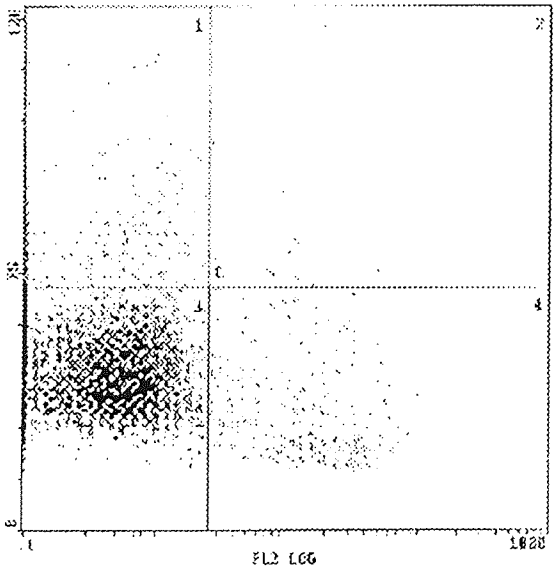


Figure 6. 8: Flow cytometry analysis of the apoptotic/necrotic state of RAW264.7 cell line in absence of bacterial products.

Table 6.2: Viability of RAW264.7 cells in the presence and absence of bacterial components

	Percent of total population (Gate A)	Percent Normal	Percent Early necrosis	Percent Late Necrosis	Percent Apoptotic
Control	89.6	91.6	3.45	0.31	4.67
WTA (100µg/ml)	92.3	94.3	2.69	0.28	2.7
Lipid S (25µg/ml)	81.2	67.9	24.6	0.86	6.58
LTA (25µg/ml)	84.5	68.5	23.9	0.79	6.78
PG (25µg/ml)	85.7	72.4	20.7	0.91	5.95
LPS (100ng/ml)	86.2	66.4	25.6	1.19	6.8

The results show that the populations of control cells and cells incubated with WTA (which has previously shown to have no effect on production of inflammatory mediators) were similar in all stages. Cells containing other bacterial products did not cause cells to go into the pre-programmed cell death known as apoptosis as little variation was detected between the control and test samples and are most likely cells undergoing natural senescence. There was also little variation between cells in the late stage of necrosis. However, a greater number of cells in early necrosis stage was seen with LTA, PG, lipid S (25µg/ml) and LPS (100ng/ml), and these numbers correlate with fewer numbers of normal cells in these samples, demonstrating that this may be the beginning of necrosis as cell size would be increasing. The consequence of this would be cell death brought about by the rupture of the plasma membrane, cellular contents would then leak out into surrounding tissues and induce an inflammatory response.

Another method of cell analysis was to measure activity of the DNA cell cycle. Propidium iodide, which binds to DNA, is a rapid and accurate means for quantitating both total nuclear DNA content and the fraction of cells in each phase of the cell cycle. The fluorescence signal intensity of the PI is directly proportional to the

amount of DNA in each cell. PI is not able to penetrate an intact membrane, so cells are first permeabilised.

RAW264.7 cells (1×10^6/ml) in the presence and absence of bacterial products were incubated in a humidified 5% CO_2, 95% air atmosphere at 37°C for 24 hours and analysed by flow cytometry, examples are shown in figures 6.8 and 6.9 and results are shown in table 6.3.

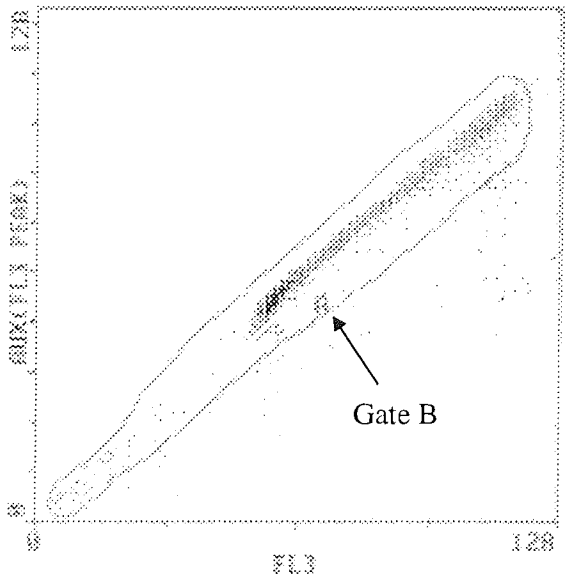


Figure 6.9: Flow cytometry analysis of the cellular DNA of RAW264.7 cell line (2×10^6/ml) in the absence of bacterial products

Cells were divided according to their distribution, a viable population of cells were selected and gated (gate A, Fig 6.8), from within this gate a count of 20,000 events was analysed to measure the DNA cell cycle of these cells using a single histogram of FL3 versus cell count (Fig 6.9).

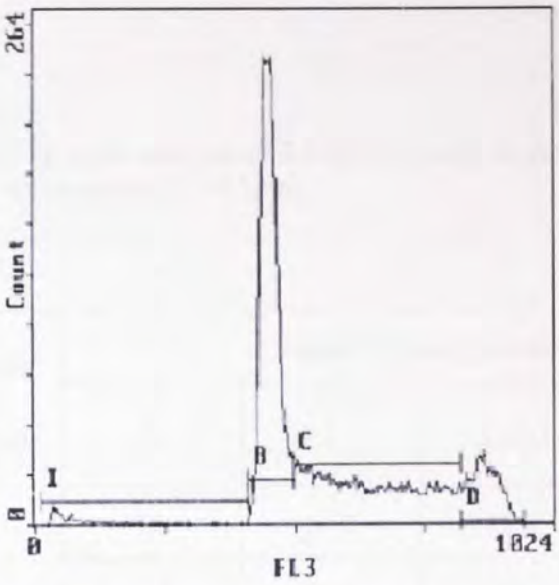


Figure 6.10: Histogram of cellular DNA from RAW264.7 cells incubated in culture media for 24 hours in the absence of any stimulus

The histogram was divided into stages of the cell cycle, region I represents cells undergoing apoptosis, region B is the G_1 stage, C represents cells in the S phase and D represents cells in the G_2/M phase. The cell cycle is an ordered set of events, culminating in cell growth and division into two daughter cells. The G_1 stage of a cell cycle is where the cell is preparing to begin DNA replication, the S stage is when cells are in the process of replicating DNA and new DNA is being synthesised. G_2 then prepares cells for the next stage of the cycle where mitosis (M phase) occurs (Fig 6.11).

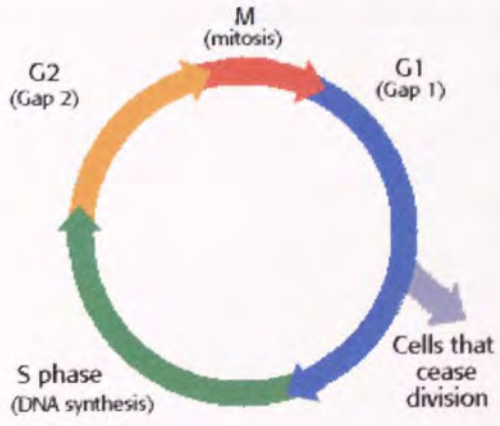


Figure 6.11: Stages of the cell cycle

Table 6.3: DNA Cell cycle analysis of RAW264.7 cells in the presence and absence of bacterial components for 24 hours

	Percentage of total population (Gate B)	Stage of cycle (percent)			
		G_1	S	G_2/M	Apoptotic
Control	97	51	34.2	12.2	2.9
WTA (100µg/ml)	97.7	50.8	33.4	13	2.84
Lipid S (25µg/ml)	96.8	63.4	23	10.2	3.66
LTA (25µg/ml)	96.9	58.1	27	13.8	1.38
PG (25µg/ml)	95.9	59.1	25	11.8	4.33
LPS (100ng/ml)	96.7	61.1	23.5	10.9	4.96

Apoptosis was generally very low with little variation between controls and cells exposed to bacterial components and there was also little variation in cells in the G_2/M phase. The most striking information these results show is the higher percentage (approximately 60 versus 50) of cells in the G_1 stage following treatment with lipid S, LTA, PG and LPS, with a corresponding reduction in the entry of cells in the S phase.

6.4 DISCUSSION

It has been suggested that the reported inflammatory activity of Gram-positive cell wall components is due to contamination with Gram-negative LPS (Morath *et al.*, 2002). To test the reliability of the results seen so far, experiments were conducted to determine if preparations of cell wall components extracted from *S. epidermidis*, contained contaminating LPS, which might be responsible for the observed activity.

Previous studies have stated that PMB can combine with LPS preventing its interaction with the macrophage but not with Gram-positive bacteria (Kengatharan *et al.*, 1996a). Preliminary experiments showed that polymyxin B (PMB) inhibited the release of NO when incubated with doses of LPS in RAW264.7 cells. A fixed concentration of PMB (5μg/ml) had little effect on high concentrations (500-1000ng/ml) of LPS, suggesting receptors were saturated with LPS and the concentration of PMB used was not high enough to bind all LPS molecules and prevent receptor binding. However complete inhibition of nitrite production was observed at lower concentrations (25-100ng/ml) of LPS (Fig 6.1). These results confirmed previous studies where PMB has been shown to bind LPS and inhibit its stimulatory actions *in vitro* (Jacobs and Morrison, 1977) and has also been used *in vivo* to improve the survival of both experimental animals and patients with septic shock (Mayumi *et al.*, 1999; Asanuma *et al.*, 1999).

Further experiments were then conducted to determine if PMB (5μg/ml) could also block the activity of LTA and lipid S from *S. epidermidis* and LTA from *S. aureus*. Previous studies have stated that PMB does not effect Gram-positive bacterial components for example De Kimpe *et al.* (1995b) demonstrated that PMB had no effect on the delayed circulatory failure caused by LTA in an anesthetised rat model. The results here show PMB did marginally reduce NO production when co-incubated with lipid S and LTA (*S. epidermidis*) at 10 and 25μg/ml respectively; however with lipid S at a concentration of 5μg/ml, PMB slightly increased production of NO. Thus this latter stimulation was unlikely to be due to endotoxin contamination. In contrast, combinations of LTA from *S. aureus* with PMB resulted in an inhibition of NO of

24% (Fig 6.2). A similar pattern emerged when the cytokine TNF-α was measured, again marginal differences in the production of TNF-α were observed for lipid S and LTA from *S. epidermidis*. LTA from *S. aureus* was again the most affected component with a 14% inhibition (Fig 6.3), suggesting it may be contaminated.

The limulus test gave negative results for all components tested, less than 0.04ng/ml of LPS was detected in all samples which were similar to the control sample of pyrogen free water (Table 6.1). Therefore all results shown in previous chapters (3 – 5) with Gram-positive components cannot possibly be due to LPS contamination and the observed activity is genuinely produced by the gram-positive bacterial component.

Some commercial LTA's including LTA from *S. aureus* have been implicated as being impure and that the extraction process is inadequate (Morath *et al.*, 2001) and further purification by phenol extraction resulted in the inability of the component to induce cytokine release (Kusunoki *et al.*, 1995). To determine if this was correct commercial LTA from *S. aureus* was subjected to the same phenol extraction (PE) process that was used to extract LTA from *S. epidermidis*. The resulting LTA was then tested alongside its original commercial form and LTA from *S. epidermidis*. The results from both the production of NO (Fig 6.4) and TNF-α (Fig 6.5) demonstrated that LTA from *S. epidermidis* and the original form of commercial LTA from *S. aureus* were very similar in their stimulatory activity. The purified commercial LTA from *S. aureus* was less potent than the unpurified LTA at high concentrations stimulated a 21% less in NO production and a 34 -50% less TNF-α. Therefore it appears likely that the commercial LTA (*S. aureus*) is not pure LTA. The fact that no endotoxin was detected in the commercial LTA suggests that it does not contain LPS as stated in another article (Gao *et al.*, 2001), but does contain some crude unknown immunostimulatory material that can induce the production of NO and TNF-α.

To investigate the possibility that cell wall components could be toxic to cells and induce the cell death mechanism of apoptosis or necrosis, the viability of the RAW264.7 cell line was tested in the presence and absence of bacterial components from *S. epidermidis* and LPS from *E. coli*. RAW264.7 cells were incubated for 24 hours in the presence of these components and viability was measured on a flow cytometer. There was no evidence of apoptosis in either of the viability tests (tables

6.2 and 6.3) as there was no significant increase in PI uptake with cells incubated with bacterial components relative to the control cells. However there are indications that cells incubated with bacterial products (with the exception of WTA) were undergoing the early necrotic pathway, approximately 20% more cells were detected in the early necrotic stage. When PI uptake was compared to cell size (measured as FS, Fig 6.6) it can be concluded that the increased PI uptake is due to a necrotic rather than an apoptotic process because necrotic cells swell before dying and apoptotic cells do not (Garcia-Ruiz *et al.*, 1997). The increase in the number of cells in early stages of necrosis correlated with a decrease of normal cells, suggesting that the bacterial products do have an effect on cells which leads to the destruction pathway of cell necrosis. Apoptosis and necrosis are two distinct forms of cell death, necrosis is observed upon death due to severe or sudden injury such as physical or chemical trauma which results in cell swelling and eventually cell rupture. The contents of cells are then released into the surrounding media and initiate an inflammatory response (Cohen, 1993) which can only further heighten the release of inflammatory mediators. Moreover, inflammation is seen more often in necrosis than in apoptosis and is mostly evidence of the phagocytosis of cell debris produced by the necrotic process (Kanduc *et al.*, 2002). However, it is not known whether it is the bacterial components themselves or the effects they have which result in the induction of inflammatory mediators and cellular injury. TNF-α and NO are known to be toxic at high concentrations to cells and peroxynitrite, formed from the reaction between superoxide radicals and nitric oxide, will be highly toxic and may cause direct cellular injury (Deakin *et al.*, 1995). During apoptosis there is no induction of inflammation because the plasma membrane remains intact with no release of toxic intracellular contents (Kolesnick and Kronke, 1998)

DNA cycle analysis of PI-stained nuclei measures apoptosis rather than necrosis and gives a view on what phase of the cell cycle cells are in. After 24 hour incubation the RAW264.7 cells were analysed and the results showed that a moderately higher percentage of cells were in the G_1 stage than the S phase. This indicates that some cells were undergoing cell cycle arrest before the S phase. No major differences were seen in the G_2/M stage, suggesting mitosis was not affected. There clearly may be some effects of some cell wall products inducing some necrosis in macrophages but *in vivo* possibly other tissue cells could be affected. It appears there may be some

inhibition of the cell cycle to prevent DNA synthesis and delaying the macrophage in the G1 phase.

Thus it can be concluded that the bacterial products do not induce apoptosis but there were signs that after 24 hours a higher percentage of macrophage cells entered early necrosis. This form of cell death is not suggestive of a control role for the cell population (Cohen, 1993). However it is not known whether this is due to autosuicide, the bacterial components alone or to inflammatory mediators being toxic to cells.

CHAPTER 7: EFFECTS OF ANTI-LTA, ANTI-CD14 AND PLATELET ACTIVATING FACTOR (PAF) ON INDUCTION OF INFLAMMATORY MEDIATORS BY BACTERIAL PRODUCTS

7.1 INTRODUCTION

It has previously been shown that antibodies can be used to identify the cell surface receptors that bind to bacterial components and can possibly also be used in therapy to reduce the inflammatory effects seen in septic shock.

CD14 is a 55kDa glycosylphosphatidylinositol-anchored glycoprotein on monocytes and neutrophils and also exists in plasma as a soluble protein. LBP binds to LPS and this complex then binds to the CD14 receptor on the surface of macrophages, LBP acts to opsonise LPS particles and mediates the attachment to macrophages (Schwandner, 1999). The CD14 receptor molecule has also been shown to bind components of the Gram-positive cell wall (Yang *et al.*, 2001) and it was found to cause a time and dose-dependent increase in nitric oxide production (Hattor *et al.*, 1997). Schimke *et al.* (1998) used an anti-rabbit CD14 mAb, which blocked LPS-CD14 binding and protected the rabbit against organ injury and death.

As CD14-dependent pathways are involved in responses to a wide variety of pathogens and microbial substances in addition to LPS and LTA the possible role of CD14 in the stimulation of NO production by lipid S in J774.2 and RAW264.2 cell lines was investigated. Therapy with anti-CD14 monoclonal antibody in patients with septic shock may be useful for both Gram-negative and Gram-positive infections, especially when the causative bacteria are unknown and where other therapies have failed to be efficacious.

A monoclonal antibody to the glycerol phosphate chain of *S. epidermidis* LTA has been developed (QED Bioscience Inc., cat no 15711) and might also have the potential to reduce the effects of Gram-positive septic shock. It was utilised here to observe the effects on responses to LTA and lipid S. A similar anti-LTA monoclonal

antibody has been manufactured to work with human cells and is on trial at the present time in America. Biosynexus Inc have reported clinical results for B5YX-A110, a chimeric anti-LTA antibody, it demonstrated outstanding safety, pharmacokinetics, and serum anti-staphylococcal opsonic activity in healthy adults. Studies are currently underway in premature infants (Biosynexus, 2002).

PAF is a highly potent phospholipid mediator of allergic and inflammatory reactions and is produced by a variety of cell types including macrophages. It has been suggested that platelet activating factor could be involved in the pathological process of inflammation and may play an important role in septic shock (Todoroki *et al.*, 1998). It can induce various responses such as platelet aggregation and the secretion and chemotaxis of leukocytes. It also activates macrophages to produce active oxygen species (Ichinose *et al.*, 1994). The aim of this present study is to determine if PAF can activate macrophages to produce NO in a similar way to LPS, LTA and lipid S and if the production of NO induced by Gram-positive and Gram-negative bacterial products can be reduced by the actions of a PAF receptor blocker.

7.2: MATERIALS AND METHODS

7.2.1: Anti-CD14 monoclonal antibody

It has long been established that LPS signals via CD14 receptors on the surface of macrophages and this is also true for LTA (Yang *et al.*, 2001). However the macrophage receptor for which lipid S interacts with has not been identified, it is assumed that because it is structurally related to LTA, it will also signal via the CD14 receptor.

Purified rat anti-mouse CD14 monoclonal antibody rmC5-3 (Pharmingen International) 0.5mg/ml was diluted in PBS (Dulbecco A) (Oxoid).

Cells were prepared as in section 2.4.6, and the antibody was diluted in culture media and final concentration in wells was 10μg/ml. Cells were preincubated for 1 hour with antibody prior to the addition of bacterial components.

7.2.2: Anti-lipoteichoic acid (LTA) monoclonal antibody

A monoclonal antibody directed against LTA from *S. epidermidis* (ATCC 55133) was obtained from QED Bioscience Inc., USA. It was provided in a solution of 1 mg Protein G-purified antibody in PBS, pH 7.4

The anti-LTA antibody was used to screen FPLC fractions from Gram-positive bacterial strains (section 2.2) by an ELISA assay. Western blots of lipid S from the same bacterial strains were performed using the anti-LTA monoclonal which was also used as a possible blocking agent in cell cultures against the stimulatory activities of LTA and lipid S.

7.2.2.1: ELISA

Samples from individual fractions from FPLC were assayed as in section 3.2.6 using both the serum from a patient with confirmed *S. epidermidis* CVC sepsis (serum SS) and the anti-LTA monoclonal antibody to detect antigenic material.

7.2.2.2: Western blot analysis

Eluted fractions from FPLC that were found by ELISA to bind the anti-LTA antibody were pooled together and analysed further by western blotting.

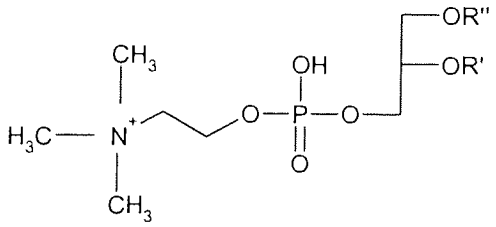
The western blotting process described in sections 3.2.7 and 3.2.8 was used. To detect bound anti-LTA monoclonal antibody, membranes were placed in anti-mouse IgG (Fab specific) peroxidase conjugate dissolved in TBS-Tween (0.5µl/ml).

7.2.2.3: Cell culture

Cells were prepared as in section 2.4.6. The antibody was diluted in culture media and the final concentration in wells was 10µg/ml. Cells were preincubated for 1 hour at 37°C with antibody prior to the addition of bacterial components.

