The Interaction Between Lipopolysaccharides and Host Leucocytes

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Declaration

I declare that this thesis has been composed by myself and that the research reported therein has been conducted by myself.

Edinburgh, 27 June 1996

Abdurrazag Urayet

I dedicate my work to all my family

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Abstract

Gram-negative septic shock is a life threatening disease associated with high mortality, particularly in immunocompromised patients. The mechanism by which Gram-negative bacteria induce septic shock is complex, with little direct experimental evidence to pinpoint a single toxic factor. Endotoxin, a lipopolysaccharide (LPS) outer membrane component found in all Gram-negative organisms, has been implicated as a primary mediator of the toxicity associated with Gram-negative infection. When purified from organisms and injected into experimental animals, this substance induces many of the same pathophysiological changes observed during Gram-negative infection. Based on an increasing body of data, it is now felt that many of the symptoms of septic shock are attributable to the action of macrophage-derived mediators released following exposure to LPS.

The aim of this project was to investigate the interaction of various forms of LPS- free, membrane associated and as a component of whole bacteria- with mammalian cells to determine the cell surface molecules involved and to study the spectrum of mediators released.

Initially the interaction of various forms of *Salmonella minnesota* Ra LPS with mouse peritoneal macrophages (MØs) was investigated. Bacteria were labelled with fluorescein isothiocyanate (FITC) and the binding to host cells measured by flow cytometry. The interaction between bacteria and cells was shown to be dose and time dependant, maximal at 37° C and required divalent cations. Varying the pH from 6 to 8 had little effect on the binding. MØs elicited by intraperitoneal injection of thioglycollate bound more bacteria than cells obtained from control animals. To investigate the role of LPS in the interaction of bacteria with MØs, different forms of

LPS were used to try to inhibit binding. Pure free LPS and LPS attached to bacterial outer membranes extracted from *S. minnesota* inhibited the binding of bacteria to MØs but whole unlabelled bacteria (*S. minnesota*) failed to inhibit the binding of labelled bacteria to the MØs. This finding led to a microscopic study of the effect of FITC labelling of bacteria on the binding to MØs. FITC labelling enhanced the binding of bacteria to the MØs as compared to the binding of unlabelled bacteria.

The production of nitric oxide (NO) and other reactive nitrogen intermediates by cytokine-activated rodent cells is an important component of antimicrobial and/or antineoplasic activity of these cells. The pathway involves the oxidation of arginine to citrulline with concomitant release of NO by an inducible form of NO synthase. Although human cells such as hepatocytes and endothelial cells have been shown to express inducible NO synthase, the presence of such a pathway in human monocytes/macrophages has been questioned by many investigators and is the subject of great controversy. Therefore an attempt was made to investigate whether human monocytes/MØs produce NO in response to different forms of LPS. NO production was assayed by measuring nitrite, a stable metabolic product of NO, in macrophage culture supernatants. Preliminary work was performed using MØs from several mice strains and different forms of LPS. An immature human MØ cell line (THP-1) was used to investigate NO production in humans. Free LPS failed to produce any measurable amounts of NO from human cells, but produced small amounts of NO from mouse cells. LPS attached to outer membranes and whole bacteria produced significant amounts of NO from both human and mouse cells. Human peripheral blood monocytes also produced significant amounts of NO when stimulated with whole bacteria and LPS attached to outer membranes.

Macrophages are the principle source of the endotoxin-induced mediator tumour necrosis factor (TNF). This cytokine is pyrogenic and when infused into animals

produces all the clinical and pathological features of septic shock. All forms of LPS used stimulated THP-1 cells to release TNF as measured by a L929 bioassay.

The characteristics of the production of TNF and NO suggest that a novel pathway independent of CD14 and lipopolysaccharide binding protein might be involved.

Contents

Chapter 1

General introduction

The study of endotoxin began at the end of the 19th century when Richard Pfeiffer, a student of Robert Koch, found that lysates of heat-inactivated *Vibrio cholerae* contained a toxic substance which was capable of inducing shock and death in experimental animals. He termed this heat-stable toxin "endotoxin" to distinguish it from the heat-labile exotoxins which were actively secreted by *V. cholerae*. Around the same time two other scientists, Eugenio Centanni and Hans Buchner, independently isolated the same toxin. Centanni made two important contributions. First, he observed that this toxin could be isolated from lysates of many different Gram-negative bacteria, but never from similar preparations of Gram-positive bacteria. Second, he drew attention to the remarkable pyrogenic properties of endotoxin. Buchner was the first to demonstrate the association of endotoxin with leucocytosis and altered host immunity.

In 1935 Boivin and Messrobeanu, using a method of trichloroacetic acid extraction, determined that the endotoxic activity of Gram-negative bacterial lysates resided in a cell wall macromolecular complex of protein, lipid and polysaccharide. Two decades later Westphal and Luderitz commenced their classic studies on the biochemistry of endotoxin. Protein-free lipopolysaccharide (LPS), prepared by phenol-water extraction and purified, possessed all the properties of crude endotoxin. Further separation of LPS into water-soluble polysaccharide fraction led to the finding that the biological activity of the endotoxin resided in the lipid moiety, now termed lipid A (Bayston and Cohen, 1990).

1.1 Lipopolysaccharides

1.1.1 Introduction

Lipopolysaccharides (LPS: endotoxins) are major components of the outer membranes of Gram-negative bacteria (Fig. 1.1) which include human pathogens such as Enterobacteriaceae and Neisseriaceae. These molecules are essential for the physical organisation and function of the membranes of each bacteria and thus for bacterial growth and multiplication. Lipopolysaccharides represent the main surface antigens (O-antigens) of Gram-negative bacteria. They are targets for bacteriophages, they harbour binding sites for antibodies and non-immunoglobulin serum factors and therefore are involved in the specific recognition and elimination of bacteria by the host's defence system. On the other hand LPS may prevent the activation of complement and uptake of bacteria by phagocytes and therefore, play an important role in bacterial virulence. Further, LPSs are endowed with a broad spectrum of biological (endotoxic) activities such as pyrogenicity and lethal toxicity (Rietschel *et al.*, 1990).

Septic shock causes approximately 175,000 deaths every year in the United States. Gram-negative sepsis may account for up to one half of the cases of septic shock. It is a syndrome characterised by refractory hypotension leading to inadequate organ perfusion, multi-organ failure, and, frequently, death. The lipopolysaccharide of Gram-negative bacteria is thought to be responsible for initiating host responses leading to septic shock. LPS is not intrinsically toxic but acts by inducing myeloid and/or non-myeloid cells to express a multiplicity of genes encoding proteins with activities that produce the haemodynamic and haematologic changes observed in septic shock. It is one of the most potent biological response modifiers known; picomolar concentrations are sufficient to stimulate cells of the immune/ inflammatory/vascular systems. A vast amount of information about the molecular mechanisms of host defence responses and the production of inflammatory mediators has been derived from studies using LPS as a stimulus. The mechanisms of LPS-induced cell activation were not well understood until recently. Two major advances have helped to bridge this gap in our knowledge. First was

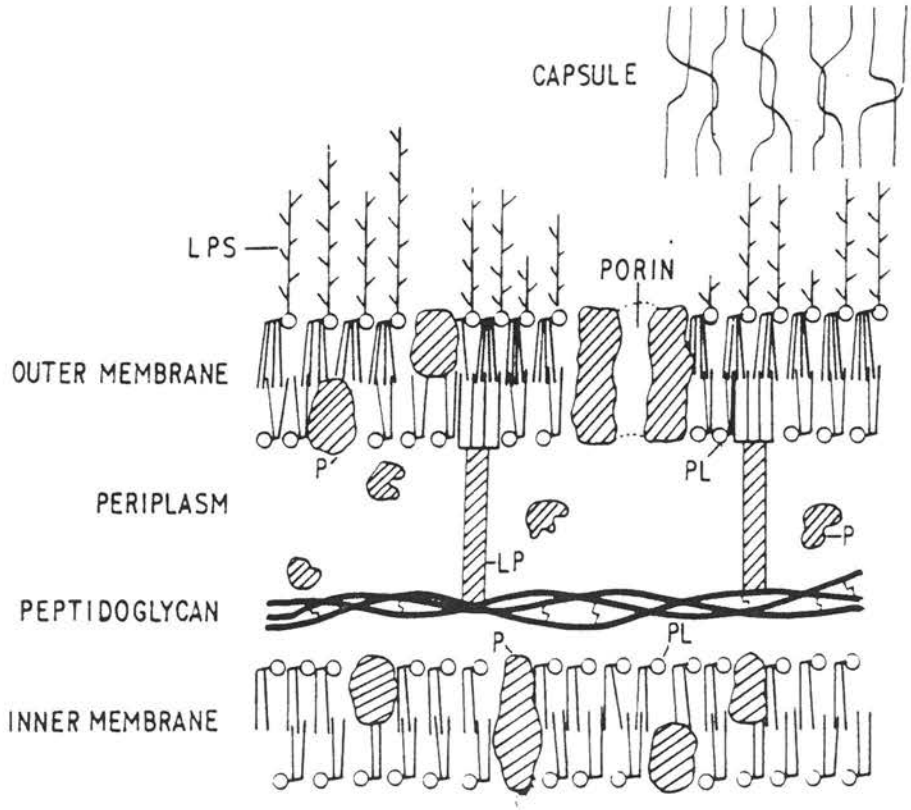

Figure 1.1 The cell envelope of a Gram-negative bacterium. LP: lipoprotein; LPS: lipopolysaccharide; P: protein; PL: phospholipid. (reproduced from Hancock and poxton, 1988)

the discovery of LPS binding protein (LBP), a 60-kDa serum glycoprotein that binds to the lipid A moiety of LPS molecule. Analysis of LBP by cDNA cloning led to the recognition of a structure-function relationship among LBP, bactericidal/permeability-increasing protein (BPI) and other proteins (Ulevitch and Tobias, 1995). This structural relationship between LBP and BPI has led to the proposed use of BPI as a therapeutic agent (Marra *et al.*, 1996). Most importantly, characterisation of LBP function in determining cellular responses to LPS revealed an unanticipated mechanism for LPS-induced cell activation that involves a membrane receptor for LPS-LBP complexes. The second major advance was the identification of this cellular receptor as CD14. CD14 is a 55-kDa glycosyl-phosphatidylinositol (GPI)-anchored membrane protein (mCD14) of myeloid cells; it is also found as a soluble serum protein (sCD14) lacking the GPI-anchor. Although other cell surface proteins have been suggested to be LPS receptors, CD14 is the only protein with fully defined structure that binds LPS and mediates LPS-induced cell activation. A role for mCD14 has been defined in LPS activation of myeloid cells, while sCD14 has been shown to participate in activation of nonmyeloid cell types such as endothelial or epithelial cells that normally do not express mCD14 (Ulevitch and Tobias, 1995).

The pathogenesis of septic shock is complex and is attributable to both microbial and host factors. Experimental and clinical data have shown that the pro-inflammatory cytokines TNF-α and IL-1 are important mediators of severe sepsis. TNF-α is present in the systemic circulation after the administration of live or heat-killed bacteria or endotoxin. Administration of TNF-α reproduces many of the physiologic changes associated with severe sepsis, and antibodies against TNF-α have a protective effect in animal models of severe sepsis. The effects of TNF-α are mediated through cell surface TNF receptors. The extracellular portions of the receptors are shed in vivo and then bind circulating TNF-α, thereby blocking its bioavailability. The presence of TNF-α and its ratio to soluble TNF receptors in plasma are correlated with mortality from sepsis (Fisher *et al.*, 1996).

Hypotension and hypoxaemia are pivotal in the pathogenesis of multi-organ failure. Within the lung, cytokine-induced changes can lead to profound hypoxaemia and respiratory failure. TNF-α, IL-8 and C5a all contribute to neutrophil chemotaxis, whilst the upregulation of endothelial cell adhesion molecules and neutrophil integrins facilitates the passage of leucocytes from the circulation into the interstitium and alveolar spaces of the lung. Acute inflammatory changes occur as a result of abnormal leucocytosis and neutrophil degranulation leading to acute respiratory distress syndrome (ARDS). Within the peripheral vasculature, TNF-α, IL-1 and IFN-γ can synergistically trigger the production of nitric oxide (NO) (Sriskandan and Cohen, 1995). Recent studies have linked NO to the development of hypotension after endotoxaemia and bacteraemia. Inhibitors of NO synthase (NOS), such as N^G-methyl-L-arginine (NMA), can partially prevent or reverse hypotension secondary to endotoxaemia. Furthermore, hypotension induced by TNF can be reversed by inhibiting NOS, suggesting that both endotoxin and TNF induce hypotension through NO-dependent pathway (Fahey *et al.*, 1996).

Bacterial LPS, which is present within the circulation or tissues of the human or animal host, is more usually referred to as endotoxin. In this study both terms (LPS/ endotoxin) were used synonymously.

1.1.2 Function of LPS in bacteria

LPS forms a hydrophobic barrier which restricts the entry of noxious substances such as bile salts, digestive enzymes and certain antibiotics and enables the bacterium to evade many host defence factors including complement, lysozyme and cationic proteins. The surface of many unicellular organisms is usually covered with a carbohydrate layer. In organisms lacking capsules, the O-side chains of LPS form this layer. One important function of the cell surface carbohydrate is to increase hydrophilicity of the cell surface. This increase of hydrophilicity is apparently crucial in enabling cells to escape phagocytosis (Cunningham *et al.*, 1975). Avoiding phagocytosis is necessary for survival of pathogens of animals such as *Salmonella* ssp.

The O-side chains might also mask cell surface components, such as lipid A, which is capable of activation of the classical complement pathway in the absence of specific antibody (Morrison and Kline 1977). O-side chains from *E. coli.* OIII and *Salmonella* species bind complement, but resistance to lysis results from the C3b component being bound preferentially by longer polysaccharide chains at a distance sufficient to prevent the insertion of the terminal membrane attack complex (C5b-9) into the bacterial outer membrane (Joiner *et al.,* 1986; Grossman *et al.,* 1987). Both the length of the O-side chains and the coverage of lipid A core appear to be important in this respect (Goldman *et al.,* 1984).

The structure of the exposed LPS (i.e. the O-side chain) often shows a tremendous diversity. The attachment of specific antibodies to bacterial surfaces enhances phagocytosis by promoting attachment to the Fc and complement receptors on the phagocyte surface (Silverstein *et al.*, 1979), and antibodies will also activate complement leading to increased phagocytosis and lysis of bacteria and stimulation of an inflammatory response and by decreasing the hydrophilicity of the bacterial surface (van Oss and Gillman, 1977). The diversity within the O-side chains is advantageous to bacteria since when each different O-antigenic type enters a host for the first time there will be no immunological memory to the organism. This will give a significant selective advantage to pathogenic bacteria, and the diversity is pronounced in animal symbionts and pathogens (Nikaido and Nake, 1979).