7.2.3: Platelet activating factor (PAF) Agonists and Antagonists

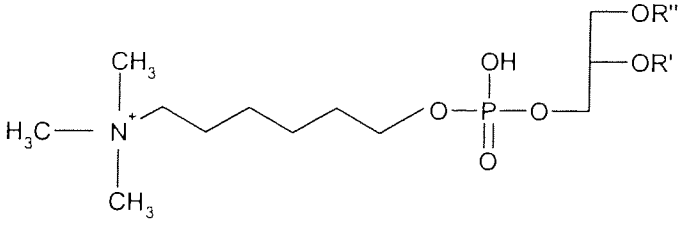
Platelet activating factor-16 agonist (Calbiochem) diluted in culture media (2.5.2)



PAF-16, R' = ether-linked palmityl, R'' = acetyl,
1-O-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine

Figure 7.1: Structure of Platelet activating factor-16 agonist

Platelet activating factor-16 antagonist (Calbiochem) diluted in culture media (2.5.2)



PAF-16 antagonist, R' = ether-linked palmityl, R'' = acetyl,
1-O-hexadecyl-2-acetyl-*sn*-glycero-3-phospho-(N,N,N-trimethyl)hexanolamine

Figure 7.2: Structure of Platelet activating factor-16 antagonist

PAF was used to determine its effects on NO production; the antagonist was used to investigate its ability to block the release of NO. Cells were preincubated for 1 hour at 37°C with antagonist prior to the addition of bacterial components.

7.3: RESULTS

7.3.1: Effect of anti-CD14 monoclonal antibody on the production of nitrite

The anti-CD14 antibody was used in combination with lipid S to establish whether CD14 was the cell surface receptor used by this bacterial component to initiate the production of nitric oxide. LPS and LTA have previously been reported to bind to CD14 and were used here as positive controls.

Cells were incubated at 37°C in 95% air, 5% CO_2 in 1ml of supplemented medium. A fixed concentration of anti-CD14 (10μg/ml) was added 1 hour before the addition of bacterial components and supernatants were collected 24 hours after incubation and assayed using the Griess reaction. Bars are standard error of the mean of triplicate samples.

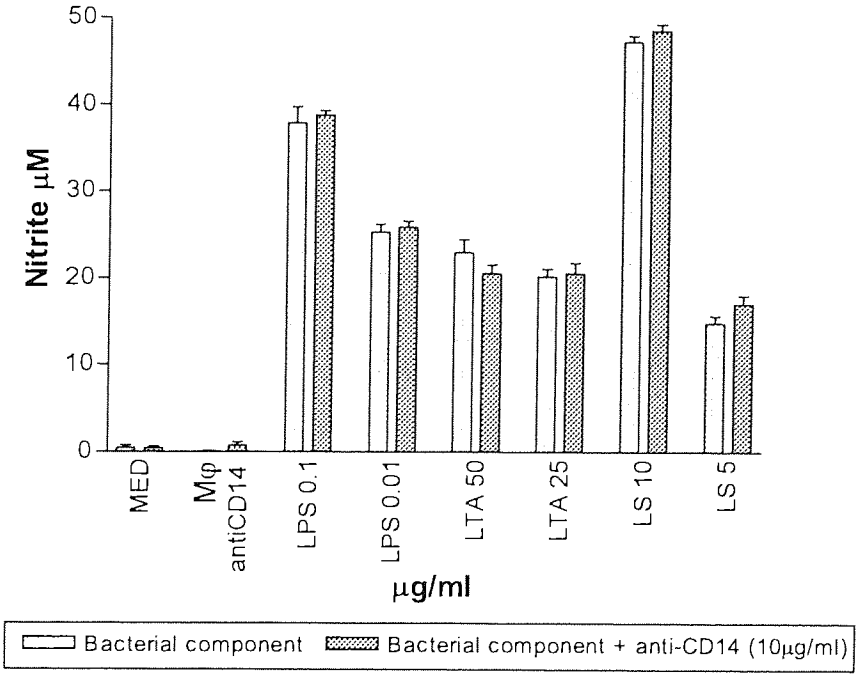


Figure 7.3: Effect of LPS, LTA and lipid S on nitrite production from RAW264.7 cells (1×10^6) in the presence and absence of anti-CD14

The anti-CD14 antibody failed to diminish the production of NO in cells with the addition of LPS, LTA or lipid S; in fact in most instances the release of NO was slightly higher in the presence of anti-CD14. It would be expected that the activity induced by LPS and LTA would be reduced as previously both have been shown to bind to CD14 (Wright *et al.*, 1990; Hattor *et al.*, 1997). However, there were no significant differences between results in the presence or absence of anti-CD14 antibody ($p = >0.05$). Similar results were observed from the J774.2 cell line even when a higher concentration of anti-CD14 (20μg/ml) was employed. One possible explanation is that insufficient antibody was present to block all CD14 receptors. Alternatively the bacterial components might displace the antibody from the CD14 receptors, or soluble CD14 receptors might have neutralised the blocking antibody leaving available CD14 receptors on the cells.

Further experiments were then set up using lower concentrations of serum in the cell culture which would therefore also reduce the amount of soluble CD14 available in the culture medium. The results showed that serum concentrations (4 – 9% of total culture medium) had little or no effect and low levels of serum (0.5 – 3%) resulted in a failure in macrophage activity (results not shown).

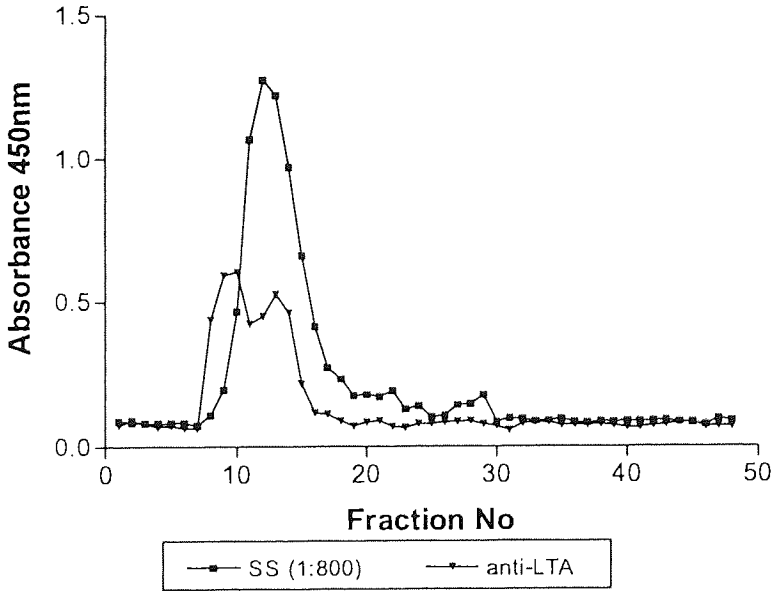
7.3.2: ELISA Analysis of FPLC Fractions from Lipid S extracts from different Gram-positive bacteria

In a series of experiments Superose 12 FPLC fractions from HHW culture supernatants containing lipid S were examined to compare their elution profiles and capacity to bind anti-LTA antibody with their capacity to bind antibody in human serum SS (patient with *S. epidermidis*, CVC sepsis). To obtain a direct comparison of the antigenic activity equal amounts of material were applied to the column (1ml of a 10mg/ml solution in distilled water).

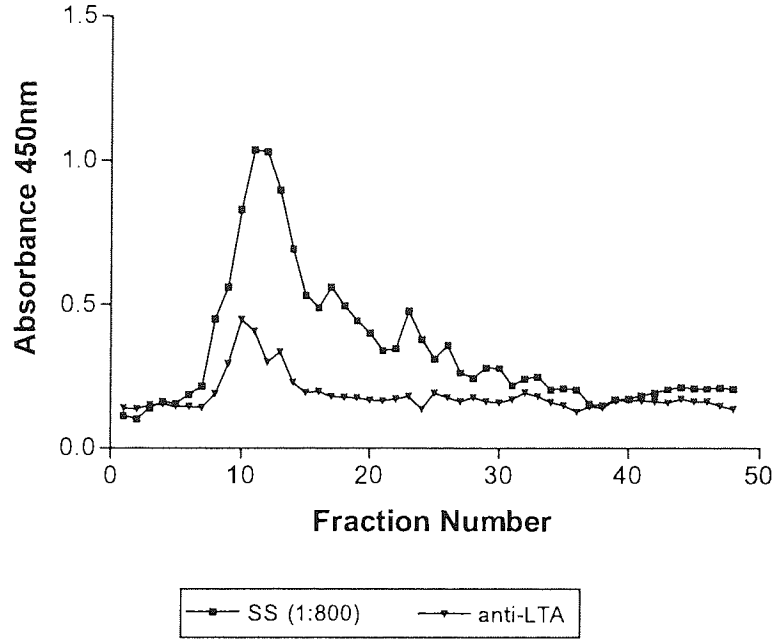
For the following experiments (figures 7.5a - 7.5e) assays were performed and observed under the same conditions. Fractions were collected as in section 3.2.1,

diluted 100-fold in bicarbonate buffer and coated onto a 96 well ELISA plate. Human serum SS was from a patient with CVC *S. epidermidis* infection and a monoclonal antibody directed against LTA were used in separate experiments for detecting the presence of antigens. The HRP-protein A conjugate for the ELISA assay was used to detect bound antibody in human serum and anti-mouse IgG-HRP for the monoclonal antibody.

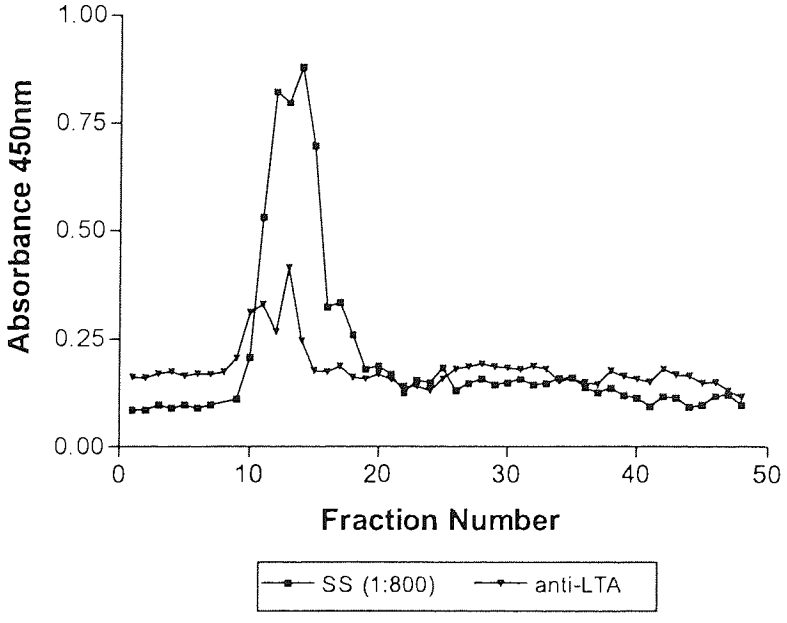
Figure 7.4: ELISA profiles of eluted HHW culture supernatant, on Superose 12 FPLC fractions 1 – 48 (Chromatography conditions as section 3.2.1).



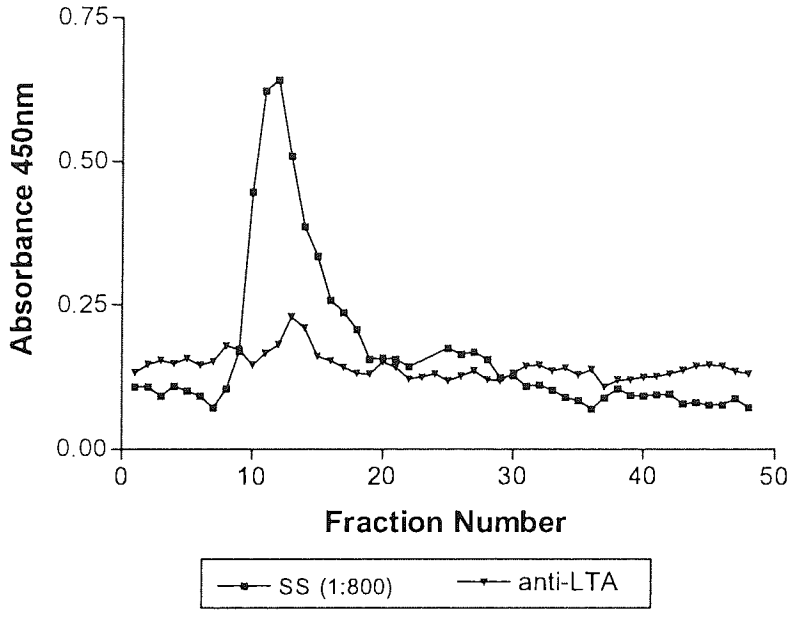
a) *S. epidermidis* NCIMB 40896 HHW culture supernatant



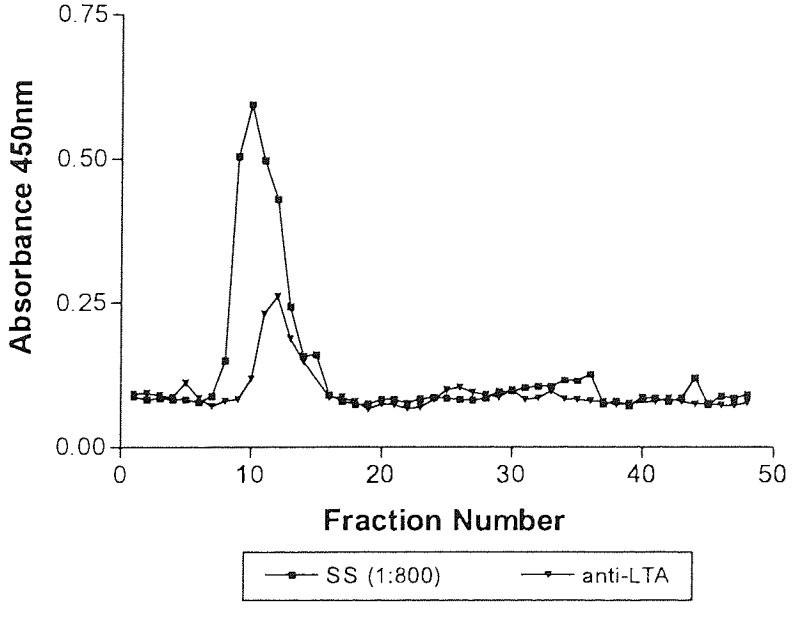
b) *S. epidermidis* NCTC 11047 HHW culture supernatant



c) *S. hominis* K ATCC 35982 HHW culture supernatant



d) *S. aureus* NCTC 6571 HHW culture supernatant



e) *S. aureus* 12598 HHW culture supernatant

The human serum IgG (SS) bound to material in fractions ranging from 9 - 16 from the Superose 12 column, although the fractions 10 – 15 gave the strongest reactions. These fractions presumably contain antigenic material released by these Gram-positive bacteria during infection which stimulated an IgG response in the patient. Profiles observed when the fractions were detected using the monoclonal antibody directed against LTA showed some differences compared to the human sera. Antigenic material was detected in fractions 10-15 for all strains studied although the monoclonal detected very little antigenic material in *S. aureus* NCTC 6571. For the two *S. epidermidis* strains and *S. hominis* the anti-LTA antibody appeared to detect two peaks of material, suggesting the presence of two species of antigen separated in the Superose column.

7.3.3: Western Blotting

To establish whether there were any differences in the electrophoretic properties of the different lipid S extracts binding to the anti-LTA monoclonal antibody, the FPLC fractions 10-15 for each strain were examined by western blotting. LTA extracted from whole cells of *S. epidermidis* with phenol was used for comparison purposes.

Lane
1 = *S. epidermidis* NCIMB 40896
2 = *S. aureus* NCTC 6571
3 = *S. aureus* 12598
4 = *S. epidermidis* NCTC 11047
5 = *S. hominis* K ATCC 35982,
6 = LTA from *S. epidermidis* NCIMB 40896
7 = Marker Ladder

Figure 7.5: Profile of lipid S extracts and LTA on a nitrocellulose membrane, detected by a reaction with monoclonal antibody, anti-LTA.

A similar pattern was observed when this experiment was repeated

With the exception of the lipid S extract from *S. aureus* 6571 the anti-LTA monoclonal antibody bound to lipid S from all other bacterial strains and to LTA

extracted from *S. epidermidis* 40896. The migration of the materials indicates that it is a low molecule weight material, negatively charged and is observed as broad immunostained bands at the bottom of the nitrocellulose membrane. These results demonstrate that the anti-LTA monoclonal antibody binds to LTA and it also cross reacts with lipid S.

7.3.4: Effect of anti-LTA on the production of nitrite or cytokines induced by bacterial components

For the following experiments (Fig 7.6 – 7.10) RAW264.7 cells (1×10^6) were incubated at 37°C in 95% air, 5% CO_2 in 1ml of supplemented medium. A fixed concentration of anti-LTA monoclonal (10μg/ml) was added 1 hour before the addition of bacterial components and supernatants were collected 24 hours after incubation and assayed for nitrite or cytokine production. Bars are standard error of the mean of triplicate samples.

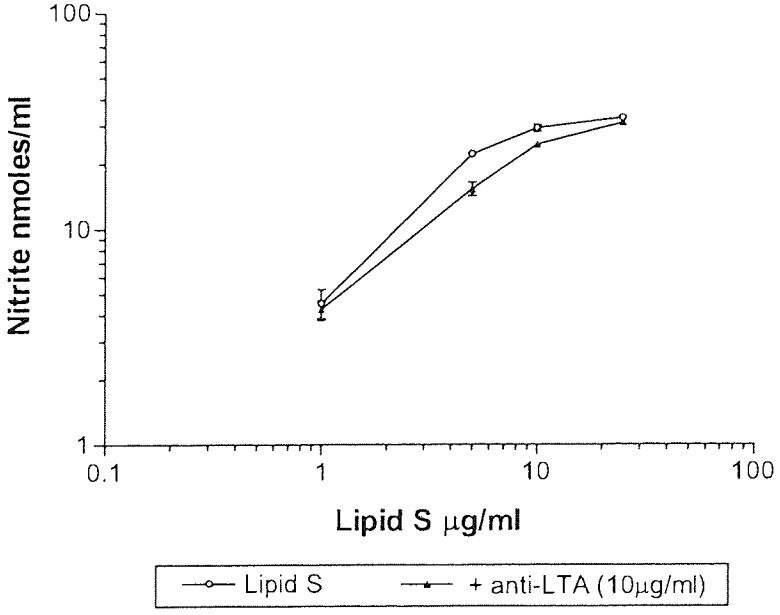


Figure 7.6: Log dose response curves of Lipid S from *S. epidermidis* NCIMB 40896 in the presence and absence of anti-LTA on nitrite production

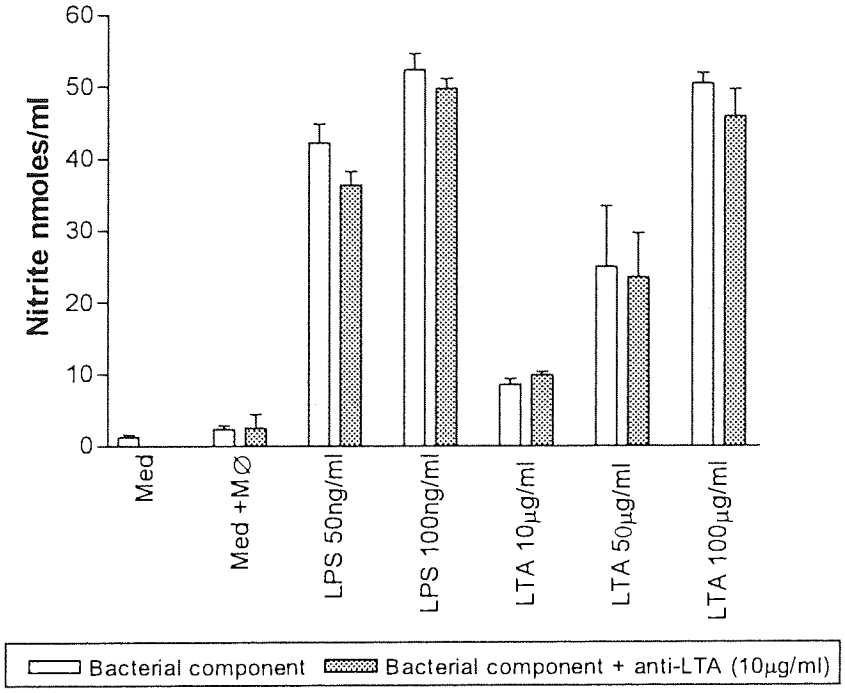


Figure 7.7: Effects of different concentrations of LPS and LTA from *S. epidermidis* NCIMB 40896 in the presence and absence of anti-LTA monoclonal on nitrite production

In most instances the anti-LTA antibody slightly reduced the stimulatory response to lipid S (Fig 7.7), LTA and LPS (Fig 7.8) in the release of NO, however the results were insignificant ($p = >0.05$). It had a similar effect on all the bacterial products tested at higher doses, giving a marginally reduced response, and a lower concentration of LTA (10µg/ml) with anti-LTA induced a slightly higher production of NO than cells incubated with only LTA. Surprisingly the anti-LTA antibody also reduced LPS which is structurally different from LTA. Possible reasons are contamination of LPS with LTA or an inhibitory component is present in the anti-LTA preparation.

However, controls of medium and cells with the inclusion of the anti-LTA antibody failed to induce or inhibit the basal level of production of NO, indicating that the antibody alone has no effect on cells *in-vitro* ($p = >0.05$).

Lipid S was then used in the presence and absence of anti-LTA to assess the blocking effect of anti-LTA on the production of cytokines TNF-α, IL-1 and IL-6 (Fig 7.8 – 7.10).

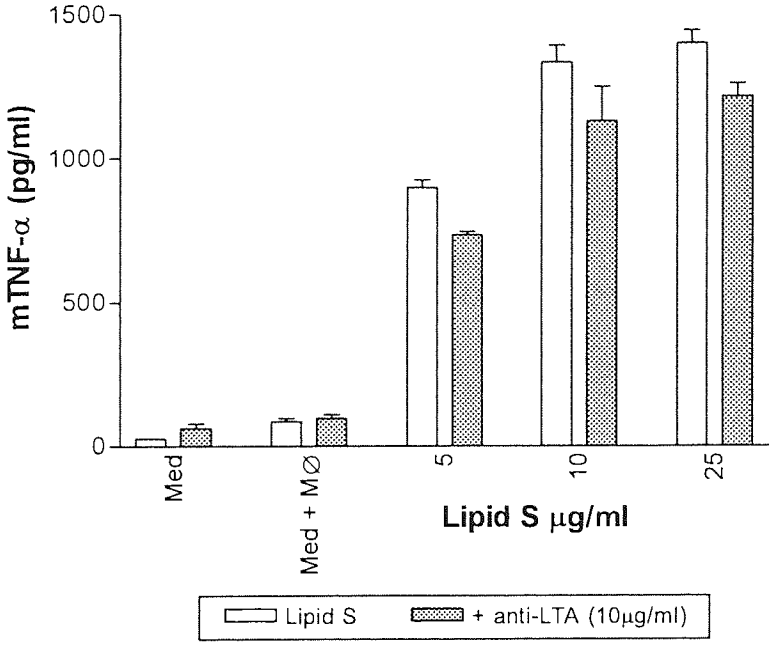


Figure 7.8: Effects of different concentrations of lipid S from S. epidermidis NCIMB 40896 in the presence and absence of anti-LTA on TNF-α production.

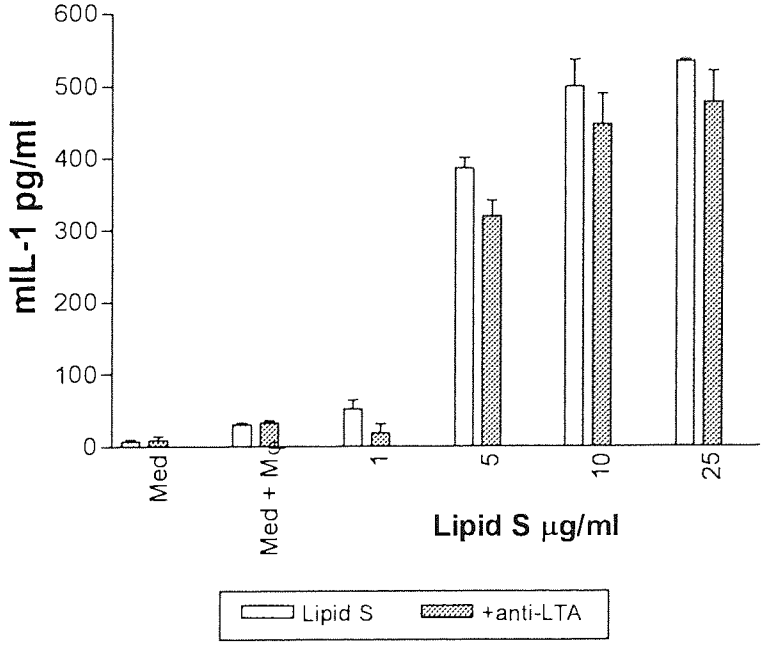


Figure 7.9: Effects of different concentrations of lipid S from *S. epidermidis* NCIMB 40896 in the presence and absence of anti-LTA on IL-1 production

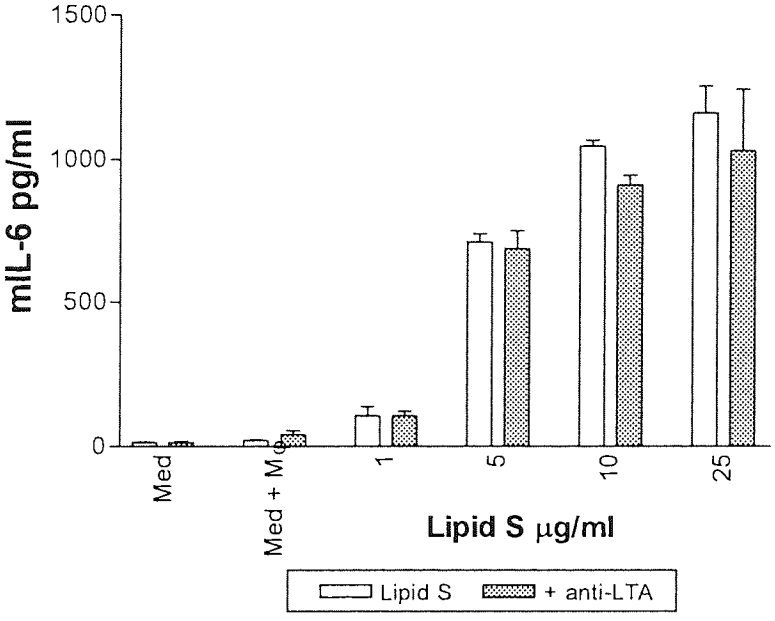


Figure 7.10: Effects of different concentrations of lipid S from *S. epidermidis* NCIMB 40896 in the presence and absence of anti-LTA on IL-6 production

Results were similar for all cytokines tested; controls confirmed that anti-LTA alone had no effect; a small reduction in cytokine activity was observed in all concentrations of lipid S incubated with anti-LTA however this was only marginal and not significant ($p = >0.05$).

A 17% maximum reduction in the production of TNF-α was seen with a lipid S concentration of 5µg/ml ($p = 0.0303$), smaller insignificant reductions were seen with 25 and 10µg/ml resulting in decreases of 14 and 16% respectively (Fig 7.8), $p = >0.05$. Again the results for the production of IL-1 showed small insignificant decreases in the presence of anti-LTA: 25, 10 and 5µg/ml of lipid S ($p = >0.05$) anti-LTA resulted in decreases of 11, 11 and 16% respectively (Fig 7.9). Similar effects were observed with the release of IL-6, the same concentrations showed decreases of 11, 12 and 4% respectively (Fig 7.10), $p = >0.05$. Lower concentrations of 1µg/ml of lipid S were similar to controls levels.

The results with anti-LTA show that it has a small effect on the production of both NO and cytokines in cell culture induced by lipid S. Perhaps the specificity of this antibody in cell culture provides only a weak binding with lipid S which is insufficient to block the induction fully.

7.3.5: Effect of PAF and PAF antagonist on the production of nitrite induced by bacterial components

For figures 7.11 - 7.13, assays were performed and observed under the same conditions; J774.2 (1×10^6) cells in 1 ml of supplemented medium were incubated with bacterial components and either PAF or a PAF antagonist at 37°C in 95% air, 5% CO_2 for 48 hours. LPS 100ng/ml and lipid S (10μg/ml) were used as positive controls (Fig 7.11), medium alone and with macrophages served as negative controls. Bars are standard error of the mean of three samples for controls and the mean of duplicate samples for antagonists.

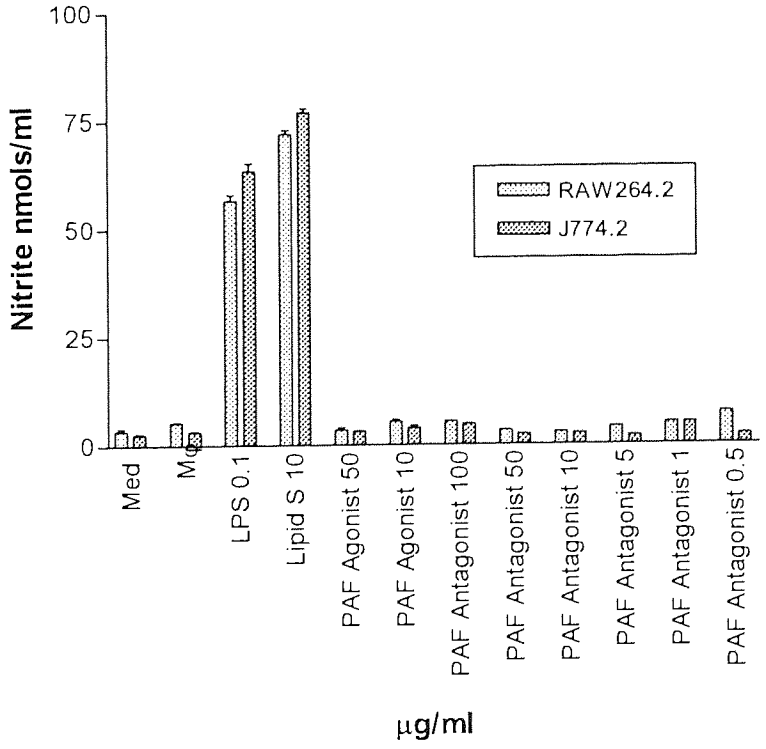


Figure 7.11: Nitrite released from J774.2 and RAW264.7 cells after 48h incubation with PAF and PAF antagonist alone.

As expected both lipid S and LPS induced measurable NO production in both cell lines. PAF and PAF antagonist did not display any stimulatory activity above those seen with negative controls.

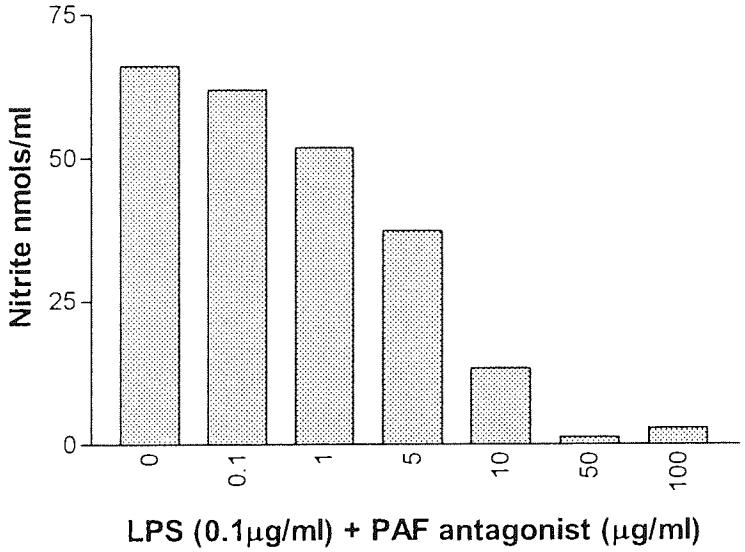


Figure 7.12: Nitrite released from J774.2 cells after 48h incubation with LPS alone and LPS with various doses of PAF antagonist. IC50 = 6.1μg/ml PAF antagonist.

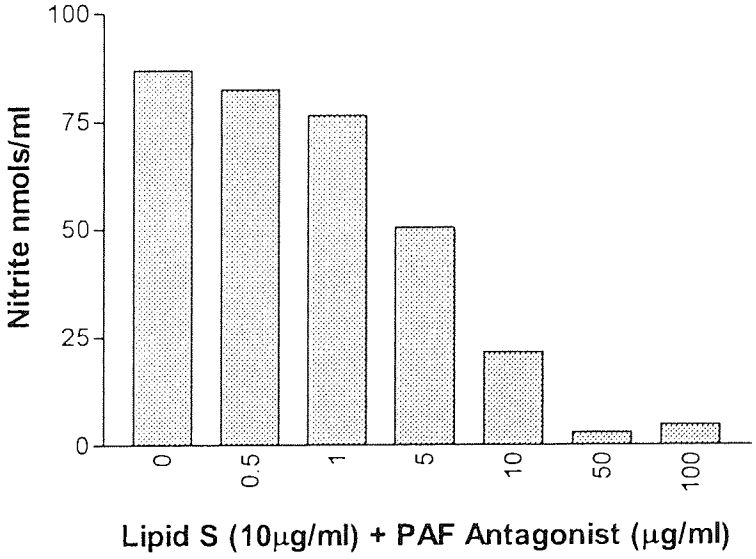


Figure 7.13: Nitrite released from J774.2 cells after 48h incubation with lipid S alone and lipid S with various doses of PAF antagonist. IC50 = 8.0μg/ml PAF antagonist.

When cells were stimulated with LPS the PAF antagonist blocked the release of NO in a dose dependent manner, higher doses (50 – 100μg/ml) completely inhibited the release of NO to that seen with control cells (Fig 7.12), and the IC50 = 6.1μg/ml. A similar inhibitory response was observed with lipid S in the presence of the PAF antagonist (Fig 7.13); where a marginally higher IC50 was determined of 8.μg/ml.

7.4 DISCUSSION

NO is generated by three different isoforms of NO synthase (NOS), two are calcium dependent and are expressed constitutively in endothelium (eNOS) and the nervous system (nNOS), whilst the other isoform is induced in macrophages independently of calcium (iNOS). Following an appropriate stimulus, the expression of iNOS can be rapidly induced and can be maintained for a prolonged period to sustain its effects. LPS was the first bacterial component to be successfully used to induce iNOS expression (Kroncke *et al.*, 1995). Many attempts to reduce the production of NO have had limited success (Cochran *et al.*, 1999). A major problem with targeting iNOS is the selectivity of blocking agents; many agents used to block the release of NO also affect the synthesis of the other two isoforms and can therefore have detrimental effects in other tissues.

There is now a plethora of data suggesting an essential role for CD14 in recognition of LPS and LTA by cells and more importantly in triggering cell activation processes. It is generally agreed that CD14 almost certainly acts in concert with additional cellular components such as toll-like receptors to initiate a transmembrane signalling cascade. CD14 is a major surface receptor for LPS (Matsuno *et al.*, 1998). A complex formed with the serum protein LPB is necessary for binding of LPS to CD14 (Wright *et al.*, 1990).

The present study examined the effects of a purified rat anti-mouse CD14 monoclonal antibody (anti-CD14). This was incubated with macrophages 1 hour before the further addition of bacterial components, presuming that this would block the CD14 binding site for bacterial components and would result in the inhibition of NO production. When LPS, LTA or lipid S were incubated in the presence of an antibody to CD14 *in-vitro* there was no blockade of the production of nitrites (Fig 7.3). This is contrary to reports from Hattor *et al.* (1997), they reported blocking of the synthesis of nitric oxide in LTA-stimulated J774 macrophages with this same antibody, however they did not test stimulation with LPS or lipid S and Pharmingen (suppliers of antibody) have not been able to reproduce the blocking effect. The synthesis of NO was also

blocked by anti-CD14 binding to mCD14 (membrane CD14) receptors in rabbits when challenged with LPS (Schimke *et al.*, 1998). Other reports have suggested that the NO production observed with high doses of LPS (>10ng/ml) could not be blocked completely by an anti-CD14 monoclonal antibody (Matsuno *et al.*, 1998)

There are a number of possible reasons for the results observed and, as suggested in the results section, a CD14-independent mechanism of bacterial stimulation may exist, indeed a number of additional receptors have been identified including CR3, CR4, CD11, CD18 and scavenger receptors (Wright *et al.*, 1990; Ingalls and Golenbock, 1995; Medvedev *et al.*, 1998; Ingalls *et al.*, 1999). The CR3 receptor has been suggested to mediate NO production provoked by Gram-positive bacteria in the macrophage cell line J774.1 (Goodrum *et al.*, 1994). Thus the lack of binding observed in this study could be due to the binding of bacterial components to these receptors.

When the monoclonal antibody to LTA was used to detect LTA in FPLC fractions from a number of different Gram-positive bacteria a similar profile emerged to that observed from fractions analysed with serum from a patient with *S. epidermidis* infection (Fig 7.4a – 7.4e). The results show that fractions 10 – 15 did contain antigenic material. As seen earlier (section 3. 3) only the material in these fractions had the capacity to bind to antibody directed against *S. epidermidis* in human convalescent sera (patient SS). The same fractions or fractions within the range 9 – 16 also bound to the anti-LTA antibody, although to a lesser extent. This was most likely due to the different concentrations of IgG present in the serum and monoclonal sample and also due to factors in serum which may also bind to antigenic material in the fractions, thus inducing a higher reaction. These results show that the anti-LTA antibody not only detects LTA but also lipid S and that cross-reactivity must occur between these components.

The results from western blotting (Fig 7.4) confirmed the ELISA test results and indicate that the anti-LTA antibody does indeed cross react with lipid S extracted from culture supernatants and therefore it seems likely that these two components share the same or some epitopes. The only organism that failed to respond to the anti-LTA antibody in western blotting was the *S. aureus* strain NCTC 6571, this same

bacterial strain also induced a smaller peak in the ELISA assay of the Superose 12 column fractions (Fig 7.4d). This suggests that the monoclonal antibody to LTA recognises an epitope that is either blocked or modified in lipid S from this strain.

Inhibition of NO and cytokines was investigated using the anti-LTA antibody in cell culture, its function may represent a mechanism by which the effect of Gram-positive bacterial antigens on the host immune response could be dampened and facilitate survival. Thus, an antibody that not only reacts with LTA but also cross reacts with lipid S may provide a potential target for therapeutic intervention when Gram-positive organisms are involved. However, the antibody only caused a small reduction in lipid S or LTA-induced NO production Surprisingly the production of NO from LPS stimulated macrophages was also slightly inhibited. Possibly indicating that LPS was contaminated with LTA (Fig 7.6 and 7.7). When concentrations of cytokines TNF-α, IL-1 and IL-6 were measured with lipid S in the presence of antibody the inhibitory effect was again marginal, a maximum inhibition was observed with lipid S at 5μg/ml resulting in a 17% reduction in the release of TNF-α, other concentrations of lipid S were also inhibited but to a lesser extent (Fig 7.8 -7.10). Other reports suggest that antibodies to LTA enhance the release of TNF-α and IL-1. Mancuso *et al.* (1994) found that the addition of anti-LTA to monocytes sensitised with LTA induced a rapid redistribution of receptors which resulted in an increase in the production of TNF-α and IL-1. Anti-LTA antibodies are normally present in the majority of human sera through exposure to Gram-positive bacteria (Markham *et al.*, 1973). Bhakdi *et al.* (1991) reported that naturally occurring antibodies to LTA were not inhibitory. Therefore macrophages would be sensitised to LTA during the course of infection and it is possible that these naturally occurring anti-LTA antibodies also contribute to the release of cytokines.

These results suggest that the anti-LTA monoclonal antibody used here is not specific enough to inhibit actions in cell culture. It could however be used as a diagnostic tool to identify if antibodies to LTA or lipid S are present in a patients serum sample. This can be measured by an ELISA assay or by western blotting.

Previous attempts to reduce cytokine production have had some success, pre-treatment with an antibody directed to TNF-α and an antagonist directed to the IL-1

receptor complex gave protection against septic shock in animal studies but are not effective once sepsis has developed (Read, 1998). They also showed no advantage in human trials (Fischer et al., 1992).

PAF has been shown to be a potent mediator of inflammatory reactions and by using a PAF receptor antagonist the mortality rate among patients with Gram-negative sepsis is significantly reduced (Dhainaut *et al.*, 1994). It was tested here to measure its effect on the production of NO. PAF failed to induce a reaction in either RAW264 or J774 cell lines (Fig 7.11). This suggests that PAF does not induce NO but needs other stimulatory material or signals to bind to PAF receptors on the macrophage surface or the concentration was not sufficiently high enough to initiate a response. However when a PAF antagonist was utilised in the presence of lipid S or LPS there was definite inhibitory action in the production of NO resulting in a PAF antagonist IC50 of 6.1μg/ml in the presence of LPS (Fig 7.12) and 8.0μg/ml for lipid S (Fig 7.13). De Kimpe *et al.* (1995c) also used a PAF antagonist (WEB2086) to successfully inhibit the production of nitrite in cultured macrophages when challenged with LTA and another PAF antagonist (ABT-299) has also been reported to be effective in preventing hypotension and gastrointestinal damage induced by LTA, although other PAF antagonists have failed to be effective (Albert *et al.*, 1996). This is probably due to the specificity of the PAF antagonist; there are structural similarities with the PAF antagonist used in this study (Fig 7.2) and that of lipid S (Fig 1.7) which suggest that both components bind to the same PAF receptor on macrophage surface and the PAF antagonist has a higher affinity for the PAF receptor effectively blocking the actions of both these compounds by competing for receptor binding.