1.1.3 Structure of LPS

Chemically LPS consists of two parts of contrasting chemical and physical properties; a hydrophilic polysaccharide and a hydrophobic lipid, termed lipid A. The polysaccharide can be subdivided into the O-specific chain (O-side chain) and the core oligosaccharide. Several bacterial mutant strains have been isolated in which the O-chain and part of the core oligosaccharide are missing as a result of a defect in the biosynthesis of the core region. Because of their colonial morphology such mutant strains are called rough (in contrast to the smooth colonies of bacteria possessing a full O-side chain). Depending

on the site of the defect, these mutant strains in *Salmonella* spp and *E. coli* are labelled Ra-Re (Fig. 1.2). Some bacteria produce naturally rough LPS. e.g. *Neisseria* spp.

1.1.3.1 O-specific chain

The O-specific chain constitutes a polymer of repeating oligosaccharide units which contain up to eight different, or in some cases identical, sugar residues which are generally interlinked by glycosidic bonds (Lüderitz *et al.,* 1982). A large diversity of the constituent sugar components of repeating units has been revealed within different enterobacterial serotypes. The nature, ring form, anomeric configuration and type of substitution of the individual monosaccharide residues, as well as their sequence within a repeating unit, is characteristic and unique for a given LPS and the parental bacterial strain, i.e. a bacterial serotype or species. Because of the diversity of constituents and their linkages, an enormous number of structures of specific chains is conceivable and verified in nature. Therefore, an immense structural variability is revealed if the O-specific chains of different bacterial species are compared. Even within a particular individual clone of bacteria, variation in O-side chains is found. When LPS samples from a specific organism are studied by polyacrylamide gel electrophoresis, the number of repeating units may vary from 0 (= unsubstituted core) to approximately 50 (Hitchcock *et al.*, 1986). The fact that in a given LPS preparation a family of molecules exists which differ in the presence or absence of O-specific chains and the degree of polymerisation of repeating units is one of the reasons for the intrinsic heterogeneity of LPS (Nowotny, 1984).

A variety of nonenterobacterial wild-type strains of pathogenic Gram-negative bacteria such as *Acinetobacter, Bordetella, Campylobacter, Neisseria, Haemophilus* and *Chlamydia* (Fig. 1.3) form LPS which lacks O-specific chains with repeating units (Griffis *et al.,* 1988). In their general architecture and behaviour in polyacrylamide gel electrophoresis these LPSs resemble those of Enterobacterial rough-(R)-mutants. Although in their fine structure (notably of the outer oligosaccharide region) they are clearly different.

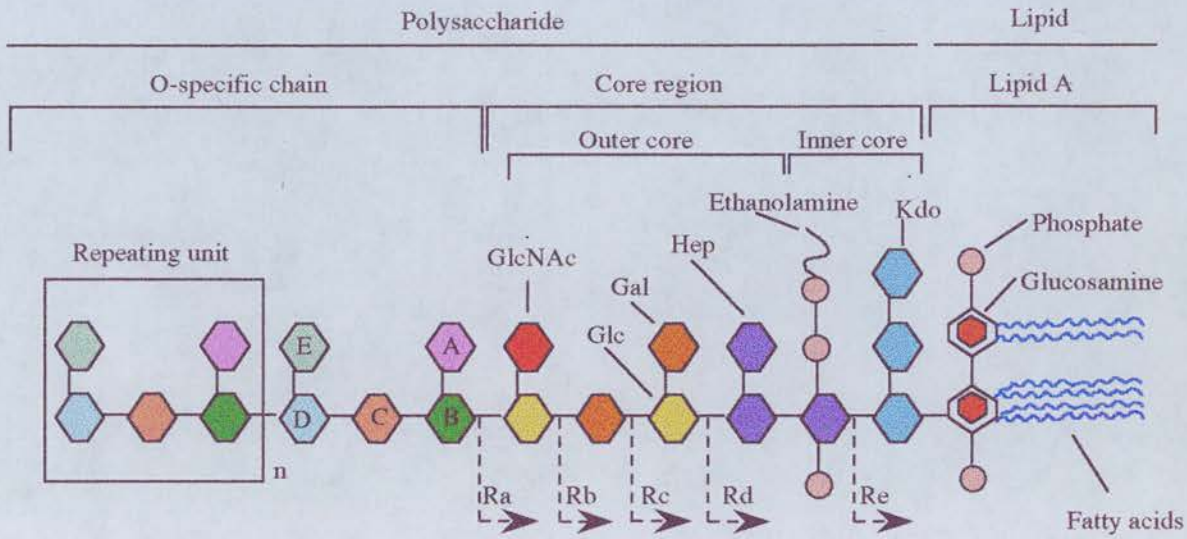

Fig. 1. 2 Chemical structure of *Salmonella typhimurium* LPS showing: A-E sugar residues; Glc, D-glucose; Gal, D-galactose; GlcNAc, *N*-acetyl-D-glucosamine; Hep, L-glycero-D-manno heptose; KDO, 2-keto-3-deoxyoctonic acid. Ra-Re are incomplete R-form lipopolysaccharide. (Reproduced with modification from Rietschel and Brade, 1992)

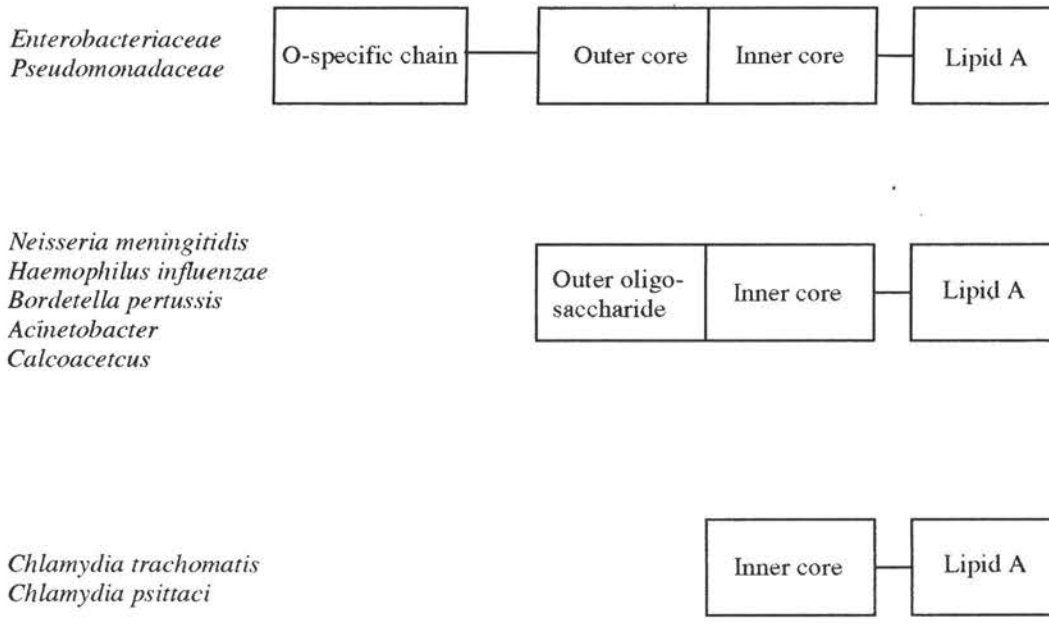

Fig. 1.3 Schematic presentation of lipopolysaccharide of pathogenic Gram-negative bacteria. (reproduced with modification from Rietschel *et al*, 1993)

1.1.3.2 Core oligosaccharide

The core of enterobacterial LPS consists of a hetero-oligosaccharide which can be formally subdivided into the O-chain proximal (outer core) and the lipid A proximal (inner core) region. The structure variability of the core within the various species of an enterobacterial genus is limited. In *Salmonella* only one core type (Ra core) exists for all serotypes and in *Escherichia coli* so far five core types (R1- R4 and K 12) have been described for over one hundred different serotypes (Jansson *et al.*, 1981). Structural differences between these core types are mainly recognised in the outer region.