Nitric oxide is postulated to be a major mediator of vasodilatation and hypotension in septic shock (Cobb and Danner, 1996). These results show there is a definite role for PAF receptors in the production of NO and by using an antagonist to block receptors could possibly provide a suitable treatment for both Gram-positive bacteria and Gram-negative sepsis.

CHAPTER 8: TOLL-LIKE RECEPTOR SIGNALLING

8.1 INTRODUCTION

A key feature of the innate immune system is its capacity to recognize a broad spectrum of pathogens using a repertoire of receptors. These receptors recognize an array of microbial components. Many different types of receptors participate in bacterial detection (Underhill and Ozinsky, 2002), and a recent development in the understanding of pathogen recognition has been the discovery that the toll-like receptor (TLR) family mediates recognition of microbial targets in several organisms including humans, mice and flies. When stimulated, these receptors activate the inflammatory response (Aderem, 2001).

In recent years there have been many conflicting reports on which TLR is linked to a particular bacterial component. However, there is now strong evidence that implicates TLR4 as the major receptor for LPS (Akashi *et al.*, 2000a). Recent studies have begun to clarify the molecular basis of LPS intracellular signalling in response to LPS. Activation of LPS-responsive cells, such as monocytes and macrophages, occurs after LPS interacts with circulating LPS-binding protein (LBP). After binding with LBP, LPS-induced cell activation depends on the presence of 3 proteins: CD14, TLR 4, and MD2 collectively comprising the LPS receptor complex (da Silva Correia *et al.*, 2001). The activation of TLR4 leads to DNA binding of the transcription factor NF-κβ, resulting in activation of the inflammatory cascade (Hallman *et al.*, 2001).

Poltorak *et al.* (1998) and Qureshi *et al.* (1999) used C3H/Hej and C57b2/10ScCr mice, which have naturally-occurring mutations in their TLR4 gene which made them hyporesponsive to LPS. A point mutation in the C3H/Hej-derived TLR4 gene resulted in the replacement of proline with histidine in the cytoplasmic portion of TLR4 protein. These mice have a normal response to Gram-positive infections but an increased susceptibility to Gram-negative infection; suggesting that different receptors

are involved in responding to different Gram-negative and Gram-positive pathogens (Brightbill and Modlin, 2000). Targeted disruption of this TLR4 gene by homologous recombination has also been shown to result in the abrogation of LPS responsiveness in macrophages and B cells (Hoshino *et al.*, 1999). In addition Takeuchi *et al.* (1999) have engineered and studied TLR2 and TLR4 gene knockout mice and their results demonstrated that TLR4 but not TLR2 mediates the response to LPS.

There is less evidence regarding cell activation by Gram-positive components, though the general consensus is that TLR2 mediates signalling in response to LTA and PG (Schwandner *et al.*, 1999). Later findings by Takeuchi *et al.* (2000) demonstrated that mice deficient of TLR2, but not of TLR4, are highly susceptible to *S. aureus* infections, supporting the relevance of TLR2 signalling in the host immunity to Gram-positive infections. By contrast other studies have indicated that TLR4 is also linked to LTA binding. This has been attributed to endotoxin contamination of commercial LTA and therefore may account for the conflicting results (Gao *et al.*, 2001). However the LTA (*S. epidermidis*) preparation in this study has been shown to be endotoxin free (section 6) and investigating the role of TLR4 in signalling may help identify if this TLR is exclusively involved in cellular activation from Gram-positive components.

Monoclonal antibodies (mAbs) to TLRs have been developed and used in cell culture studies. Previous reports have shown that the production of TNF-α with human peripheral blood mononuclear cells and monocytes were inhibited by the use of mAbs anti-TLR4 HTA125 and HTA1216 (Akashi, *et al.*, 2000a). Potentially antibodies developed against TLR's could be important in therapy to reduce the effects of septic shock.

The TLR linked to lipid S has never been investigated and therefore using these mAb's and the LPS low-responder mice (C3H/Hej) may identify if TLR4 is the receptor used on macrophage cells to respond to lipid S. For purposes of comparison and to confirm previous reported findings, LPS and LTA were also investigated.

8.2 MATERIALS AND METHODS

8.2.1: anti-TLR4 monoclonal antibodies

TLR4 HTA125 and TLR4 HTA1216 have both been used in experiments neutralising LPS response and have their greatest effect at 20μg/ml (Akashi *et al.*, 2000a). The effects of these anti-TLR4 mAbs were investigated as possible blocking agents in human THP-1 cell cultures against the stimulatory actions of LPS, LTA and lipid S.

The following monoclonal antibodies were a kind gift from Dr Kensuke Miyake, Department of Immunology, Saga Medical School, Saga, Japan.

MAb Human TLR4 HTA125 mouse IgG2a/κ 1.0mg/ml

MAb Human TLR4 HTA1216 mouse IgG1/κ 1.7mg/ml

Isotype controls, mouse IgG2a and IgG1 were used as controls

THP-1 cells have been reported to express TLR4 and MD-2 as detected by flow cytometry analysis using anti-TLR4 mAbs and rabbit anti-MD-2 (Viriyakosol *et al.*, 2001).

THP-1 cells were prepared as in section 2.4.3, and the antibodies were diluted in culture media and final concentration in wells was 20μg/ml. Cells were preincubated for 1 hour with antibody prior to the addition of bacterial components.

8.2.2: Mice

C3H/HeJ mice have an impaired ability to respond to LPS due to a mutation in the gene that encodes Toll-like receptor 4 (TLR4). To determine whether TLR4 was

critical in the release of pro-inflammatory mediators comparisons were made investigating the stimulatory effects of LPS, LTA and lipid S using peritoneal macrophages isolated from TLR4-defective C3H/HeJ mice and TLR4-sufficient C3H/Hen sub-strain mice.

C3H/Hen - Normal strain wild type (Charles River laboratories, Germany)

C3H/Hej - TLR4 deficient mice (Charles River laboratories, Germany)

The C3H parent strain was developed by LC Strong in 1920 from a cross of a Bagg albino female with a DBA male. A spontaneous mutation occurred in the substrain C3H/Hej mice at the LPS response locus (mutation in TLR4 gene, *Tlr4*^{lps}) making this strain endotoxin resistant whilst the other substrains including C3H/Hen are endotoxin sensitive.

Mouse peritoneal macrophages were isolated and prepared as in section 2.4.1.

8.2.3: Measurement of Nitrite

NO analysis of peritoneal macrophages was performed as in section 4.2.1

8.2.4: Measurement of Cytokines

TNF-α, IL-1 and IL-6 was measured by enzyme-linked immunosorbent assay (ELISA) using the appropriate human or mouse TNF-α, IL-1β or IL-6 Duoset ELISA development system kits (R and D Systems).

The ELISA cytokine assays were carried out as in section 5.2

8.3 RESULTS

8.3.1: Effects of monoclonal antibodies directed against TLR4 on the ability of bacterial cell wall components to elicit cytokine release

The anti-TLR4 monoclonal antibodies (mAbs) were used in this study as potential neutralising agents to establish whether TLR4 was the receptor used by bacterial components to initiate the production of cytokines. Both have been reported to inhibit the release of TNF-α in LPS-stimulated human monocytes (Akashi *et al.*, 2000a).

For figures 8.1 – 8.4, THP-1 cells (1×10^6) were incubated with PMA (10^{-7}M) at 37°C in 95% air, 5% CO_2 in 1ml of RPMI supplemented medium. A fixed concentration of anti-TLR4 (20μg/ml) was added 1 hour prior to the addition of bacterial components. Controls of medium alone, medium with macrophages, macrophages with PMA alone and with non-specific mouse IgG1 and IgG2a isotype controls and anti-TLR4 were assayed for comparison. Supernatants were collected 24 hours after incubation and assayed for cytokine production. Bars are standard error of the mean of triplicate samples from a single experiment.

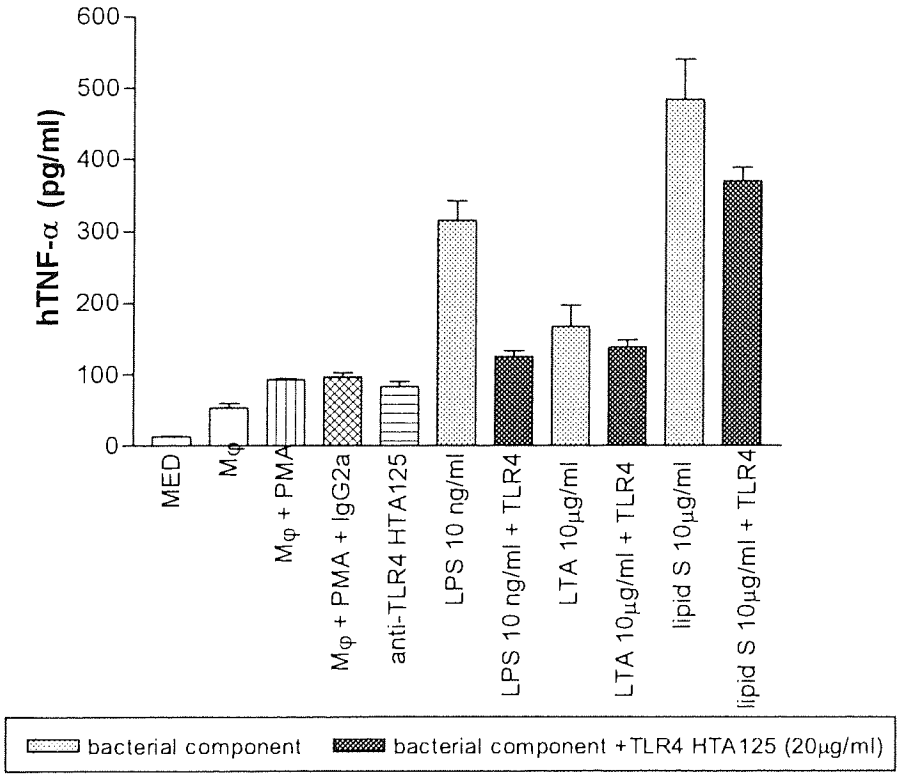


Figure 8.1: Effect of LPS from *E. coli*, LTA and lipid S from *S. epidermidis* on TNF-α production from THP-1 cells (1×10^6/ml) in the presence and absence of anti-TLR4 mAb HTA125 (20μg/ml). Bars are standard error of the mean of triplicate samples

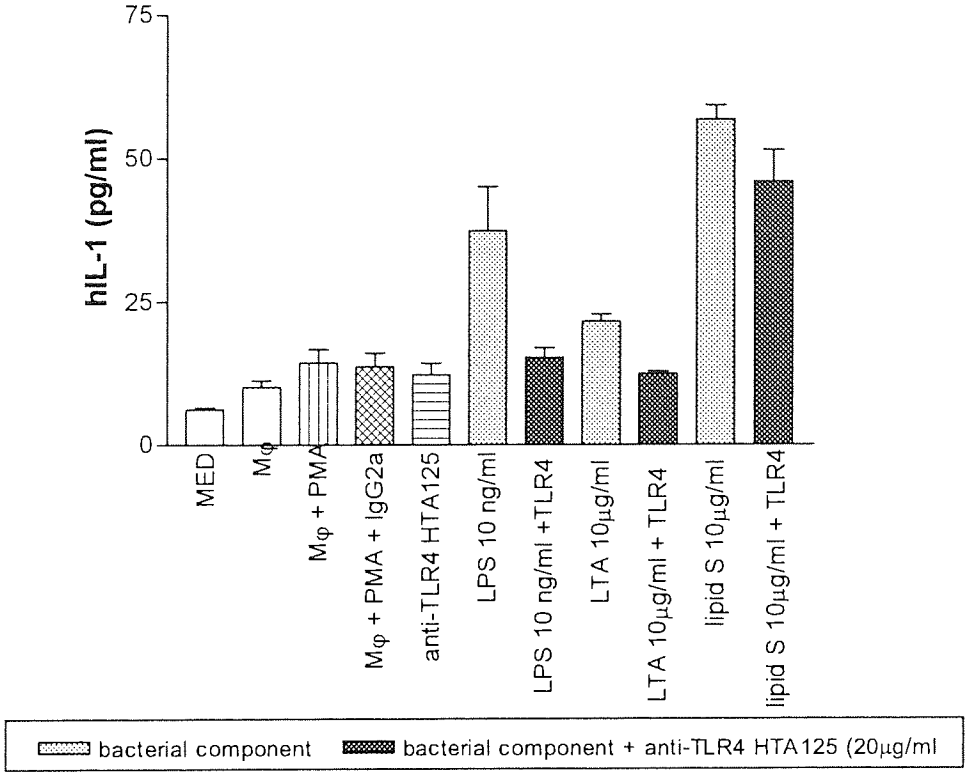


Figure 8.2: Effect of LPS from *E. coli*, LTA and lipid S from *S. epidermidis* on IL-1 production from THP-1 cells (1×10^6/ml) in the presence and absence of anti-TLR4 mAb HTA125 (20µg/ml). Bars are standard error of the mean of triplicate samples

Control samples of macrophages alone demonstrated that these cells release small amounts of TNF-α and IL-1 without any stimulus (Figures 8.1 and 8.2). With the addition of PMA, the THP-1 cells showed a marginal increase in both TNF-α and IL-1 production, suggesting PMA has some stimulating actions to induce the release of these cytokines. Although this was proved to be insignificant ($p = >0.05$). The isotype control IgG2a and the antibody control both incubated with macrophages and PMA but without bacterial products showed little variation in TNF-α and IL-1 production to that of cells with PMA, alone, indicating they had no effect on cells *in-vitro* ($p = >0.05$).

The results from samples containing bacterial components with the inclusion of the mAb HTA 125 showed that the antibody could bind to the TLR4 receptor and inhibit the production of TNF-α and IL-1, though to varying degrees. The mAb had its

greatest effect when incubated with LPS, a 60% reduction in TNF-α ($p = 0.001$) and a 59% decrease in IL-1 production was observed ($p = 0.001$). However if the controls are taken into account then the anti-TLR4 mAb completely abolished the stimulatory effect of LPS on TNF-α and IL-1 production, but only reduced the effect of LTA and lipid S by 17 and 24% respectively for TNF-α and 42 and 19% reduction in IL-1 production ($p = >0.05$). However the results from samples containing LTA were low, only marginally above control samples and therefore are not accurate enough to draw any firm conclusions.

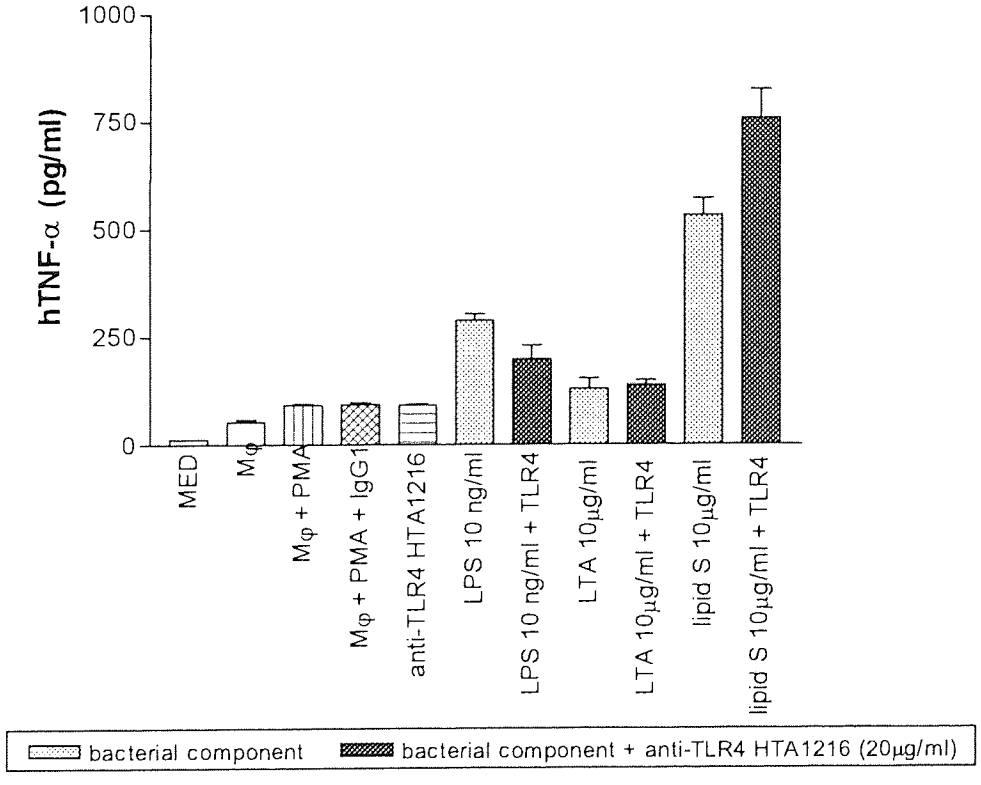


Figure 8.3: Effect of LPS from *E. coli*, LTA and lipid S from *S. epidermidis* on TNF-α production from THP-1 cells (1×10^6/ml) in the presence and absence of anti-TLR4 mAb HTA1216 (20μg/ml). Bars are standard error of the mean of triplicate samples

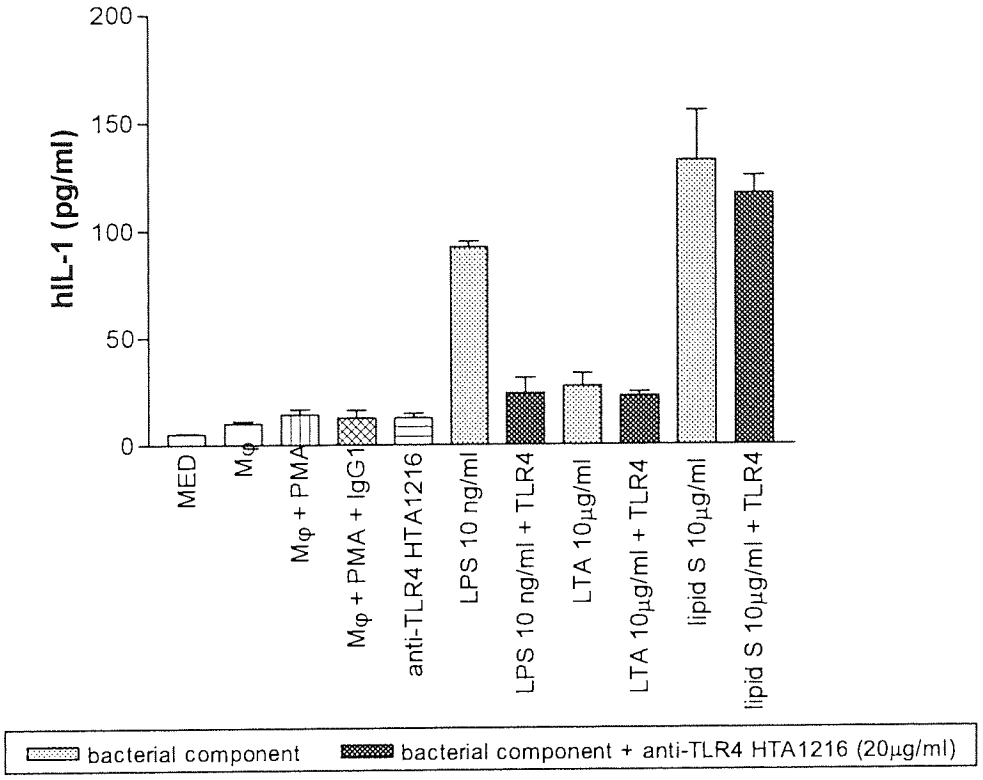


Figure 8.4: Effect of LPS from *E. coli*, LTA and lipid S from *S. epidermidis* on IL-1 production from THP-1 cells (1×10^6/ml) in the presence and absence of anti-TLR4 mAb HTA1216 (20µg/ml). Bars are standard error of the mean of triplicate samples

All control samples show very low residual TNF-α and IL-1 release, only marginally above background (Figures 8.3 and 8.4). Overall the anti-TLR4 mAb HTA1216 inhibited the response to LPS but not Gram-positive components. An inhibition of TNF-α and IL-1 production was observed with LPS, resulting in a 32% ($p = >0.05$) and 73% ($p = 0.001$) reduction respectively, taking controls into account an almost complete inhibition of IL-1 production would be evident. In contrast the response from incubating lipid S with the antibody showed a 42% ($p = 0.001$) increase in TNF-α and a 12% ($p = >0.05$) decrease in the release of IL-1. Similarly to experiments above (Figures 8.1 and 8.2) LTA failed to induce a response much above control levels and therefore results are not conclusive.

8.3.2: The effect of bacterial components on cytokine and NO production from LPS- resistant C3H/Hej mouse macrophages.

The inbred mouse strain C3H/Hej has been identified as being a low responder strain to LPS due to a mutation in their TLR4 gene. The wild-type strain mouse, C3H/Hen has a normal response to LPS and was used here as a positive control. To further examine the TLR associated with Gram-positive bacteria the response of murine peritoneal macrophages to LTA and lipid S was measured along with LPS to discover whether TLR4 is the transmembrane receptor used by these components to transducer the signal from inflammatory mediators.

For the following experiments (Fig 8.5 – 8.7) a count of 5×10^5/ml peritoneal macrophages were incubated at 37°C in 95% air, 5% CO_2 for 24 hours in 1 ml of supplemented medium. Cell wall components LTA, lipid S from *S. epidermidis* and LPS from *E. coli* were added to wells at time 0. Controls of medium alone and medium with macrophages were assayed for comparison. Supernatants were removed after 24 hours and assayed for cytokine production or after 48 hour incubation and assayed for nitrite. Bars are standard error of the mean of triplicate samples and results are from a single experiment.

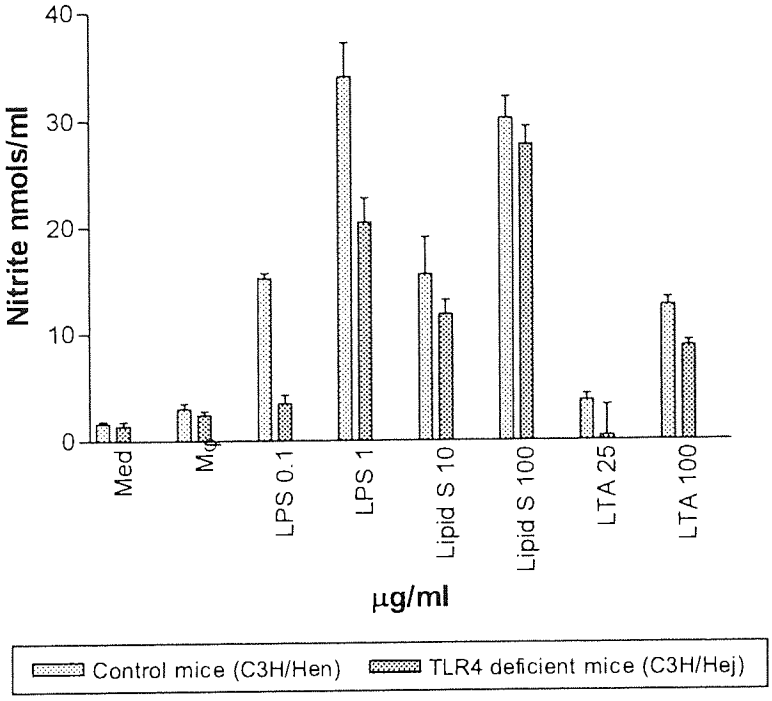


Figure 8.5: Effects of different concentrations of bacterial components on nitrite production from mouse peritoneal cells 5 x 10^5/ml after 48 hours. Bars are standard error of the mean of triplicate samples

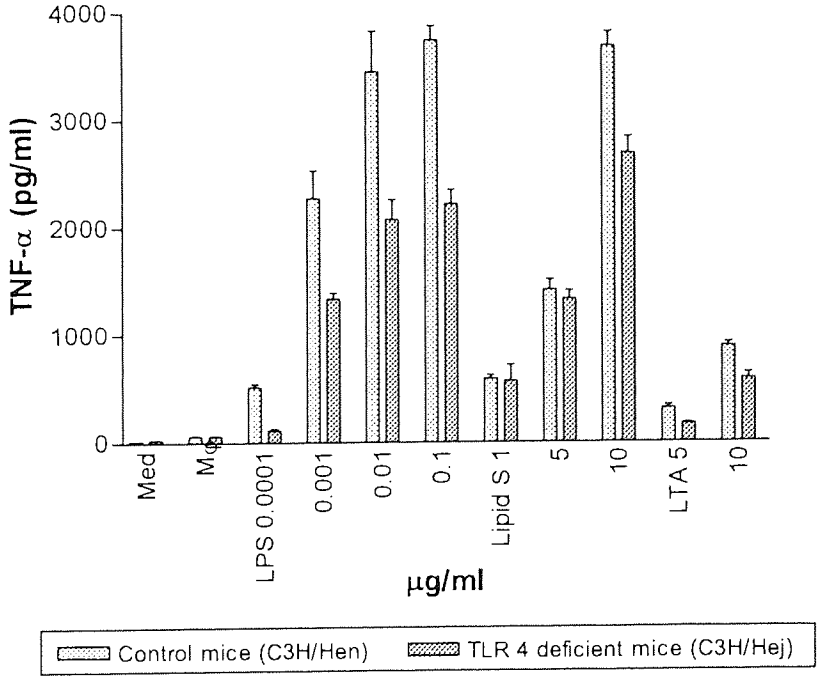


Figure 8.6: Effects of different concentrations of bacterial components on TNF-α production from mouse peritoneal cells 5 x 10^5/ml after 24 hours. Bars are standard error of the mean of triplicate samples

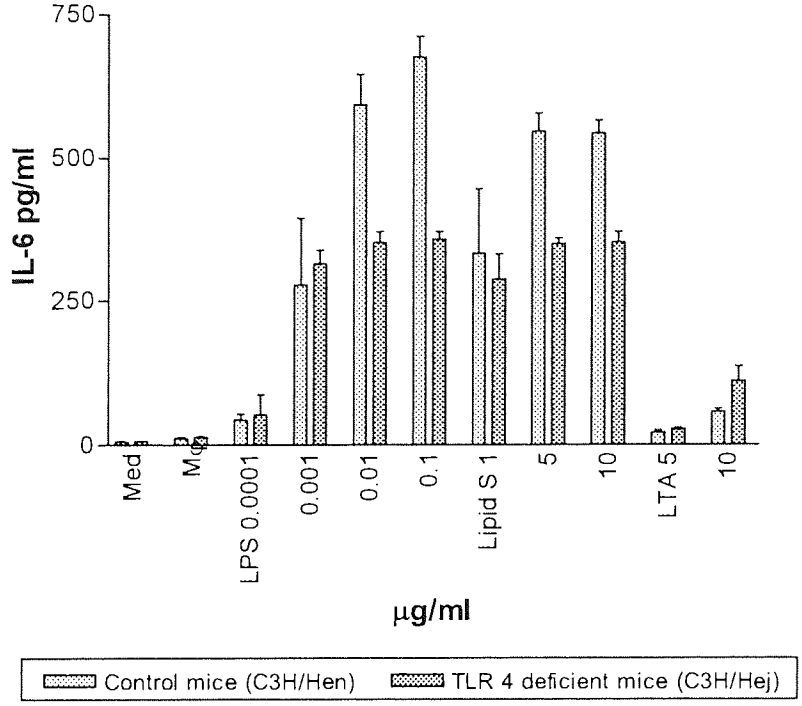


Figure 8.7: Effects of different concentrations of bacterial components on IL-6 production from mouse peritoneal cells 5 x 10^5/ml after 24 hours. Bars are standard error of the mean of triplicate samples

Mice with defective TLR4 receptors certainly showed diminished responses (compared to wild-type) to LPS in terms of NO, TNF-α and IL-6 production. In contrast responses to Gram-positive components were relatively unimpaired.

LPS at a high dose of 1μg/ml showed a significant 40% ($p = 0.0249$) decrease in stimulating activity and a lower dose of 0.1μg/ml induced a very significant 78% ($p = 0.0002$) reduction in nitrite production from C3H/Hej compared to C3H/Hen macrophages. Very little insignificant differences were observed between the two strains when lipid S was incubated at a dose of 100μg/ml ($p = >0.05$). A lower concentration of lipid S (10μg/ml) resulted in a 23% ($p = >0.05$) reduction and a high dose of LTA (100μg/ml) resulted in a significant 30% ($p = 0.013$) decrease, lower concentrations of LTA failed to induce a reaction above control levels (Fig 8.5).

When the production of TNF-α was measured a similar pattern emerged. LPS incubated at doses 0.1 – 0.001μg/ml resulted in a diminished response in the C3H/Hej mouse strain of between 39 – 41% ($p = <0.01$) compared to the C3H/Hen strain. A lesser, although significant reduction of 27% ($p = 0.0011$) was observed with lipid S at 10μg/ml and similar insignificant levels of TNF-α was produced by both strains at lower doses of lipid S (5 and 1μg/ml, $p = >0.05$). The response to LTA was also reduced by 34% ($p = 0.007$) at a dose of 10μg/ml in the C3H/Hej strain and a lower dose gave inconclusive results as levels were barely above those observed for controls (Fig 8.6).

The results gained from the experiments measuring IL-6 showed that a maximal reduction of 47% ($p = 0.0001$) occurred with cells containing LPS at 0.1μg/ml, a slightly lower reduction of 41% ($p = 0.0015$) was observed for 0.01μg/ml. The response from lipid S at 10 and 5μg/ml was diminished by 35% ($p = <0.001$) and an insignificant increase in production of IL-6 of 13% ($p = >0.05$) was seen at 1μg/ml. Low doses of LPS and LTA failed to induce measurable responses above control levels in both mouse strains.

8.4 DISCUSSION

TLRs are emerging as the transmembrane protein receptors responsible for microbial recognition and signalling cell activation. Comparative studies of TLRs from mice and humans suggest that TLR4 interacts directly with LPS (Poltorak *et al.*, 2000). There is also a general consensus that TLR2 plays a key role in cell signalling to Gram-positive components, such as PG, LTA and various lipopeptides (Lien *et al.*, 1999; Wong, 2001). Dziarski *et al.* (2001) also reported that TLR2 in the presence of MD-2 also responds to Gram-negative bacteria. In addition TLR2 and TLR6 have been shown to work together (Ozinsky *et al.*, 2000) and TLR5 has also been implicated in anti-bacterial host defence (Takeuchi *et al.*, 2001).

Inhibition of cytokines was investigated using the anti-TLR4 antibodies in cell culture. Adding anti-TLR4 might be expected to compete for available TLR4 and inhibit cellular activation of pro-inflammatory cytokines.

The results presented here showed that blockade of the TLR4 with mAbs HTA125 and 1216 strongly reduced the TNF-α and IL-1 response to LPS demonstrating these antibodies have a high affinity for the TLR4. and are consistent with previous observations (Akashi *et al.*, 2000a), confirming that LPS mediates through TLR4. Indeed if controls are taken into account then almost complete antibody inhibition is observed with LPS. Viriyakosol *et al.* (2001) also found by incubating THP-1s with anti-TLR4 mAbs HTA1216 and HTA125 inhibited the induction of IL-8.

LTA was a poor inducer of TNF-α and IL-1 in monocytes from these mice, even at a moderate dose (10μg/ml). It is not known why this occurred as it had previously been shown to induce the production of both these cytokines in various mouse cell lines. Nevertheless, despite the low production of cytokines produced the results did not show a clear inhibition of LTA.

Lipid S with both HTA125 and HTA 1216 mAbs showed that generally cytokine production was inhibited slightly compared to lipid S alone. However a 42% increase in the production of TNF-α was observed with HTA1216. These results are

conflicting, possibly suggesting that the HTA1216 antibody was not as specific as the HTA125, alternatively it might bind to lipid S and accentuate the production of TNF-α. Although the data obtained here supports the role of TLR4 as the key component for LPS it seems probable that lipid S also utilises this receptor, though not exclusively.

Much evidence for a differential role of TLRs in bacterial recognition has come from the analysis of TLR4-deficient mice and TLR2 knockout mice. Indeed the activation of signalling pathways induced by LPS is completely eliminated in TLR4 deficient mice, whilst TLR2 knockout mice showed a normal response. Conversely in the TLR2 knockout mice the cellular responses to Gram-positive bacteria were severely impaired (Takeuchi *et al.*, 1999). Notably the results shown here demonstrate that the mouse strain C3H/Hej, allegedly resistant to LPS, did respond by producing nitrite (Fig 8.5), TNF-α (Fig 8.6) and IL-6 (Fig 8.7) to all bacterial products tested, including LPS. However measurable levels were lower from this mouse strain than to the control LPS-responsive mice, C3H/Hen.

The maximal inhibition of both nitrite and cytokine production occurred when C3H/Hej peritoneal macrophages were incubated with LPS compared to LTA or lipid S. Higher concentrations of LPS (0.01 – 1μg/ml) were most affected, with a decrease in production of nitrite and cytokines between 40 - 78%. The results show that LPS can initiate a response in both mouse strains and therefore does not specifically only activate via the TLR4 receptor and must use other mechanisms, possibly binding to other toll-like receptors such as TLR2, previously suggested by (Yang *et al.*, 1998).

This is in contrast to results reported from many sources that TLR4-defcient macrophages, which did not produce any detectable levels of cytokines in response to LPS (Poltorak *et al.*, 1998; Hoshini *et al.*, 1999; Akira, 2000). However, other studies have demonstrated that under certain conditions TLR4-deficient mice have been observed to be responsive to LPS. High doses of LPS have been shown to have a stimulatory effect on these mice (Rosenstreich *et al.*, 1982) and some types of LPS from different strains of Gram-negative organisms also activated these mice (Tanamoto *et al.*, 1997).

Interestingly the response by C3H/Hej cells to produce IL-6 (Fig 8.7) showed that there appears to be a set fixed response to all bacterial components at higher concentrations. This indicates that IL-6 can be produced by other signalling pathways but that only a limited amount is produced, possibly suggesting that the involvement of other receptors is required for a maximal response.

Similar results were found with the two mouse strains for low concentrations of LTA (5 – 25 μg/ml) and lipid S (1 – 5 μg/ml) whereas higher concentrations resulted in a lower production of nitrite and cytokines in the C3H/Hej macrophages. This demonstrates that the response from these bacterial products was reduced in the absence of TLR4, but not completely abrogated, indicating possible involvement of other TLRs such as TLR2. Takeuki *et al.* (1999) also stated that there is possibly an overlap in the TLR function and response as TLR4 could also be linked with LTA.

The results failed to demonstrate a clear inhibition by the mouse strain C3H/Hej in the production of TNF-α, IL-6 or nitrites with the addition of LPS suggesting that other TLRs are involved or that other mechanisms within cells are operating to induce the release of these pro-inflammatory mediators. However it is clear that Gram-positive components LTA and lipid S are not affected by defective TLR4 or blockage of that receptor and further experiments with a TLR2 defective receptor and anti-TLR2 mAb may identify the toll-like receptor linked to these components.

Clearly the mechanisms underlying responses to bacterial components are very complex and probably involve multiple factors that determine the specificity of the TLR responsiveness. Based on the lack of compelling binding data, TLRs might well operate downstream of the initial step or steps of bacterial recognition. It could be that TLR4 is recruited later on and is required to achieve maximum response.

Lastly, Gram-positive and Gram-negative sepsis is one of the major causes of death even in the present day. Recent studies revealed that the TLR signalling pathway is a critical mediator of sepsis. An understanding of TLRs and their signalling pathway may reveal a therapeutic target for sepsis.

CHAPTER 9: TETRACYCLINE ANTIBIOTICS AS ANTI-INFLAMMATORY AGENTS

9.1: INTRODUCTION

Treatment for sepsis currently involves, where possible, surgical removal of any septic tissues combined with antibiotic therapy (Vincent, 1998). However, some antibiotics, depending on their mode of action can cause release of bacterial components which can then induce the release of pro-inflammatory mediators (Nau and Eiffert, 2002) and further complicate the condition. Despite aggressive management of septic shock, the mortality rate is 40–60%. Investigations are now focussing on the use of anti-inflammatory therapies to improve outcome. Previous studies have indicated that, in addition to their anti-microbial effects, a number of antibiotics have anti-inflammatory properties (Khan *et al.*, 2000).

Tetracyclines have a long standing history as broad spectrum antibiotics (Lorian, 1991). This family of drugs is represented in the clinic mainly by three established compounds: tetracycline, doxycycline, and minocycline. These drugs were developed specifically as antibacterial agents and their use for many years provides a clear understanding of their safety and efficacy. However, in addition to their use as anti-bacterials, the tetracyclines demonstrate a number of other clinically useful properties including anti-inflammatory activity. This anti-inflammatory activity is thought to be independent of their anti-bacterial action and includes inhibitory effects on enzymes responsible, such as matrix metalloproteases, nitric oxide synthase, cyclooxygenase-2, phospholipase and inflammatory cytokinessuch as TNFα, IL-1β, (Amin *et al.*, 1996; Golub *et al.*, 1997; Patel *et al.*, 1999; Greenwald and Golub, 2001).

In a mouse model of lethal Gram-negative sepsis tetracyclines have been shown to have a protective effect (Milano *et al.*, 1997). In this case the tetracyclines affected early events triggered by LPS. LPS-treated mice protected by tetracycline showed significant reductions in the levels of TNF-α, IL-1 and nitrate in blood, events that were directly correlated to survival (D'Agostino *et al.*, 1998a; D'Agostino *et al.*, 1998b). While the precise mechanism by which the tetracyclines are able to modulate

this wide range of inflammatory factors is unknown, it suggests that the molecule is able to intervene at a key step in the inflammatory cascade process. This suggests that the tetracyclines could be candidates for the treatment of septic shock, having both antibacterial and anti-inflammatory activity.

In practice the anti-inflammatory activity of the tetracyclines is already being taken advantage of clinically to treat a variety of disorders including periodontal diseases, acne and rheumatoid arthritis (Akamatsu *et al.*, 1991; Golub *et al.*, 1997; Langevitz *et al.*, 2000). However, no reported attempts have been made to systematically screen a large set of tetracyclines for anti-inflammatory activity and to use the knowledge gained by this screening effort to discover an improved anti-inflammatory tetracycline drug.

For comparison purposes other antibiotics previously reported to have anti-inflammatory effects were also investigated. Isoniazid is an anti-tuberculosis agent, but has also been reported to have anti-inflammatory properties as it has been shown to interfere with myeloperoxidase and impairs the production of the oxidizing agent, hypochlorous acid (HOCl) (van Zyl *et al.*, 1989). This has an effect by reducing oxidative burst activity and free radical damage to surrounding cells and tissues. Other antibiotics reported to have anti-inflammatory action include ciprofloxacin, a quinolone antibiotic and phosphomycin, a pyruvate transferase inhibitor. Both have been reported to decrease the production of TNF-α and IL-1 by LPS stimulated human monocytes (Labro, 2000; Morikawa *et al.*, 1996).

9.2 MATERIALS AND METHODS

To investigate the potential use of antibiotics in suppressing the inflammatory response and thus the initiation of septic shock, a number of antibiotics were employed. Experiments were conducted to investigate their ability to inhibit the release of nitrite and TNF-α from two macrophage cell lines (RAW 264.7 and J774.2). Compounds that showed some inhibition were subjected to further analysis using primary cultures of murine macrophages.

9.2.1: Antibiotics used to investigate possible modulatory effects on nitrite and TNF-α production

The antibiotics (Figures 8.1 and 8.2) tetracycline, minocycline, oxytetracycline, doxycycline, chlortetracycline, phosphomycin, isoniazid and ciprofloxacin (Sigma) were each provided in powder form and stock solutions of 10 mg/ml were prepared by dissolving in sterile distilled water. Further dilutions were prepared in cell culture media (see section 2.5). Stock solutions were stored in sterile 1.5 ml eppendorf tubes (Sarstedt) at -20°C until ready for use.