The outer core of enterobacterial LPS contains the common hexoses D-glucose (Glc), D-galactose (Gal) and N-acetyl-D-glucosamine (GlcNAc) and N-acetyl-galactosamine (GalNAc) in the pyranosidic ring form. The outer core is, therefore, also termed the hexose region (Fig 1.4 a). In the case of *Salmonella* and *E. coli*, the outer core mostly consists of a branched pentasaccharide which carries Glc (Hex I) at the reducing position (Fig 1.4 b). Through this Glc residue the outer core is α (1-3)- linked to the inner core region. The outer core functions as a receptor for core-specific bacteriophages and it harbours those structures which determine the serological R-specificities. Furthermore, the outer core, probably through Gal residues, mediates binding of LPS or bacteria to lectin-like receptors of activated T-lymphocytes. It has been suggested that this interaction plays a role in the differentiation of T-helper cells or the induction of suppressor cells (Lehmann *et al.*, 1980), and the removal of bacteria by lymphocytes (Jirillo *et al.*, 1990).

The inner core region of enterobacterial LPS is characterised by the unusual sugars, heptose (Hep), mainly in the L-*glycero*-D-*manno* form though occasionally in the D-*glycero*-D-*manno* configuration, and notably 3-deoxy-D-*manno*-octulosonic acid (2-keto-3-deoxyoctonic acid, KDO). LPS of *Enterobacteriacae* and the majority of other bacteria studied (including those which lack the O-specific chain) contain heptose whereas all LPS, independent of their bacterial origin, harbour at least one α-bound pyranosidic or furanosidic KDO residue (or a derivative) with free carboxyl group. The latter residue occupies the lipid A-proximal and reducing position of the inner core region (Holst and

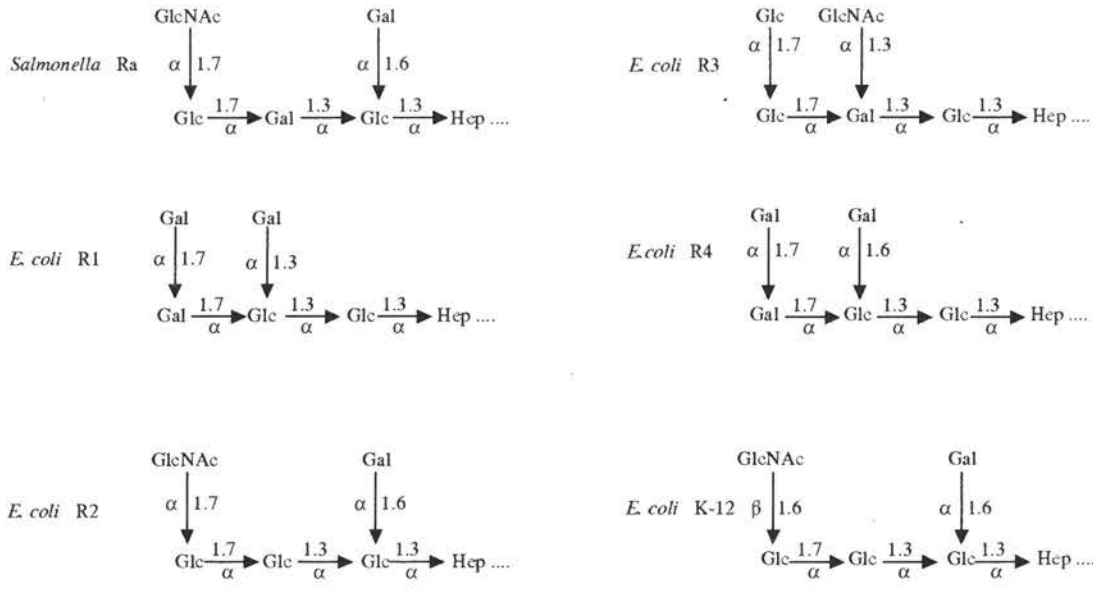

Fig. 1.4 a

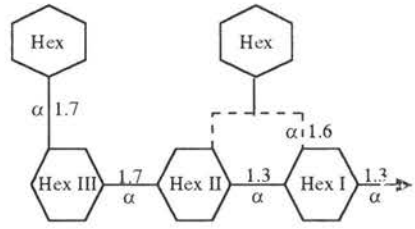

Fig. 1.4 b

Figure 1.4 Structure of the hexose region of *Salmonella* and *E. coli* core types. In *E. coli* K-12, GlcNAc is a β(1-6)-linked to Hexose III (a). A common linear hexose trisaccharide is recognised in the outer core of *Salmonella* and *E. coli* with Glc (Hex I) at the reducing end (b). (Reproduced from Rietschel *et al*, 1990)

Brade, 1992). In this respect the inner core region is structurally rather conserved. Figure 1.5 shows the proposed chemical structure of the inner core region of salmonella LPS.

In recent years, the KDO-containing inner core has been recognised to be of great significance for the biological activity and function of LPS_t. Bacteria with a defect in KDO biosynthesis are not viable showing that KDO (and LPS in general) is essential for growth and multiplication (Osborn, 1979).

1.1.3.3 Lipid A

Lipid A constitutes the covalently-linked lipid component of LPS. Enzymes which cleave the polysaccharide-lipid bond are not known and therefore, polysaccharide-deprived free lipid A can only be prepared by chemical degradation (e.g. by acid catalysed hydrolysis of LPS). The ketosidic linkage between KDO and lipid A is particularly acid labile and therefore, free lipid A can be prepared by treatment of LPS with mild acids (Rietschel *et al.*, 1993). The application of mild organic or mineral acids may cause some degradation and a certain heterogeneity of free lipid A (Takayama and Qureshi, 1992). Lipid A exhibits also intrinsic heterogeneity which is due to the presence of partial structures resulting from incomplete biosynthesis (Nowotny, 1984). Despite this, the primary structure of enterobacterial and some nonenterobacterial lipid As has been elucidated in great detail (Takayama and Qureshi, 1992). These structures were, in general, analysed by using R-form LPS. Figure 1.6 shows the primary structures of the predominant components of *E.coli* and *Salmonella minnesota* lipid A (Rietschel *et al.*, 1984). In both cases lipid A is composed of a β-D-glucosaminyl-(1-6)-α-D-glucosamine disaccharide which carries two phosphoryl groups, one in position 4' (of the distal glucosaminyl residue, GlcN II) and one in position 1 (of the reducing glucosaminyl residue, GlcN I). This hydrophilic lipid A backbone is, in both cases, acylated by four primary acyl groups i.e., four (R)-3-hydroxytetradecanoic acid residues [14:0(3-OH)] at positions 2, 3, 2' and 3'. As a further common feature, both lipid A molecules contain two unsubstituted hydroxyl groups at position 4 and 6'. The latter primary hydroxyl group is only free in polysaccharide-deprived free lipid A since in LPS it represents the attachment site of