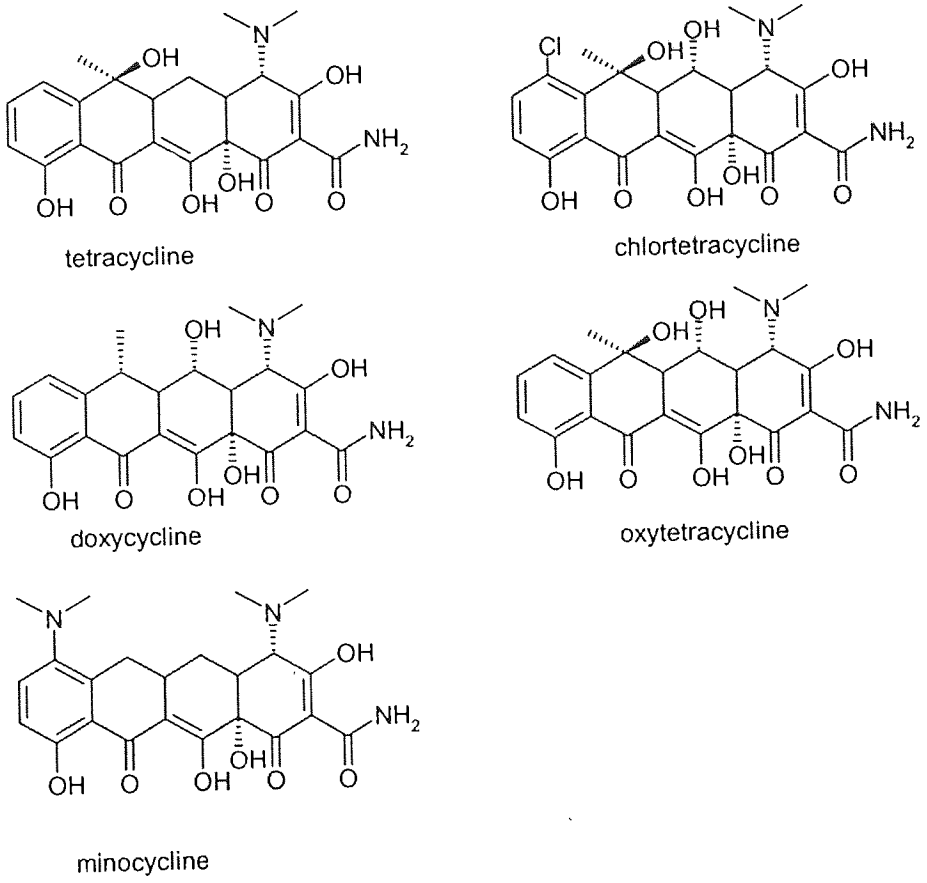


Figure 9.1: Structure of clinically useful tetracyclines. Tetracyclines have a common 4-ring carbocyclic structure with different substitutions at carbons 5, 6, or 7.

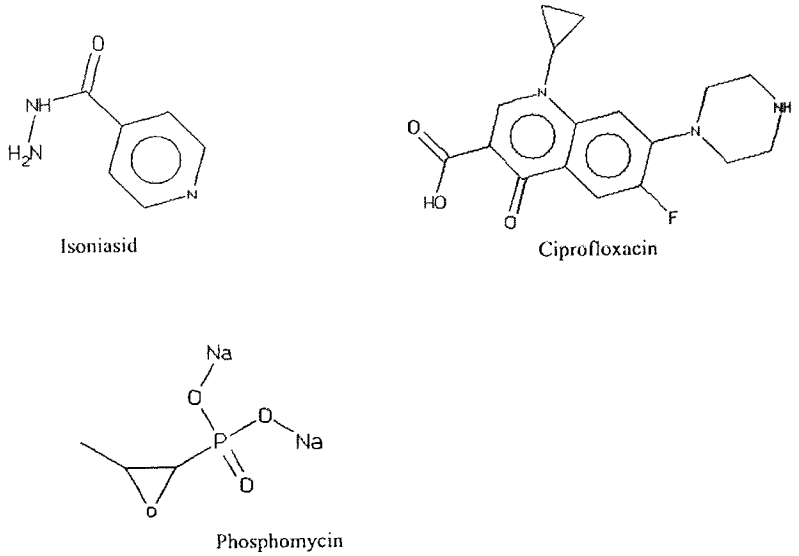


Figure 9.2: Structure of isoniasid, ciprofloxacin and phosphomycin

9.2.2: Novel tetracyclines

A number of non-antibacterial tetracycline derivatives (Paratek Pharmaceutical, Boston, USA) were provided in powder form and were dissolved in dimethyl sulphoxide (DMSO) to obtain a stock solution of 10 mg/ml and stored at -70°C until ready for use. Further dilutions were prepared in cell culture medium (section 2.5). The final concentration of DMSO in each well was 1% of the total volume.

Due to confidentiality, the compounds are not named and their structures can not be disclosed but they are all analogues of the tetracycline antibiotic. The Paratek library mainly contains derivatives of doxycycline and minocycline with various substitutions at positions 2, 5, 6, 7, 8 and 9 on the molecule (see figure 9.3). The molecular weight of the compounds varies between 430 and 650 depending upon the R group

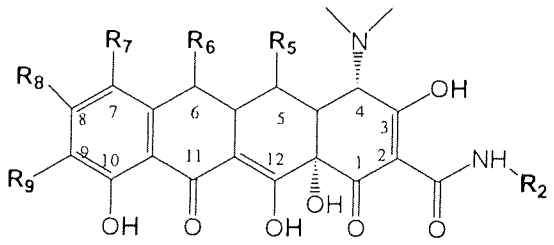


Figure 9.3: Schematic representation of the Paratek Pharmaceuticals tetracycline molecules showing positions for derivatization.

Table 9.1: Paratek code numbers of non-antibacterial tetracyclines

Compounds	
P000003-HCl-2	P000714-HCl-1
P000005-HCl-2	P000745-HCl-1
P000006-HCl-2	P000790-2HCl-2
P000303-HCl-2	P000840-HCl-2
P000351-HCl-2	P000960-2HCl-1
P000439-HCl-1	P001084-2HCl-1
P000632-3HCl-1	P001114-TFA-1
P000642-HCl-2	P001128-3HCl-1
P000643-HCl-2	P001183-3HCl-1
P000669-2HCl-1	P001229-TFA-1

Due to the activity shown by some of the compounds tested initially, further tetracycline analogues were tested (Table 9.2).

Table 9.2: Further novel non anti-bacterial tetracycline analogues (supplied by Paratek)

Compound	Molecular Weight
P000705-2HCl-1	577.59
P000766-2HCl-2	590.62
P000973-2HCl-1	543.57
P001032-HCl-1	708.64
P001044-2HCl-1	633.69
P001069-2HCl-1	572.61
P001081 -TFA-1	529.50
P001104-2HCl-1	564.54
P001109-3HCl-1	632.71
P001151-2HCl-1	550.56
P001181-2HCl-1	563.60
P001206-3HCl-1	646.70
P001233-2TFA-1	570.59
P001238-3HCl-1	540.61
P001228-2TFA-1	570.27
P002072-2TFA-1	570.63
P002092-3TFA-1	554.59
P002303-no salt-1	304.18

All tetracyclines were > 85% pure, determined by mass Spectrometry (LC-MS) Column: (Phenomenex Luna 5 µm C18 (2) 50 x 4.6 mm) Mobile Phase: (HPLC grade H_2O and 0.1% Formic acid) (HPLC grade Acetonitrile and 0.1% Formic acid). UV detection @ 280 nm and 254 nm. ELSD and LC/MS-TOF (Positive Electrospray).

9.2.3: Treatment of cells

MF1 mouse peritoneal macrophages (5×10^5) were isolated and prepared as in section 2.4.1 and cell lines RAW264.7 and J774.2 (1×10^6) were prepared as in section 2.4.2. Cells were seeded (250 µl per well) at an appropriate concentration in a 96-well tissue culture plate (Nunclon, Denmark). These were incubated at 37°C in an atmosphere of 95% air and 5% CO_2 for a period of two hours to allow cells to settle. Cells were then preincubated with a range of concentrations of a given antibiotic for 1 hour prior to the addition of stimulant (LPS, lipid S, LTA or PG). All cell treatments were performed in triplicate wells and incubated at 37°C in an atmosphere of 95% air and 5% CO_2. Supernatants were collected after 48 hours for analysis of nitrite and 24 hours for cytokines. All antibiotic compounds and stimulants were prewarmed to 37°C prior to use.

9.2.4: Measurement of Nitrite

nitrite released by the peritoneal macrophages or the cell lines was analysed as in section 4.2.1.

9.2.5: Measurement of TNF-α

TNF-α was measured by enzyme-linked immunosorbent assay (ELISA) using the TNF-α mouse cytokine Duoset ELISA development system kits (R and D Systems).

The ELISA cytokine assays were carried out as in section 5.2

9.3: RESULTS

9.3.1: Modulation of NO and TNF-α production in RAW264.7 and J774.2 cell lines

The effects of a range of concentrations of various tetracycline antibiotics were investigated to determine if they were able to reduce the production of nitrite and TNF-α in J774.2 and RAW264.7 cells when exposed to bacterial components. Other antibiotics were also used to determine their potential to modulate nitrite and TNF-α.

Cells of the murine macrophage-like cell lines J774 and RAW264.7 (1×10^6) in 250μls of supplemented medium are simulated with bacterial components, LPS isolated from *E. coli* (O111:B4) and LTA, lipid S and PG isolated from *S. epidermidis* (NCIMB 40896). The concentrations of bacterial components used have previously been shown to stimulate inflammatory mediators from the cell lines. After 48 hours samples of the culture supernatants are collected and stored at -20°C for subsequent analysis of release of nitric oxide. A range of concentrations of the test compound (100, 50, 10, and 1 μg/ml) are added to the cell suspensions 1 hour prior to addition of bacterial components. Wells containing medium alone, medium with cells and cells with antibiotics but no bacterial stimulants were used as controls. Assays are carried out in triplicate and data is recorded as the average IC50 value obtained (i.e. the concentration required for 50% inhibition) from a single experiment.

Because of the huge number of results obtained from testing various antibiotics on the different cell wall products a table summarising their inhibition of nitrite is presented.

Table 9.3: Concentration of antibiotic required to inhibit nitrite production by 50% (IC50) in J774.2 and RAW264 cells following stimulation by bacterial products.

Compound	IC50 (µg/ml)							
	LPS (100ng/ml)		Lipid S (10µg/ml		LTA (25µg/ml)		PG (25µg/ml)	
Cell line	J774.2	RAW264	J774.2	RAW264	J774.2	RAW264	J774.2	RAW264
Tetracycline	96	>100	85	93	84.5	79.5	97	92
Doxycycline	40	35	53	33	39	26.5	39	43
Oxytetracycline	12	23.5	8	21	18	20	19	18.5
Minocycline	35	28.5	18.5	16.5	24	14	30	9
Chlortetracycline	82	86	85	93.5	89	83	90.5	85
Phosphomycin	>100	>100	>100	>100	>100	>100	>100	>100
Isoniazid	>100	>100	>100	>100	>100	>100	>100	>100
Ciprofloxacin	>100	>100	>100	>100	>100	>100	>100	>100

Controls of the antibiotics alone had no effect on nitrite production when compared to the macrophage control, therefore demonstrating that they are not stimulatory themselves (results not shown). All bacterial components induced the production of nitrite in the absence of antibiotics. Also the antibiotics did not appear to discriminate between Gram-negative or Gram-positive bacterial components or between the two cell lines, with similar results obtained for each antibiotic tested.

None of the antibiotics completely inhibited the production of nitrite, indeed the non-tetracycline antibiotics phosphomycin, isoniazid and ciprofloxacin had no effect, even at the highest concentration used (100µg/ml), indicating they do not act as anti-inflammatory drugs.

Each of the tetracycline drugs suppressed the production of nitrite in a concentration dependent manner. Tetracycline and chlortetracycline were slightly more effective, though the IC50 concentrations were very high, ranging from 79.5 to over 100µg/ml, suggesting these antibiotics were not suitable candidates as anti-inflammatory agents.

The most successful compounds were oxytetracycline and minocycline, examples of these are shown below (Fig 9.4 and 9.5), followed by doxycycline, which in the majority of tests resulted in an average IC50 of 38.5μg/ml.

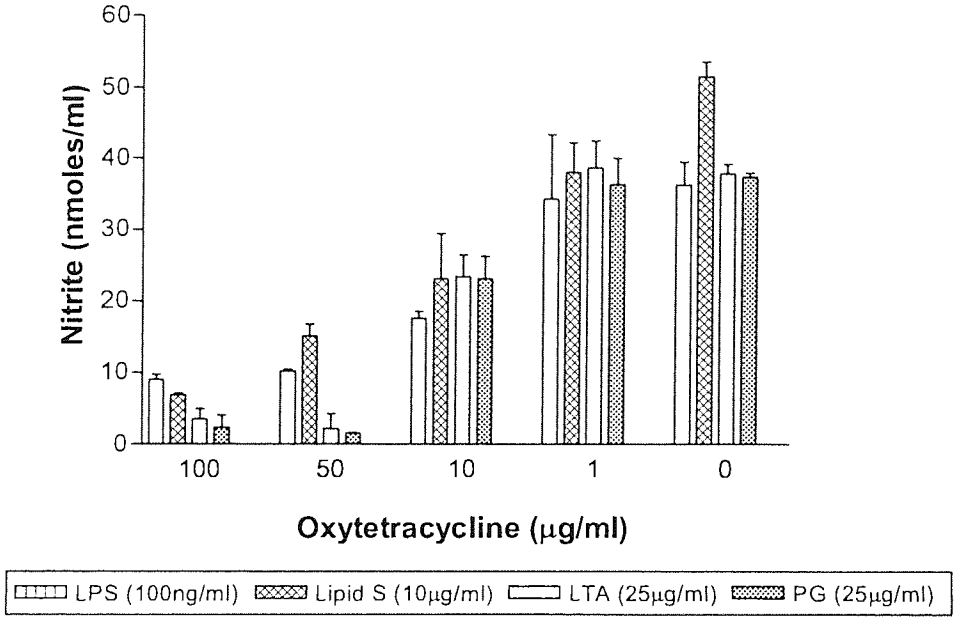


Figure 9.4: The inhibitory action of oxytetracycline on the release of nitrite from J774.2 cells exposed to LPS (100ng/ml), lipid S (10μg/ml), LTA and PG (25μg/ml).

Figure 9.4 illustrates a dose dependent reduction of nitrite on all bacterial component-stimulated J774.2 cells, reducing the effects of all bacterial components at higher doses. Oxytetracycline had a maximal effect with high doses of 100 and 50μg/ml, in all instances, $p = <0.05$. A moderatly significant inhibition was observed at a dose of 10μg/ml, resulting in 39, 38, 52 and 55% ($p = <0.05$) inhibition of LTA, PG, LPS and lipid S respectively. The low dose of oxytetracycline (1μg/ml) when compared to macrophage controls had no effect on reducing nitrite production; levels were equivalent to those observed with cells containing no antibiotic ($p = >0.05$).

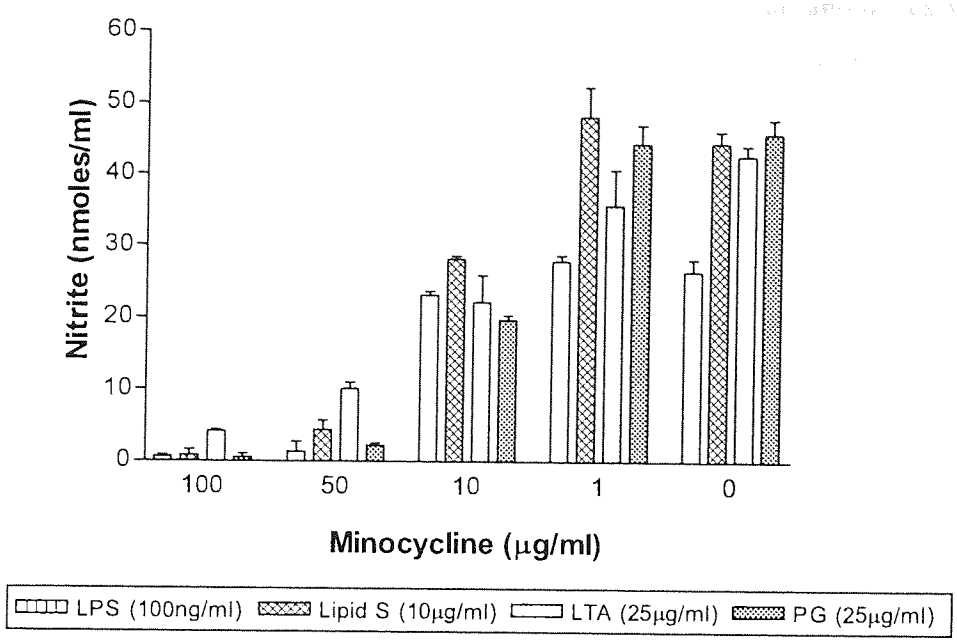


Figure 9.5: The inhibitory action of minocycline on the release of nitrite from RAW264.7 cells exposed to LPS (100ng/ml), Lipid S (10μg/ml), LTA and PG (25μg/ml).

Figure 9.5 depicts the effect of minocycline on nitrite production in bacterial component-stimulated RAW264.7 cells. Again a dose dependent inhibition was observed, reducing the effects of all bacterial components at higher doses. A high concentration of minocycline (100μg/ml) had the greatest effect in reducing nitrite production and in most instances this concentration significantly abrogated nitrite production completely ($p = <0.001$). A lower concentration of minocycline (50μg/ml) also showed almost complete inhibition, by at least 75% in all samples tested ($p = <0.001$). A lower dose of 10μg/ml was less effective, though LTA and PG were the most affected components, resulting in a reduction in activity by at least 50% ($p = < 0.001$). A low dose of 1μg/ml only showed a minimal insignificant capacity to reduce the effects of LTA, whilst levels of nitrite production in LPS, lipid S and PG-stimulated cells were not inhibited at this concentration ($p = >0.05$).

Following these results further investigations with the tetracycline antibiotics were conducted to measure the production of TNF-α. Cells of the murine macrophage-like cell line RAW264.7 (1×10^6) in 250μl of supplemented medium were simulated with bacterial components. After 24 hours samples of the culture supernatants were collected and stored at -20°C for subsequent analysis of release of TNF-α. A range of concentrations of the antibiotic test compound (100, 50, 10, and 1 μg/ml) was added to the cell suspensions 1 hour prior to addition of bacterial components. Wells containing medium alone, medium with cells and cells with antibiotics but no bacterial stimulants were used as controls. Assays were carried out in triplicate and values represent the mean +/-SEM (bars) of a single experiment.

The effect of tetracycline antibiotics on the production TNF-α was investigated, using the following compounds; tetracycline, minocycline, oxytetracycline, doxycycline and chlortetracycline. The results showed that levels obtained from control samples of all antibiotics incubated with cells caused increases in TNF-α production above those obtained from control samples containing only cells. In all assays the antibiotics had very little effect on inhibiting the production of TNF-α and all IC50 values were above the highest concentration used (100μg/ml). A typical example of the effects of the tetracycline antibiotics on TNF-α production is illustrated in Figure 9.6.

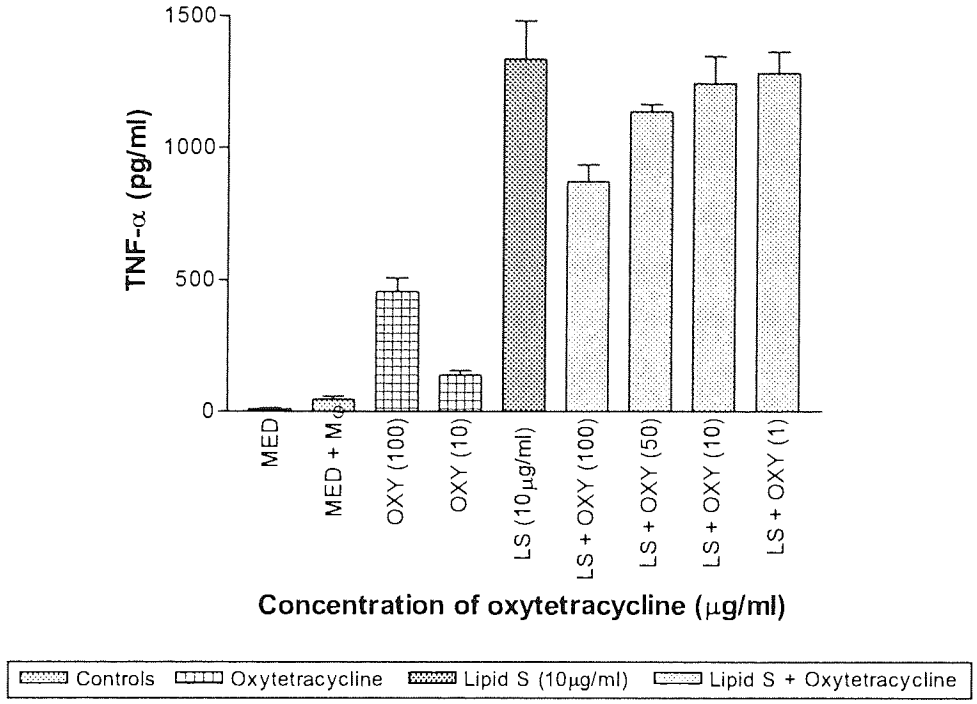


Figure 9.6: The inhibitory action of oxytetracycline on the release of TNF-α from RAW264.7 cells exposed to Lipid S (10μg/ml)

Controls of medium alone and medium with cells showed little TNF-α production. In contrast the oxytetracycline-only control samples 100 and 10μg/ml increased the production of TNF-α by 9.8 ($p = <0.05$) and 3-fold ($p = >0.05$) respectively when compared with the macrophage only control. The greatest decrease in TNF-α was seen with the highest concentration of oxytetracycline used at 100μg/ml, resulting in a decrease of 35% ($p = <0.05$) when compared to results from lipid S in the absence of the antibiotic. The effects of lipid S on TNF-α production was only marginally reduced with lower doses of oxytetracycline (50, 10 and 1μg/ml), resulting in a decrease of 15, 7 and 4% ($p = >0.05$) respectively.

9.3.2: Modulation of NO using novel tetracyclines

Following the success of some of the tetracyclines to diminish the production of nitrite further investigations were then carried out, investigating the inhibitory action

of a select group of novel non-antibacterial derivatives novel tetracyclines to reduce the production of nitrite in the J774.2 cell line.

J774 cells (1×10^6) in 250µl of supplemented medium were simulated with bacterial components, LPS isolated from *E. coli* (O111:B4) and lipid S isolated from *S. epidermidis* (NCIMB 40896). After 48 hours samples of the culture supernatants are collected and stored at -20°C for subsequent analysis of release of nitric oxide. A range of concentrations of the test compound (100, 50, 10, and 1 µg/ml) was added to the cell suspensions 1 hour prior to addition of bacterial components. Wells containing medium alone, medium with cells and cells with antibiotics but no bacterial stimulants were used as controls. Assays were carried out in triplicate and data recorded as the average IC50 value obtained from a single experiment.

Table 9.4: Concentration of antibiotic required to inhibit nitrite production by 50% (IC50) in J774.2 cells following stimulation by bacterial products.

	Nitrite IC50 (µg/ml)	
Compound	**LPS (100ng/ml)**	**Lipid S (10µg/ml)**
P000003-HCl-2	9.5	4.5
P000005-HCl-2	6	3
P000006-HCl-2	13	4
P000303-HCl-2	<1	<1
P000351-HCl-2	12	8
P000439-HCl-1	3.5	4.5
P000632-3HCl-1	77.5	87
P000642-HCl-2	7	7
P000643-HCl-2	4	2.5
P000669-2HCl-1	5	<1
P000714-HCl-1	16.5	24
P000745-HCl-1	7.5	6
P000790-2HCl-2	36	21
P000840-HCl-2	4	<1
P000960-2HCl-1	>100	>100
P001084-2HCl-1	<1	<1
P001114-TFA-1	>100	83
P001128-3HCl-1	>100	93
P001183-3HCl-1	>100	95
P001229-TFA-1	>100	98

A total of 20 analogues of tetracycline were investigated for their ability to diminish the production of nitrite. Table 9.4 displays the results of IC50's obtained for the various compounds when tested with stimulants LPS or lipid S.

Control samples of macrophages alone and macrophages with antibiotics and without any stimulus had very little effect on the production of nitrite. The results show that 30% of the compounds had little or no effect at the highest concentration used (100μg/ml). However, half of the drugs did notably inhibit nitrite production of both LPS and lipid S stimulated macrophages, with IC50 values of below 10μg/ml. Furthermore the IC50's obtained from two of these compounds P000303 and P001084 were below the lowest concentration used (1μg/ml). These results can be seen in Figures 9.7 and 9.8.

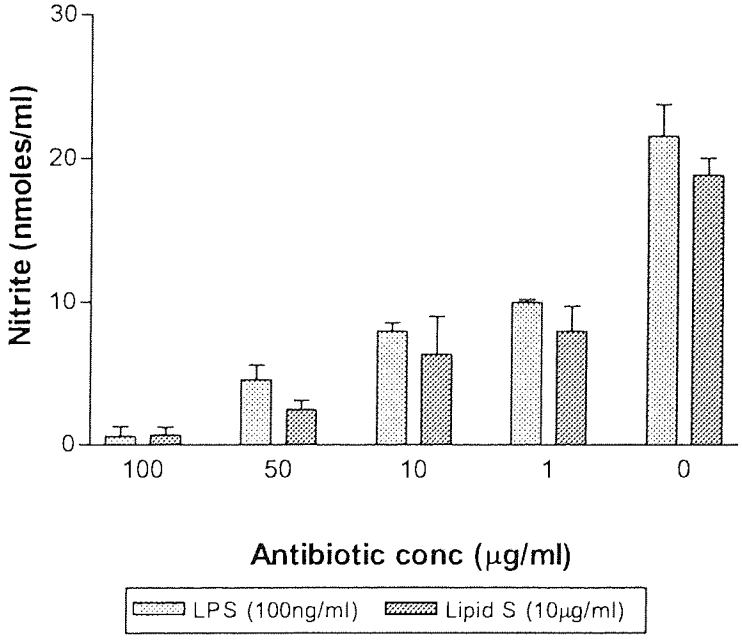


Figure 9.7: The inhibitory action of P000303 on the release of nitric oxide from J774.2 cells exposed to LPS (100ng/ml) and lipid S (10μg/ml)

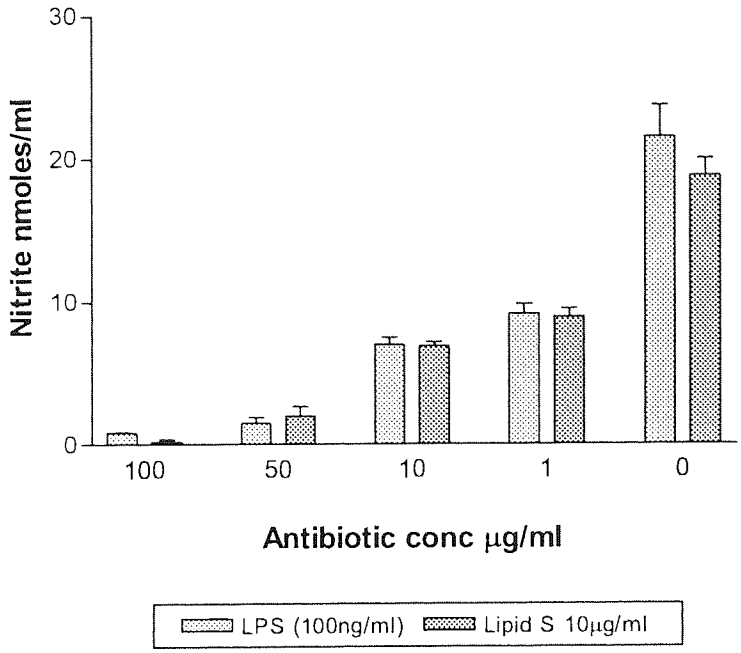


Figure 9.8: The inhibitory action of P001084 on the release of nitric oxide from J774.2 cells exposed to LPS (100ng/ml) and lipid S (10µg/ml)

Figures 9.7 and 9.8 illustrate the effects of two antibiotics (P000303 and P001084) on nitrite production induced by J774.2 macrophages. With the addition of LPS and lipid S the macrophages induced the greatest production of nitrite. When antibiotics were introduced into cell culture prior to stimulants they had a marked effect on the synthesis of nitrite. Each drug suppressed the synthesis of nitrite by LPS and lipid S-stimulated macrophages in a concentration-dependent manner. High doses of 100µg/ml had the greatest significant effect, inhibiting the production of nitrite to levels observed with control samples ($p = <0.001$). Doses of 50 and 10µg/ml were also effective resulting in an inhibition of between 63 - 93% ($p = <0.01$) compared to controls of LPS and lipid S. The amount of nitrite produced with a dose of 1µg/ml of both compounds was moderately reduced by 52 - 57% ($p = <0.05$) resulting in IC50 values below the lowest dose used.

The promising results obtained for these novel non-antibacterial tetracyclines led to further experiments on a further batch of 18 compounds supplied by Paratek Pharmceutical..

J774.2 and RAW264.7 cells (1×10^6) in 250µls of supplemented medium were stimulated with LPS or lipid S. A range of concentrations of the test compound were added to the cell suspensions 1 hour prior to addition of bacterial components. Samples were collected for analysis after 48 hours. Wells containing medium alone, medium with cells and cells with antibiotics but no bacterial stimulants were used as controls. Assays were carried out in triplicate and data was recorded as the average IC50 value obtained from a single experiment.

Table 9.5: Effect of different non-antibacterial tetracycline derivatives on the production of nitrite.

	IC50 (µg/ml)			
	Lipid S (10µg/ml)		**LPS (100ng/ml)**	
Compound	**RAW264**	**J774.2**	**RAW264**	**J774.2**
P000705-2HCl-1	>100	>100	>100	>100
P000766-2HCl-2	>100	65	>100	>100
P000973-2HCl-1	>100	>100	>100	>100
P001032-HCl-1	10	24	17.5	30
P001044-2HCl-1	72	65	45	>100
P001069-2HCl-1	57	80	31	25.5
P001081 -TFA-1	>100	>100	>100	>100
P001104-2HCl-1	>100	>100	79	>100
P001109-3HCl-1	41	49	54	62.5
P001151-2HCl-1	80	>100	>100	67
P001181-2HCl-1	3	7	<1	6.5
P001206-3HCl-1	>100	>100	>100	>100
P001233-2TFA-1	>100	>100	>100	>100
P001238-3HCl-1	3	5	<1	4.5
P001228-2TFA-1	<1	10	<1	5.5
P002072-2TFA-1	45	>100	38	>100
P002092-3TFA-1	>100	>100	>100	>100
P002303-no salt-1	6	24	7	36

Table 9.5 shows the IC50 values obtained from this batch of tetracycline derivative antibiotics on LPS- and lipid S-challenged J774.2 and RAW264.7 macrophages. Unlike the previous set of antibiotics (Table 9.4) this group as a whole were not as effective in inhibiting the production of nitrite. Over half the compounds had little or no effect, even at the highest dose used (100μg/ml) and there were some differences in the results between the cell lines. For example, P002303 inhibited the LPS- and lipid S-stimulated RAW264.7 cells to a greater extent than the J774.2 cells, with resulting IC50's of 6 and 7μg/ml respectively for the RAW264.7 cell line and 24 and 36μg/ml with J774.2 cells. However 3 of the compounds (P001238, P001228 and P001181) out of 18 showed substantial ability to inhibit the production of nitrite, with low doses of 10μg/ml or below resulting in at least a 50% inhibition compared to control samples.

To confirm the results obtained from cell lines the most successful compounds from tables 9.4 and 9.5 were selected and their inhibitory action on the production of nitrite using murine peritoneal macrophages *in-vitro* was investigated. Because of the limited number of macrophages isolated from a single mouse, which in turn limits the number of assays, to maximise cell usage macrophages from 10 MF1 mice were pooled together and a lower number of cells per ml were used. A count of 5×10^5 pooled peritoneal macrophages per ml, isolated by adherence were incubated at 37°C in 95% air, 5% CO_2 for 24 hours in 250μl of supplemented medium. A range of concentrations of the test compound were added to the cell suspensions 1 hour prior to addition of bacterial components. Controls of medium alone and medium with macrophages were assayed for comparison. Supernatants were removed after 48 hour incubation and assayed for nitrite production using the Griess reaction. Results obtained from duplicate samples from a single experiment and are displayed in table 9.6

Table 9.6: Effects of non-antibacterial tetracycline derivatives on the production of nitrite in murine macrophages

	IC50 (µg/ml)	
Compound	**Lipid S (10µg/ml)**	**LPS (100ng/ml)**
P000005-HCl-2	2.5	5
P000303-HCl-2	6	<1
P000439-HCl-1	6.5	5.5
P000642-HCl-2	7	1.5
P001084-2HCl-1	7.5	4.5
P000669-2HCl-1	5	<1
P000745-HCl-1	24	15
P001181-2HCl-1	1	<1
P001238-3HCl-1	2	<1
P001228-2TFA-1	4.5	12.5

The majority of compounds tested in murine macrophages displayed similar levels of inhibition activity to the results observed with the cell lines, demonstrating that values obtained from the cell lines are reproducible with murine macrophages. With the exception of one compound (P000745) all compounds gave IC50 values below 10µg/ml. In contrast, P000745 did not diminish the production of nitrite to the same extent as with the cell lines (see table 9.4) where the resulting IC50 values were below 10µg/ml, incubation with murine macrophages resulted in an IC50 of 24 and 15µg/ml for lipid S and LPS respectively. Two compounds, P001181 and P001228 displayed the greatest significant ability to reduce the production of nitrite, with IC50 values obtained from incubation with LPS and lipid S were below 2µg/ml. The results of P001181 are illustrated in figure 9.9.

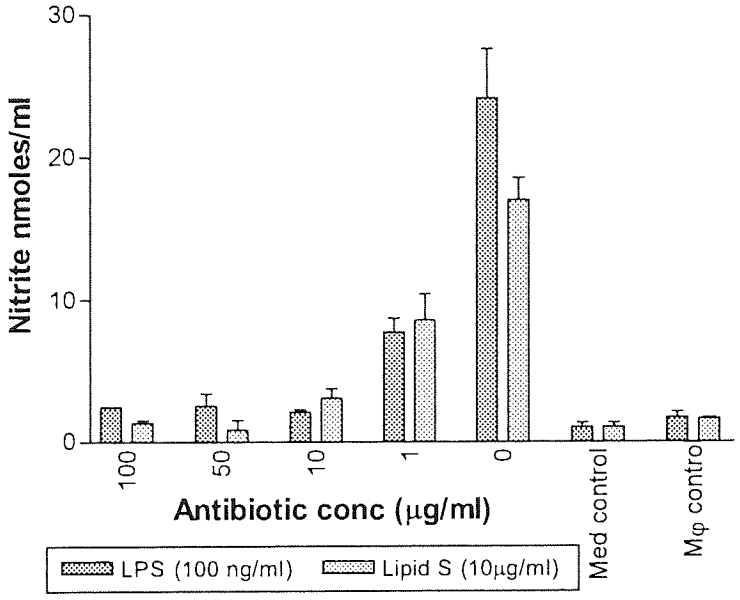


Figure 9.9: The inhibitory action of P001181 on the release of nitric oxide from murine peritoneal macrophages exposed to LPS (100ng/ml) and lipid S (10μg/ml).

Control samples containing macrophages alone had very little effect on the production of nitrite compared to the samples containing only culture medium. Marginally higher levels of nitrite production were detected for doses of 100, 50 and 10μg/ml of compound P001181 with both LPS and lipid S stimulated macrophages. This resulted in an inhibition of nitrite production by 82-95% ($p = <0.01$) compared to bacterial stimulated macrophages in the absence of antibiotics. A low dose of 1μg/ml also diminished the synthesis of nitrite by 50% ($p = < 0.05$) with lipid S-stimulated cells and 65% ($p = <0.01$) with LPS-stimulated cells.

9.4 DISCUSSION

Traditionally, sepsis was taken to be the consequence of a bacterial infection and was treated empirically with antimicrobial agents. It is now recognized that bacteria trigger pathophysiological events, resulting in an uncontrolled host response characterized by hypotension, fever, tissue necrosis, vascular damage leading to multiple organ failure and ultimately death.

Despite vast amounts of research on immunomodulatory drugs for the treatment of sepsis and septic shock, no drugs have yet proven clinically useful and poor knowledge of the intricate pathophysiological events in the clinical setting limits therapeutic approaches. Antibiotics can further complicate the condition by enhancing the release of bacterial cell wall products and as a consequence cause rapid release of pro-inflammatory or toxic components from both Gram-positive and Gram-negative bacteria (Nau and Eiffert, 2002). β-lactams have been reported to enhance the release of LTA and PG from bacteria (van Langevelde *et al.*, 1998). However some antibiotics have been reported to have both antibiotic and anti-inflammatory effects (Labro, 2000) and can modulate the secretion of inflammatory mediators, such as NO and cytokines, as discussed in section 9.1. On the basis of the anti-inflammatory properties of some antibiotics, experiments were designed to test their abilities to reduce nitrite and TNF-α production induced by bacterial components from Gram-positive and Gram-negative bacteria in two cell lines, J774.2 and RAW264.7.

Inhibition of nitrite stimulated by bacterial components was first investigated in cell cultures using established antibiotics, tetracycline, minocycline, oxytetracycline, doxycycline, chlortetracycline, phosphomycin, isoniazid and ciprofloxacin (Table 9.3).

The non-tetracyclines, phosphomycin, isoniazid and ciprofloxacin had no effect on nitrite production even at the highest dose used (100μg/ml) when measured with each of the bacterial components. In contrast previous studies from other groups have shown them to have anti-inflammatory properties (see section 9.1).

The results from the tetracycline antibiotics showed that some had inhibitory actions whereas others did not. However there was little variation with results obtained with either of the cell lines (J774.2 and RAW264.7) or between the bacterial components LPS, lipid S, LTA or PG. This demonstrates that the antibiotics do not discriminate between cell lines nor between the different bacterial components.

The tetracycline group of antibiotics had a greater effect upon nitrite secretion, all inducing a dose-dependent inhibition of nitrite. Oxytetracycline and minocycline proved to be the most effective followed by doxycycline. Tetracycline and chlortetracycline were less effective with resulting IC50 values above 80μg/ml. These results confirm previous findings that doxycycline and minocycline inhibit NO synthesis (D'Agostino *et al.*, 1998b; Patel *et al.*, 1999). Amin *et al.* (1996) also reported that doxycycline and minocycline inhibit the activity of murine macrophage iNOS. They suggest that tetracyclines inhibit iNOS activity not via a direct inhibition at the enzyme level but through an inhibition of NOS mRNA expression, which leads to a decrease in protein expression and NOS activity.

Each of the tetracycline antibiotics were then examined for their abilities to inhibit the secretion of TNF-α from LPS and lipid S stimulated RAW264.7 cells. In contrast to inhibiting nitrite none of the tetracyclines demonstrated any significant activity upon the secretion of TNF-α and all resulting IC50 values were above the highest dose used (100μg/ml) (see for example in Figure 9.6). Altogether the data here indicate that tetracyclines at high doses (50 and 100μg/ml) had a small inhibitory effect, whereas lower doses (1 and 10μg/ml) were not capable of acting on the production of TNF-α. Similar results were reported from Milano *et al.* (1997), they also found tetracyclines inhibit NO synthesis in splenic and peritoneal macrophages but were unable to decrease the release of TNF-α and IL-1. These results conflict with reports from Shapira *et al.* (1996) where tetracycline was found to inhibit TNF-α secretion from LPS-stimulated monocytes. This difference may well be due to the high concentrations of tetracycline used in their experiments, ranging from 100 – 500μg/ml compared to 1 – 100μg/ml used in this study. These doses are considered to be in excess of the *in vivo* physiological parameters used in antibiotic therapy. For example, extracellular *in vivo* concentrations of tetracycline are approximately 10 – 17μg/ml

and cellular concentrations range from 13 – 35μg/ml, dependent upon the site (Lorian, 1991). Indeed, oxytetracycline alone used in this study at a dose of 100μg/ml induced a 9.8 fold increase in TNF-α production, compared to macrophage control samples (Figure 9.6); suggesting higher doses would be detrimental. Given that the tetracyclines all have similar molecular weights then 20μg/ml of any of the compounds would inhibit NO production.

The mechanism of action of tetracyclines is not clear; however the inhibitory mechanism of nitrite by tetracyclines appears to be specific since the same tetracyclines were not able to inhibit TNF-α production. Shapira *et al.* (1998) also stated that inhibition of nitrite is not due to inhibition of TNF-α and vice versa. However the down regulation of nitrite secretion could be one of the mechanisms by which tetracyclines control inflammation.

The developement of bacterial resistance to tetracyclines has had consequences on the use of tetracyclines as therapeutic agents (Chopra and Roberts, 2001). There is no question that with the increase in antibiotic resistance that new therapeutic agents need to be found. New derivatives of the tetracyclines that can be used as anti-inflammatory agents could potentially be an alternative use for the tetracycline group of antibiotics. Furthermore, use of tetracycline derivatives with no anti-bacterial activity would not encourage resistance development.