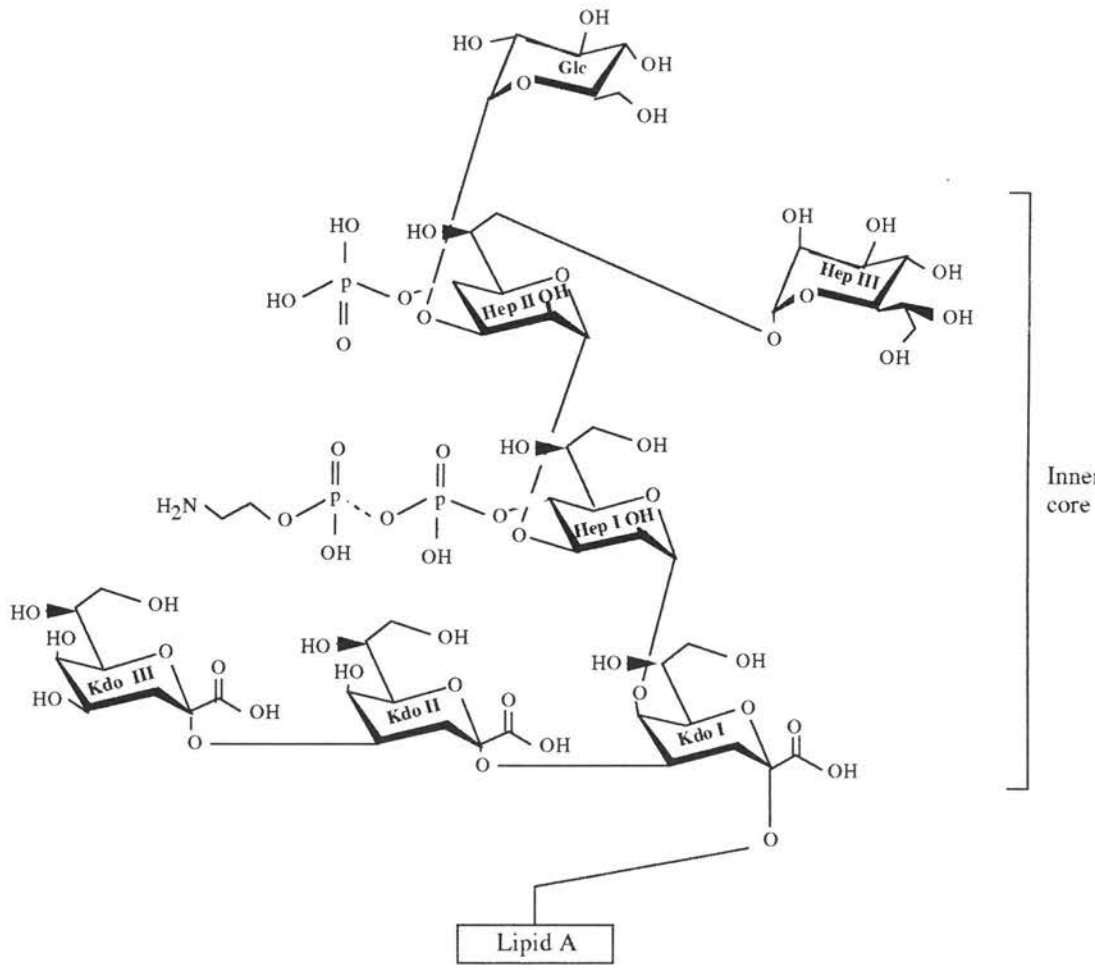

Figure 1.5 Chemical structure of the inner core of *Salmonella* LPS. Dashed lines indicate nonstoichiometric substitution. (Reproduced with modification from Rietschel *et al*, 1993)

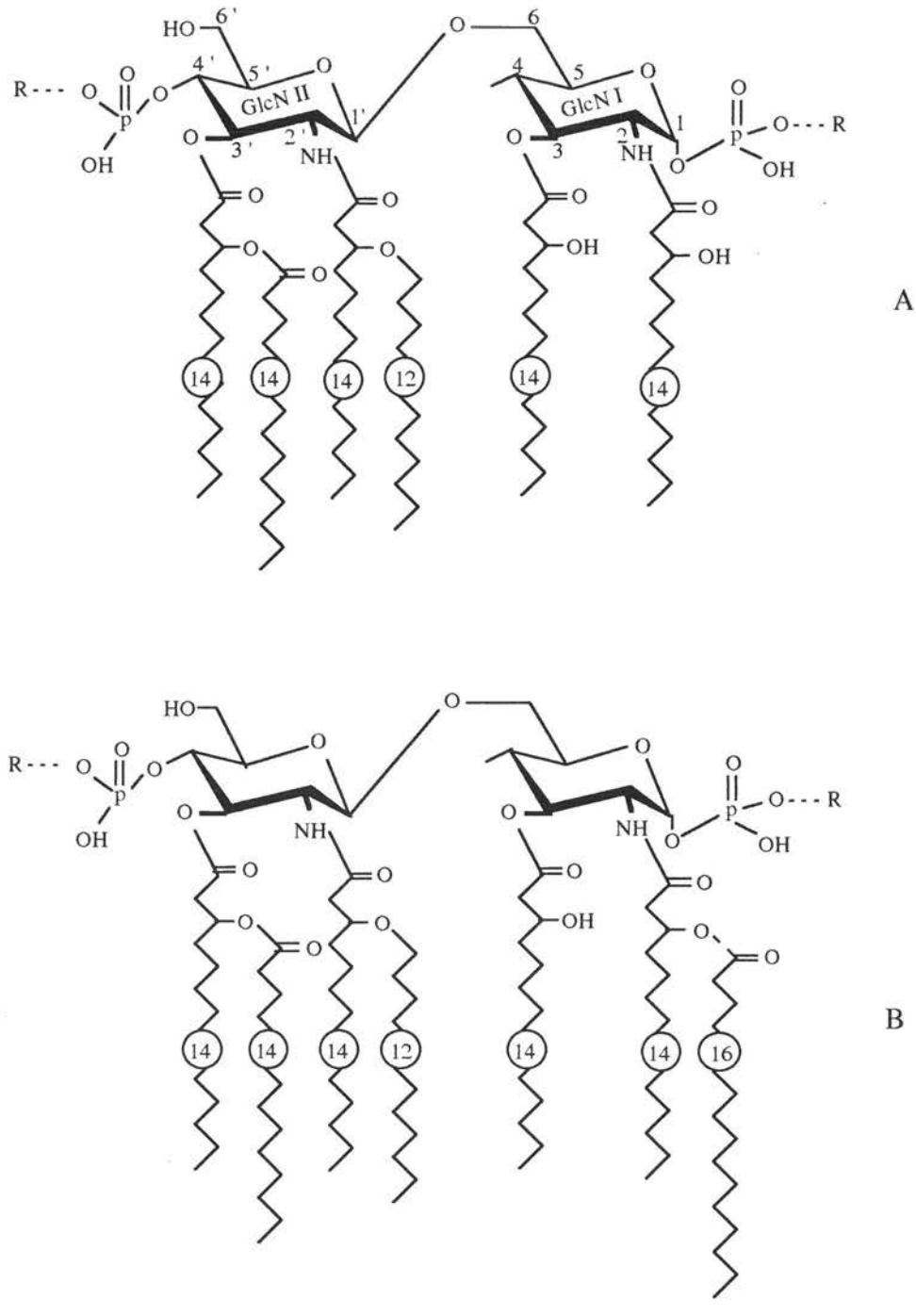

Figure 1.6 Chemical structure of the lipid A component of *E. coli* (A) and *Salmonella minnesota* (B). The hydroxyl group in position 6' (GlcN II) represents the attachment site of KDO. Numbers in circles refer to the number of carbon atoms in acyl chains. Dashed lines indicate nonstoichiometric substitution. (Reproduced from Rietschel *et al*, 1993)

KDO i.e. of the polysaccharide component (Fig. 1.6). In both *E. coli* and *S. minnesota* lipid A, the hydroxyl groups of the two 14-0(3-OH) residues bound to GlcN II (position 2' and 3') carry, as secondary acyl groups, dodecanoic (12:0) and tetradecanoic acid (14:0), respectively. (In *Salmonella*, the latter is some times present as *(S)*-2-hydroxytetradecanoic acid). In *E. coli*, the two 14:0(3-OH) residues at GlcN I are not 3-O acylated whereas in *S. minnesota* the amide-linked 14:0(3-OH) of GlcN I is either not acylated or carries hexadecanoic acid (16:0) (as indicated by the dashed line in Figure 1.6). Therefore, in *S. minnesota* lipid A two molecular species are recognised, differing in the substitution of 14:0(3-OH) at GlcN I and therefore, in the number of acyl groups present. Obviously, one of these species structurally corresponds to hexaacyl *E. coli* Lipid A. In addition to the main structures shown in Figure 1.6 these lipid A preparations also contain molecules which possess the phosphorylated glucosamine backbone but a smaller number of acyl groups (Qureshi *et al.*, 1985). Such acyl-deficient structures are particularly found in enterobacterial S-form LPS (Jiao *et al.*, 1989). The phosphoryl residues in position 1 and 4' may be substituted (residue R in Fig. 1.6), and phosphate (*E. coli*), 2-aminoethyl(pyro)phosphate and 4-amino-4-deoxy-L-arabinopyranose (*S. minnesota*) have been identified as phosphate substituents. These substituents are not present in stoichiometric amounts which represents a further facet of the intrinsic heterogeneity of lipid A. The same structure as shown for *E. coli* lipid A has been identified in *S. typhimurium* (Takayama *et al.*, 1984) and this type of lipid A is also present in other enterobacterial (Rietschel *et al.*, 1984), and nonenterobacterial genera such as *Haemophilis* (Helander *et al.*, 1988). Both hexaacyl *E. coli* lipid A and heptaacyl *S. minnesota* lipid A have been chemically synthesised (Galanos *et al.*, 1986) and shown to be identical in all chemical and physical properties to their purified bacterial counterpart.

1.2 Sources of endotoxin

Endotoxin is not detectable in the circulation in healthy individuals. The most obvious association is with Gram-negative septicaemia. Gram-negative bacilli in culture will

liberate endotoxin into the supernate to varying degrees; in the case of meningococci, it appears that the extent of endotoxin release correlates with virulence (Anderson and Solberg, 1984). Spontaneous endotoxin release may well occur *in vivo* during bacteraemia, perhaps reflecting cell division and multiplication. Endotoxin might also seep into the circulation from an enclosed abscess, even if bacteraemia is absent. An additional major source of endotoxin is the gut, and it is clear that damage to the mucosal barrier might lead to endotoxaemia independently of bacteraemia (van Deventer *et al.*, 1988). Several investigators have reported a high incidence of portal endotoxaemia, suggestive of endotoxin transport (translocation) through the intestinal mucosal barrier. However, patient groups investigated comprised patients with gastrointestinal ulcers, jaundice (Prytz *et al.*, 1976), liver disease and bowel disease (Jacob *et al.*, 1977). These studies therefore do not provide conclusive evidence for endotoxin uptake through an intact intestinal mucosa. In a well designed study using a sensitive chromogenic Limulus assay and excluding patients with bowel disease revealed no detectable endotoxin in 17 preoperatively collected blood samples (Brearly *et al.*, 1985). Hence, it is unlikely that significant portal endotoxaemia is physiological in humans without bowel and liver disease.

Another interesting possibility is that endotoxin might be released into the circulation as a result of bacterial lysis, either complement-mediated or as a result of antibiotic administration. Since most bacteraemic strains of Gram-negative bacteria are serum resistant (Roantree and Rantz 1960), it must be assumed that antibody and complement-mediated killing is not an important source of endotoxin in septicaemia. It is more difficult to judge the potential importance of antibiotic-mediated release of endotoxin. Clinical observations dating back to the 1940s had led to the suggestion that the sudden lysis of bacteria, when first exposed to antibiotic, might cause endotoxin release and symptoms of septic shock. Studies have shown that *in vitro* exposure of bacteria to certain classes of bactericidal antibiotics causes significant amounts of endotoxin to be released promptly into the supernate (Cohen and McConnell, 1986; David *et al.*, 1991). What was more difficult and is of greater importance is to try and establish if this

phenomenon is of significance *in vivo.* Animal studies have produced conflicting results. In a rabbit model of *E. coli* sepsis, the use of gentamicin had increased the circulating free endotoxin in animals receiving the antibiotic up to 2000 fold as compared to control animals receiving placebo. However the study does not provide evidence pertinent to the biological significance of antibiotic induced endotoxin liberation (Shenep and Mogan 1984). In contrast, other investigators reported a study in piglets in which gentamicin -induced endotoxin release was clearly associated with adverse effects on cardiac output and pulmonary-artery pressures (Rokke *et al.,* 1987). In both studies gentamicin was used and the contrasting results of these studies might be because of the use of different animal models.

Pure LPS can only be considered a laboratory artefact as when it is released from the bacterium it will always be associated with other outer membrane components. Once in the host it is normally complexed immediately with host proteins and lipoproteins. Only in meningococcal sepsis, where there is an overwhelming mass of endotoxin present, does the lipooligosaccharide (LOS) appear to be present as micelles or aggregates free from host molecules (Brandtzaeg *et al.,* 1992).

The physical state of the LPS used in the laboratory also has important consequences. Neutralisation of acidic LPS obtained by electrodialysis with a base yields the corresponding uniform salt of the LPS (Galanos and Lüderitz, 1975). Different salts of a given LPS exhibit characteristic differences in their solubility in distilled water and physiological buffers such as phosphate buffered saline and subsequent different biological activities in vivo and in vitro.

1.3 Clinical Association

Endotoxins have been implicated in the pathogenesis of a variety of different clinical disorders such as Gram-negative septic shock (Morrison and Ryan, 1987), liver disease [fulminant hepatic failure (Liehr, 1982), cirrhosis (Clemente *et al.,* 1977) and renal failure in obstructive jaundice (Bailey, 1976)], inflammatory bowel disease (Wellmann *et al.,*

1984), acute renal failure (Wardle, 1975), glomerulonephritis (Tomosugi *et al.*, 1989), adult respiratory-distress syndrome (Brigham and Meyrick, 1986), major abdominal trauma (Woodruff *et al.*, 1973), neonatal necrotising enterocolitis (Scheifele *et al.*, 1985), radiation injury (Maxwell *et al.*, 1986), graft-*versus*-host disease (Moore *et al.*, 1987) and toxic shock syndrome (Stone and Schlievert, 1987). Of these Gram-negative septic shock is the most familiar, and is the setting in which the role of endotoxin is most clearly established (Morrison and Ryan, 1987).

Advances in medical therapy, and increased use of invasive medical procedures and devices are factors contributing to a growing population of chronically ill, immunocompromised and seriously ill patients at increased risk for sepsis. Sepsis can occur as a primary disease process resulting from bacterial infection of the mammalian bloodstream or secondarily complicate other disease states. Gram-negative bacillary sepsis is a life-threatening infection associated with high mortality, particularly in immunocompromised patients such as those with malignancy, trauma or AIDS. It is estimated that in the United States alone more than 90 000 deaths per year are secondary to Gram-negative sepsis. It is of interest that other organisms including Gram-positive bacteria, spirochaetes, rickettsiae, mycoplasmas, parasites, fungi and viruses may also cause a shock-like syndrome indistinguishable from that induced by Gram-negative bacteria. Recent evidence indicates that, whilst endotoxin is perhaps the most potent stimulus to mediator production, factors derived from other organisms may also do so (Bate *et al.*, 1988 ; Jupin *et al.*, 1988).