In an assay investigating the inhibitory action of a selected group of non-antibacterial tetracycline derivatives on the release of nitric oxide in J774.2 and RAW264.7 cell lines exposed to LPS from *E. coli* and lipid S from *S. epidermidis*, several compounds with improved activity were identified (Tables 9.4 and 9.5). Results indicate that the non-antibacterial tetracyclines were potent inhibitors of nitrite production in a dose–dependent manner. Of the 38 compounds tested 37% gave IC50 values below 10μg/ml for both LPS and lipid S stimulated macrophages, furthermore two compounds P000303 and P001084 were active at a dose of 1μg/ml, the lowest concentration tested to date (Figure 9.7 and 9.8). Moreover the cytotoxicity of the P001084 compound, as judged from an *in vitro* assay of cytotoxicity against mammalian cells, was >25ug/ml (personal communication with Dr Michael Draper,

Paratek), indicating it is well within the boundaries for use in anti-inflammatory therapy.

An assay was then performed on murine peritoneal macrophages, using ten of the most active tetracycline compounds (Table 9.6). The results demonstrate that the majority of compounds had similar activity upon nitrite production to that observed with the cell lines, thus confirming that cell lines are suitable for studies of this nature and maybe used as a realistic screening test for mice and eventually after further tests possible for man. Most of the compounds were active below 10μg/ml and one compound in particular, P001181, gave an IC50 value of 1μg/ml for both LPS- and lipid S-stimulated murine macrophages (Figure 9.9). With the increase in antibiotic resistant bacteria, drugs that have no anti-bacterial properties but do possess immunomodulatory actions that suppress inflammatory mediators might help prevent the onset of septic shock.

Many tetracycline derivatives tested here have been shown to inhibit the production of nitrite in both macrophage cell lines and mouse macrophages. These concentrations suggest that effects of these anti-inflammatory drugs are within physiological parameters and therefore may reflect effects exerted *in-vivo*. However there are more variables to account for when investigating possible sites of action of drugs *in vivo*.

It is at present unclear how tetracyclines modulate nitrite secretion. The results presented show that some tetracyclines greatly inhibit the production of nitrite in murine peritoneal macrophages and two cell lines J774.2 and RAW264.7. It is reasonable to believe that tetracyclines targeting NO block one of the most important mediators involved in the pathogenesis of septic shock. It has been suggested that NO regulates IL-1 (Hill *et al.*, 1996). Thus by using an inhibitor to nitrite the release of IL-1 could also be inhibited, unfortunately IL-1 wasn't investigated with these non-anti-bacterial tetracyclines.

The data presented here are encouraging for the use of non-antibacterial tetracyclines in the management of inflammatory diseases such as septic shock. However, further extensive studies on their use in clinical human cases is required.

CHAPTER 10: FINAL DISCUSSION

Septic shock is a common problem encountered in the intensive care unit; it involves a complex interaction between bacterial factors and the host immune system producing a systemic inflammatory state that may progress to multiple organ failure and death (Cohen, 2002). Septic shock can be initiated by Gram-negative and Gram-positive bacteria and both have been reported to stimulate the immune system in a similar manner. The proportion of patients with sepsis caused by Gram-positive bacteria has increased, and today Gram-positive bacteria account for almost half of the incidents of sepsis. LPS has been identified as a potent, prototypical stimulus of the immune response to Gram-negative bacterial infections (Nau and Eiffert, 2002). It is unclear which component(s) of Gram-positive bacteria elicits an immune response that results in septic shock. LTA and PG have been identified as possible mediators; however the precise contribution of LTA or PG to inflammatory responses has not been defined. *S. epidermidis* is increasingly becoming more prominent as the major cause of septic shock, particularly in patients with indwelling devices (Elliott, 1988). The exocellular component termed lipid S that has recently been described by Lambert *et al.* (2000), is released during growth of *S. epidermidis* and is structurally related to LTA. High titres of IgG which can bind to lipid S have been found in patients with a *S. epidermidis* infection (Elliott *et al.*, 2000). Determining the components of bacteria that are responsible for initiating septic shock is important to identify potential therapeutic targets. As lipid S is released from the cell wall it has a higher probability than other bacterial components to encounter cells of the immune system and promote the inflammatory state seen in septic shock and could be the major mediator of Gram-positive infections.

The aims of this study were to:

- Isolate lipid S and determine its antigenic properties.
- Compare lipid S with other known bacterial cell wall components.
- Identify inflammatory mediators.

The Gram-positive bacteria, *S. epidermidis* (strain NCIMB 40896) isolated from a patient with a CVC infection was grown in a chemically defined media to reduce the risk of contaminating any antigenic compound with high molecular weight compounds found in other growth mediums. Analysis of fractions of the supernatant reacting with anti-serum from a patient with sepsis due to *S. epidermidis* infection revealed the presence of a short chain length form of LTA, previously described as lipid S (Lambert *et al.*, 2000). IgG in the immune serum released during *S. epidermidis* infection clearly bound to lipid S in an ELISA assay and was also detected in western and dot blotting. In contrast little reaction was obtained when the fractions were analysed using non-immune human serum. LTA, PG and WTA were extracted from the bacteria cell walls and analysed in the same manner. IgG in the immune serum also bound to LTA, showed a small reaction with PG and showed no evidence of interaction with WTA. This demonstrates that IgG antibodies in serum had been manufactured and produced *in vivo* due to the presence of antigens, lipid S and LTA and can cross react with them both. Furthermore other strains of staphylococci also released lipid S into culture supernatant as detected with IgG in patient serum, suggesting that lipid S is common in this genus and possibly all Gram-positive bacteria. However a larger study on other strains of Gram-positive bacteria would need to be investigated. Also a larger population of patients serum with or without infection would need to be investigated.

Serological tests to diagnose Gram-positive infections utilising lipid S have recently been developed to detect anti-lipid S IgG antibodies in ELISA tests, these have been developed to diagnose catheter related sepsis, prosthetic joint infections and to diagnose endocarditis caused by Gram-positive cocci (Elliott *et al.*, 2000; Connaughton *et al.*, 2001). A test using IgG antibodies directed to lipid S has its disadvantages; they can only determine that the patient has been exposed to *S. epidermidis* and is not conclusive evidence that an infection is currently taking place as many individuals would already have basal levels of antibody to the common antigens of staphylococci (Rafiq *et al.*, 2000). Therefore as a diagnostic tool, titres of IgG could be measured during the course of infection to determine the degree of severity of the infection and assess response to therapy; low titres would indicate no infection or that the infection had resolved and higher titres would indicate that the infection was still occurring and possibly worsening. IgG is the predominant

immunoglobulin class in serum, however IgG is not normally detected in serum until approximately 10 - 14 days after an infection has started. It is not known how IgG binds to lipid S as IgG normally only binds to proteins and not lipids. It is therefore possible that lipid S binds to a protein in serum or alternatively a protein excreted by the bacteria and then in this complex binds to IgG. A development of the serological test utilising IgM instead of IgG would be more beneficial in the early stages of an infection. Lipid antigens generally elicit IgM production and IgM is the first serum antibody produced during an infection and it can be detected within days of an invading bacteria. Another strategy would be to develop humanised anti-lipid S antibodies which could be administered to patients with a Gram-positive infection to bind to lipid S and suppress its antigenic activity. Alternatively analogues of lipid S with no antigenic capabilities could be synthesised and used as competitive antagonists. However, despite compelling evidence of the critical importance of LPS in the pathogenesis of Gram-negative bacterial sepsis the utility of anti-LPS approaches to significantly reduce the mortality rate in human septic shock remains unproven.

The pathogenesis of sepsis involves not only microbial toxins but also activated host inflammatory mediators. Macrophages are the first line of defence to combat infection and large numbers of macrophages are found at sites of infection. The inflammatory response, normally induced by infection or tissue injury, is crucial in controlling and ultimately eliminating infectious agents, as well as in promoting wound healing for restoration of tissue integrity. However, if the immune system is inappropriately stimulated or normal regulation is lost; the inflammatory response can cause injury to host tissues. These uncontrolled inflammatory responses contribute to the pathogenesis of septic shock. Therefore, investigations were performed to detect the stimulatory effects of lipid S and other cell wall components to induce macrophages *in vitro* to release the inflammatory mediators NO, TNF-α, IL-1, IL-6 and superoxide anions.

NO is normally produced in the endothelium by the constitutive isoform of the NO synthase. This physiological production of NO is important for blood pressure regulation and blood flow distribution. Several lines of evidence suggest that a hyperproduction of NO by the inducible form of NO synthase (iNOS) produced by

macrophages has detrimental effects in inflamed tissues, and promotes the inflammatory process which contributes to the hypotension, cardiodepression and vascular hyporeactivity observed in septic shock. Macrophages are important regulatory and effector cells in innate immune reactions and have been found to produce NO via the iNOS pathway. Once iNOS is induced, it produces large amounts of NO for prolonged periods, up to several days.

Lipid S, LTA, PG and LPS but not WTA all stimulated murine macrophages and cell lines to produce significant secretion of NO which increased over time up to 48 hours in cell culture. The amount of nitrite produced was dependent upon the concentration of the bacterial component. In most instances LPS was 100 times more potent than lipid S, however lipid S proved to be the most potent out of the Gram-positive samples tested. In an *in vivo* context the induction of NO stimulated from bacterial components would cause vasodilation and increased vascular permeability seen in septic shock. In non-inflamed healthy tissue iNOS is normally not expressed and is only expressed almost exclusively under inflammatory conditions, therefore using selective inhibitors to iNOS would reduce the production of NO. However due to the structural similarities of the constitutive and inducible NOS isoforms, even the most iNOS-selective of pharmacological agents can have some effect on eNOS, which could then cause additional problems. Results from previous studies of NOS inhibition in animal models are conflicting and there is evidence that this inhibition may be harmful. Therefore prior to iNOS becoming a therapeutic target much work remains to characterize all of the effects of iNOS over the wide range of inflammatory responses which affect the human microvasculature.

Macrophages recognise sugar moieties on the zymosan particles via their mannosyl-fucosyl receptors. An additional coating with C3b or C3bi engages complement receptors CR1and CR3 and enhances the oxidative burst. Lipid S, LTA and PG had no effect on macrophages alone but in the presence of OZ the oxidative burst was heightened compared to a positive control. This increase in burst may be due to increased affinity of the oxidase complex for NADPH. *In vitro* bacterial products will sensitise macrophages in isolation. In an *in vivo* context in addition to the bacterial secretory products, which clearly have direct effects on the macrophage they might also induce other lymphocytes to release cytokines, which could also further

potentiate macrophage activity. Many of the detrimental effects of NO are due to the reaction between NO and superoxide, which results in the formation of peroxynitrite, a highly reactive and cytotoxic molecule. Excessive production of peroxynitrite leads to nitrated proteins, inhibition of mitochondrial respiration, DNA damage, apoptosis and necrosis, resulting in cell and tissue damage (Kim et al., 1998). Possible targets for pharmacological agents would be the intracellular signalling mechanisms that exist for the formation of both NO and superoxides, thus preventing the consequential production of peroxynitrite.

The pro-inflammatory cytokines TNF-α, IL-6 and IL-1 have a wide spectrum of biological activities that help to coordinate the body's responses to infection, however if overproduced they cause extensive tissue damage. The findings reported in this study demonstrated that lipid S, LTA, PG and LPS all promoted the release of TNF-α, IL-1 and IL-6 from macrophages and undoubtedly would induce fever and tissue damage seen in inflammatory diseases. In a clinical situation it is the sum of all these agents that determines the outcome in a patient with a Gram-negative or Gram-positive infection by their direct and indirect effects. Many studies have previously been conducted to block or attenuate cytokine production but have so far been unsuccessful. One practical problem for inhibition of cytokines is that they occur early in an infection; this was evident in the results when after only 1 hour the production of TNF-α was evident and after 2 hours this was doubled. Patients often come to medical attention relatively late in the disease and blocking these cytokines at this stage may be too late. However it may be possible to identify patients that are more susceptible to sepsis such as immunocompromised and burns patients and deliver anti-cytokine therapy as a prevention strategy. Alternatively TNF-α, IL-1 or IL-6 derived from or activated by macrophage cells could be used as markers of an inflammatory response, future work could discover whether these correlate with the severity of inflammation and the course of the disease. Knowledge of the mechanisms involved in the regulation of cytokines is important in the search for potential targets in the treatment of inflammatory diseases such as septic shock.

To eliminate the possibility that the Gram-positive components extracted in this study were not contaminated with sufficient LPS to provoke the secretion of NO and cytokines they were subjected to a sensitive limulus amoebocyte lysate assay which

detects LPS content in samples. The results showed minute traces of LPS, equivalent to controls, which would not be sufficient to induce a stimulatory response, therefore the stimulatory activity is due to the Gram-positive cell wall components and not LPS.

Toll-like receptors have been found to participate in the activation events of the immune system in mammals (Medzhitov *et al.*, 1997; Yang *et al.*, 1998). Activation of TLR's with bacterial components requires prior binding of CD14, this complex then leads to activation of intercellular signalling cascades which results in the activation of transcription factors such as NF-κB. The activation of NF-κB induces the expression of several inflammatory mediators, such as the production of NO and cytokines. Using LPS resistant mice with a defective TLR4 receptor clearly showed that lipid S and LTA do not utilise this receptor as they were not affected by the absence of it. The stimulation of NO production with LPS was more affected, though not completely inhibited, demonstrating that LPS signals via TLR4 but perhaps not exclusively. Although not investigated in this study, it is likely that lipid S and LTA induce the production of NO through TLR2. An understanding of TLRs signalling pathways may reveal a therapeutic target for sepsis.

There is little doubt that excess levels of inflammatory mediators lie behind the clinical manifestations and mortality associated with septic shock. Therefore, any intervention that inhibits the release of NO or cytokines or neutralizes their effects could provide potential targets for drug therapy not only in septic shock but in several diseases associated with excessive production, such as inflammatory bowel disease and rheumatoid arthritis. In this study a number of potential targets were investigated to inhibit inflammatory mediators. Monoclonal antibodies directed to LTA, CD14 and toll-like receptors were utilised, PAF is also a potent inflammatory molecule and therefore PAF antagonists were also investigated. In macrophage cell culture the anti-LTA and the anti-CD14 mAb's failed to markedly attenuate the production of NO, TNF-α, IL-6 or IL-1 with any of the bacterial components tested. However the anti-LTA antibody did detect lipid S and LTA in ELISA and Western blots experiments and could therefore be used to diagnose the presence of these components in patients serum. In contrast the anti-TLR4 antibody did greatly inhibit the ability of LPS to stimulate TNF-α and IL-1 production. A smaller inhibition was observed with lipid S. Therefore targeting TLR using mAbs and in particular TLR4 mAbs can reduce the

production of cytokines and could after extensive human studies be used in Gram-negative sepsis. However it must be noted these were results from a single experiment and should be viewed with care. Further work needs to examine the participation of TLRs and in particular TLR2 and its involvement with Gram-positive bacteria. The PAF antagonist used in this study successfully inhibited the production of NO stimulated by lipid S and LPS, thus showing that these bacterial components not only bind to TLR to produce NO but also to PAF receptors demonstrating at least two receptors are involved in activation of pro-inflammatory mediators. Therefore another route for septic shock therapy would be the use of PAF receptor antagonists, however the toxicity of this compound was not tested and therefore although it greatly inhibited NO secretion future work would need to establish if this was a result of cell damage or receptor blockade.

Extensive studies in animals and humans have indicated that NO, TNF-α, IL-6 and IL-1 are the principal toxic secondary mediators and it seems logical to develop and evaluate molecules that specifically block these cytokines. However previous studies examining anti-TNF-α mAb's have been tested in large clinical studies but they were reported to have no overall benefit. Other future studies could also examine the effects of soluble receptors such as soluble TNF-α receptor which may modify the effects of TNF-α, or the effects of IL-1 could be reduced by using an IL-1 receptor antagonist.

Presently treatment for septic shock involves the use of antibiotics although the emergence of new pathogens and the increase in anti-microbial resistance has led to unsatisfactory therapy. Tetracyclines and other antibiotics have also been reported to modulate production of host inflammatory mediators. In this study antibiotics previously described to have anti-inflammatory properties were investigated. The antibiotics showed various capacities to block the production of NO but there was no evidence of inhibiting cytokine release. The tetracyclines oxytetracycline and minocycline proved to be the most effective compounds though their toxicity needs to be determined whereas other non-tetracyclines showed no inhibitory capabilities. A large group of synthetic tetracyclines with no anti-bacterial activity was then investigated. Many of these compounds were active at low concentrations and showed efficacy to inhibit both lipid S and LPS stimulated macrophages to produce NO. In particular two compounds were exceptionally active at 1μg/ml, the lowest

concentration used. Also the novel tetracyclines demonstrated they worked with all bacterial components tested suggests they potentially would be equally useful in both Gram-positive and Gram-negative infections and could yet prove to be the most effective treatment of septic shock and its complications. Non anti-bacterial tetracyclines could therefore be used as a conjunctive therapeutic option along with the use of anti-bacterials and any surgical interventions.

The results presented in this study must be interpreted with care as mouse macrophages and macrophage-like cell lines were studied *in vitro*, therefore results may not give conclusive information on macrophage activity *in vivo*. In the body they are part of a unique system of cells and signalling molecules and may act considerably differently to bacterial cell wall components when present within a living system. Nevertheless murine macrophages are capable of secreting NO, cytokines and ingesting particulate matter and these *in vitro* circumstances must also occur *in vivo*

It must also be noted that *in vivo* there are many more variables to account for when investigating possible mediators of infection. The immune system is highly sophisticated and complex. It is able to respond in a variety of different ways depending on the pathogen involved and can recruit a variety of different cell types to produce the appropriate response. As a consequence of infection, a complex series of reactions are implemented by the host in an attempt to avert ongoing tissue damage, isolate and destroy the infective organism and activate the repair processes so as to return the organism to normal function as soon as possible. If a heightened response occurs during this process, which results in the unopposed over production of inflammatory mediators then the reaction can result in septic shock, which can have the ultimate unfavourable consequence.

These experiments have shown that lipid S is a potent antigen and is potentially the principal mediator in the onset of a Gram-positive infection. Lipid S is a small diffusible molecule that is released during growth of a Gram-positive organism and would have systemic effects. Recently a group in Germany have synthesised a molecule, which they reported to be the active component within LTA (Deininger *et al.*, 2003). The structure of the molecule they synthesised is exactly the same as the lipid S structure described in this report, which confirms the significance of the

discovery of lipid S. LTA and PG also proved to be provocative agents in inducing the release of NO and cytokines, and evidence here suggests that they may act in synergy. These agents along with lipid S will thus compound the septic shock situation.

Presently two pharmaceutical companies, Biosynexus and Glaxo Smith Kline have entered into partnership to develop and commercialize staphylococcal antibodies and vaccines to prevent and treat infections caused by staphylococcal bacteria (http://www.biosynexus.com/products/index.html). Currently clinical trials of a humanized anti-LTA monoclonal antibody (BSYX- A110) are being conducted for preventing staphylococcal infections in neonates and adults. BSYX-A110 has been reported to bind to all staphylococci and promotes ingestion and killing of the bacteria by white blood cells. In addition, studies have shown that it also binds to enterococci and group A streptococci. The compound is currently in clinical trials in premature babies at multiple sites across the United States. Phase I adult trials met safety and tolerability targets, and also demonstrated outstanding pharmacokinetic and pharmacodynamic capabilities. They also plan to develop a staphylococcal vaccine using Biosynexus antigen. This mAb that binds to LTA to provide protection against staphylococci would also cross react and bind to lipid S and could prove to be a breakthrough in therapy for Gram-positive sepsis.

The production of NO and cytokines seems to be interrelated, that is they appear to work in concert, therefore inhibition of any of the components would have an effect on others. However there are numerous pathways involved in the inflammatory response, some have been described here and unless these are all identified then stopping the inflammatory response could be futile. An antibiotic might, however, be designed to bind lipid S, decrease inflammatory cytokine production, or inhibit the synthesis of NO and superoxides the clinical value of such dual-action antibiotics is largely theoretical. The important potential problem with the clinical use of anti-lipid S or anti-LPS agents is getting such drugs to the patients early enough before the proinflammatory cytokine expression has occurred.

Approaches to treatment fall into three broad categories: strategies directed against bacterial components, those directed against host-derived inflammatory mediators,

and those designed to limit tissue damage. Because septic shock is a dynamic and evolving condition, different strategies may be needed at different stages in the pathogenesis of sepsis. Through carefully performed trials and thoughtful selection of combination therapy aimed at the several different points in the pathological process, it may be possible in the future to modify the course of this serious condition

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