The mortality associated with Gram-negative septic shock is high. Estimated figures varying from 31 to 55 percent according to three independent studies carried out in the United States of America (31 % -Bone *et al.*, 1987), in the United kingdom (49 % -Ispahani *et al.*, 1987) and in a Swiss-Dutch study (55 % -Calandra *et al.*, 1988). Several factors have been shown to influence the mortality rates; *e.g.* age, underlying disease and the appropriate use of antibiotics (Kreger *et al.*, 1980a). Septic shock is a syndrome characterised by hypotension, oligurea, hypoxia, acidosis, the development of

microvascular abnormalities and disseminated intravascular coagulation (Hamill and Maki, 1986). Multiple organ failure is a common sequel. Studies at necropsy reveal widespread tissue damage with particular involvement of the liver, lungs, kidneys and adrenal glands. Tissue lesions include oedema, haemorrhage, inflammatory infiltrates, fibrin thrombi and areas of tissue necrosis. Identical physiological and pathological changes may be seen in experimental animals receiving lethal doses of endotoxin (Bayston and Cohen, 1990).

1.4 Septic shock pathogenesis

Much published data based on *in-vitro* and *in-vivo* studies testify to the biological havoc that endotoxin can cause. It exerts a profound influence on the formed elements of the blood and, in particular, on the coagulation system. Endotoxin causes metabolic and pharmacologic effects which account in large part for the pathophysiological changes seen in shocked patients, and is a powerful immunostimulant with the ability to influence both cellular and humoral limbs of the immune response (Rapson, 1988).

Much, if not all, of the toxicity of endotoxins is brought about by a series of mediators rather than by endotoxin itself. These molecules all play important roles in the normal host response to insult. Their controlled production leads to eradication of foreign material and tissue repair; however, their inappropriate or over production can have important consequences for the host. Several of these mediators have been recognised for some years; the anaphylatoxins C3a and C5a, arachidonic acid derivatives, reactive oxygen intermediates, endorphins, coagulation factors and platelet activating factor. Other more recently described procoagulant molecules, such as tissue factor and intracellular adhesion molecule-1 (ICAM-1), probably also contribute to the development of shock and, in particular, disseminated intavascular coagulation. What has caused most interest in the field of endotoxin research in recent years is the discovery of the role of cytokines in septic shock. Tumour necrosis factor α (TNF α), interferon γ (IFN- γ), interleukin 1 (IL-1), interleukin 2 (IL-2), and more recently interleukin 6 (IL-6) have all been incriminated. Of these, TNF has received most attention.

The alternative complement pathway can be activated by LPS and Gram-positive cell wall components. The classical pathway is mainly activated by certain classes of antibodies; however, certain bacterial cell wall components including LPS are active in this respect. The anaphylatoxins C3a and C5a that result from activation of these pathways are responsible for a series of inflammatory events that have been implicated in the pathophysiology of septic shock. These events include vasodilation and increased vascular permeability, which may be partly responsible for haemodynamic changes, platelet aggregation, and aggregation and activation of granulocytes. All these processes have also been implicated in the pathogenesis of the adult respiratory-distress syndrome (Jacobs, 1981). An important effect of the stimulation of complement system is the activation of neutrophils. The subsequent release of arachidonic acid derivatives, cytotoxic products of molecular oxygen and lysosomal enzymes produces additional local vasoactive effects on the microvasculature. This in addition to endothelial cell cytotoxicity results in capillary leakage. Increased concentrations of complement have been associated with fatal outcome in septic shock of Gram-positive and Gram-negative origin (Hack *et al.,* 1989).

It is well known that the derivatives of arachidonic acid metabolism that cause vasodilation, platelet aggregation, and neutrophil activation may contribute to the pathogenesis of septic shock. Such derivatives are found in increased concentrations after experimental endotoxin challenge and in humans with septic shock (Reines *et al.,* 1981). Activated neutrophils, a key element in the inflammatory response, probably play an important part in the pathogenesis of septic shock in that they contribute to vascular and tissue injuries. Activated leucocytes adhere to each other, to endothelial cells, and to tissues through interactions of receptors (on endothelial cells) and ligands (on inflammatory cells) that are mediated by specific adhesion molecules. The adhesion process is essential for most functions of leucocytes such as chemotaxis, phagocytosis and cytotoxicity (Springer, 1990).

Factor XII (Hageman factor) of the coagulation cascade has long been known to have a

central role in the pathogenesis of septic shock. It is activated by peptidoglycan residues and teichoic acid from the cell wall of Gram-positive organisms as efficiently as by LPS and lipid A from Gram-negative bacilli (Springer *et al.*, 1983). Activated factor XII not only triggers the intrinsic coagulation pathway (through activation of factor XI) but also stimulates endothelial cells and macrophages to produce tissue factor, which in turn activates the extrinsic coagulation pathway. The activation of these pathways may lead to consumption of coagulation factors and to disseminated intravascular coagulation (DIC). Tissue factor produced upon stimulation of macrophages and endothelial cells by LPS has been shown to have a major role in inducing DIC, since anti-tissue factor antibodies prevented LPS-induced DIC in rabbits (Warr *et al.*, 1990). Tumour necrosis factor (TNF) is also an activator of the extrinsic pathway of coagulation, and, therefore, may contribute to the perturbation of coagulation in septic shock (van der Poll *et al.*, 1990).

In addition to being induced by activation of complement and the arachidonic acid cascade, hypotension in septic shock may also result from LPS-activated factor XII that converts prekallikrein into kallikrein. Kallikrein in turn cleaves high molecular weight kininogen to release bradykinin, a potent hypotensive agent (Colman, 1989). Hypotension also results from release of another potent vasodilator, endothelium-derived relaxing factor, recently identified as nitric oxide (Palmer *et al.*, 1987). Generation of nitric oxide occurs in macrophages and in cultured endothelial cells. While it appears that LPS-induced nitric oxide release by macrophages takes several hours, endothelial cells react within minutes, a phenomenon that might contribute to the rapid fall in blood pressure associated with endotoxic shock (Vane *et al.*, 1990).

Endogenous opioid peptides might play a part in septic shock because opioid peptide secretion can be induced by LPS. In addition the administration of an opioid antagonist, naloxone, reverses LPS-induced hypotension under some experimental conditions. However, the importance of endorphins in the pathophysiology of shock is still incompletely understood (Hackshaw *et al.*, 1990).

Platelet activating factor (PAF) is produced by many cell types, including neutrophils,

basophils, mononuclear phagocytes, platelets and endothelial cells. It has diverse biological activities including platelet aggregation and secretion, degranulation of neutrophils, smooth muscle contraction, increased vascular permeability, hypotension and can cause death. It plays an important role in anaphylactic shock and might play a role in endotoxic shock as well (Morrison and Ryan, 1987). Intravenous LPS infusion into rats results in hypotension within five minutes and detectable levels of PAF in the circulation in less than ten minutes (Doebber *et al.*, 1985). The direct effects of PAF on endothelial cells are very likely of great clinical significance. PAF induces the margination of neutrophils on to endothelial cells. Many of the effects of PAF may stem from its ability to influence calcium fluxes and thus modulate endothelial cell function (Bussolino *et al.*, 1985). There may also be more complex interactions between endotoxin-stimulated PAF and other mediators of inflammation. Interleukin-1 (IL-1) is a potent stimulator of PAF from human endothelial cells. This stimulation occurs following activation of a membrane-localized acetyltransferase, an enzyme utilized in the generation of biologically active PAF (Bussolino *et al.*, 1986).

A group of molecules known as colony stimulating factors (CSFs) are a collection of glycoproteins that regulate the proliferation of bone marrow-derived precursor cells into functionally active cells and modify the effector functions of the mature cells. Four separate CSFs have been described in mice (Metcalf, 1986), each of which is both functionally and biochemically distinct. Granulocyte macrophage-colony stimulating factor (GM-CSF) stimulates the proliferation and differentiation of both granulocytes and monocytes. Some of the major effects of CSF on macrophages and monocytes include stimulation of phagocytic activity, synthesis of prostaglandins, secretion of proteases and expression of cytolytic activity. It is, therefore, of relevance that bacterial endotoxins are potent inducers of CSF from both macrophages and B lymphocytes. An indirect mechanism for endotoxin induced CSF production has also been described in which endotoxin stimulated monocytes cause T lymphocytes, endothelial cells and fibroblasts to produce GM-CSF (McCall and Bagby, 1985). The stimulating factor has been identified as IL-1 in the fibroblast system (Zucali *et al.*, 1986). In addition, endotoxins enhance the

capacity of CSF to induce colony formation in cultures of macrophage progenitor cells (Moore *et al.*, 1986). Since endotoxins can also induce the formation of prostaglandins in these cells, this enhancement is subject to potential feedback inhibition. Thus there are both direct and indirect means for endotoxins to induce the production of CSFs and, therefore, the diverse biological activities associated with these glycoproteins.

Tumour necrosis factor (TNF) was orginally characterised as a factor that could be detected in the serum of BCG-pretreated experimental animals shortly after challenge with endotoxin, and that could cause lysis of some tumour cell lines (Old, 1985). It is now generally accepted that TNF may be produced by endotoxin-stimulated macrophages in the absence of additional external stimuli (Beutler *et al.*, 1985a). It has been established that TNF is identical to the modulator protein cachectin, which has also been shown to be produced by mononuclear cells in responce to endotoxin (Beutler *et al.*, 1985b). Cachectin was orginally recognised for its potent capacity to inhibit lipoprotein lipase (Beutler *et al.*, 1985a). The spectrum of biological and immunoregulatory activities ascribed to TNF/cachectin molecule is impressive and is likely to continue to grow.

TNF is synthesized and secreted very rapidly by macrophages in response to endotoxin and may be detected in the circulation within one hour of experimental animals being given endotoxin. Following synthesis, TNF becomes widely distributed in the tissues and is then rapidly degraded (Beutler *et al.*, 1985c). Dexamethasone inhibits the production of TNF by endotoxin-stimulated macrophages; however, pretreatment of macrophages with dexamethasone prior to endotoxin stimulation was essential for effective inhibition (Beutler *et al.*, 1986). It is of significance that rabbit antibody to TNF partially protects mice against the lethal effects of endotoxin (Beutler *et al.*, 1985d). Optimal results were obtained in these studies when the antiserum was administered six hours prior to endotoxin challenge. This data supports a role for TNF in mediating the lethal effect of endotoxin and they reveal the macrophage as a critical target cell for the production of mediators of endotoxic activity.

TNF may exert indirect as well as direct activities in mediating endotoxic activities. TNF is intrinsically pyrogenic, similar to interleukin 1 (IL-1). It also has the ability to induce IL-1 production (Dinarello *et al.,* 1986a) with its consequent spectrum of potent biological activities. TNF induces IL-1 from both mononuclear cells and endothelial cells. Furthermore, the interaction of TNF with endothelial cells alters their coagulant activity (Nawroth and Stern, 1986) and therefore provides an amplification mechanism for endotoxin elicited TNF and IL-1 in the induction of coagulation. This mechanism can operate independently of macrophage tissue factor activity.

Interleukin-1 (IL-1) represents a family of cytokines produced and secreted by mononuclear phagocytes and other cells upon stimulation by a variety of substances including bacterial endotoxin (Dinarello, 1986). Endotoxin may be one of the more potent inducers of IL-1 in macrophages with activity at picogram level. A variety of experiments have strongly suggested that IL-1 plays a central role in mediating host responses to bacterial infections. This is due both to the intrinsic activity of IL-1 and to the impressive number of additional mediators that are stimulated by the action of IL-1 on responsive cell populations. There appear to be at least two IL-1 molecules in humans, distinguishable on the basis of isoelectrical points and unique amino acid sequences. Experiments proved that endotoxin induces IL-1 in a uniform manner from cultured human peripheral monocytes (Bayne *et al.,* 1986).

The biological activities of IL-1 are nearly as complex as the activity of endotoxin itself. The two classic activities associated with IL-1 are fever production and lymphocyte activation. These two activities have been confirmed by researchers using a well-defined recombinant form of human IL-1 (Dinarello *et al.,* 1986b). Many other activities associated with IL-1 may play a role in mediating the pathophysiologic sequelae following exposure to endotoxin. IL-1 interaction with endothelial cells promotes procoagulant activity and increases endothelial adhesiveness (Bevilacqua *et al.,* 1985). IL-1 also induces an inhibitor of tissue plasminogen activator, which may contribute to the intravascular coagulation observed during acute inflammatory processes. IL-1 enhances

the hepatic production of acute-phase proteins such as serum amyloid A and C-reactive protein, which may play immunoregulatory roles during infection. Recently, IL-1 was found to be responsible for the stimulation of cells such as T lymphocytes, endothelial cells and fibroblasts to produce granulocyte macrophage colony stimulating activity. This data combined with the well described T lymphocyte and B lymphocyte stimulatory activities of IL-1, make it increasingly clear that IL-1 often plays a key positive role in enhancing immunity.

On the other hand, a number of mediators that may be deleterious to the host are also induced by IL-1 and may therefore be viewed within the framework of second-order effects of endotoxin. Platelet activating factor and arachidonic acid metabolites, both of which are also generated directly in response to endotoxin, represent very potent vasoactive compounds stimulated by IL-1. Therefore IL-1 may be viewed as one of the more important mediators induced by endotoxin, and may be directly responsible for the production of other mediators playing critical roles in the host`s response to bacterial infections.

Nitric oxide (NO) has recently been recognised as a new class of messenger molecule that serves a variety of functions in different tissues (Moncada *et al.*, 1991). It is diffusible and activates biochemical processes inside the producer cell as well as within neighbouring cells. Nitric oxide is the final product of the oxidation of the guandino nitrogen of L-arginine by nitric oxide synthase (NOS) (Reiling *et al.*, 1994).

Several NOS isoforms have been identified, representing the products of three separate genes on distinct chromosomes; these genes have been cloned in rodents and humans (Morris and Billiar, 1994). The isoforms vary considerably in their intracellular location, structure, regulation and functions. Two constitutive NOS (cNOS) isoforms, which are spontaneously expressed in various tissues, have been demonstrated: the neuronal cNOS (type I NOS) was first identified in the cytosol of central and peripheral neurons; the endothelial cNOS (type III NOS) is generally membrane bound and is found in

endothelial cells. Following stimulation by transient calcium fluxes, cNOS isoforms generate small amounts of NO for short period of time.

Another isoform of NOS, usually absent in resting cells, can be induced by a variety of stimuli, such as cytokines and lipopolysaccharides, and has been termed inducible NOS (iNOS; type II NOS). Once expressed, this enzyme generates large amounts of NO over an extended period. The regulation and function of iNOS are mainly associated with inflammatory and immune responses (Moncada *et al.,* 1991) and macrophages appear to play a pivotal role in this process (Nathan, 1987). The production of inducible NO synthase by endothelial cells or vascular smooth muscle after exposure to LPS, TNF, IL-1 or IFN-γ leads to an over production of NO in the vasculature and profound vasodilation. This might be the cause of sepsis-related hypotension which is characterised by low vascular resistance and is often refractory to vasopressor therapy (Parker *et al.,* 1987).

Nitric oxide production plays a role in the induction of nonspecific immunity and the antimicrobial response to a variety of extracellular parasites and some tumour cells. The importance of the macrophage and various cytokines such as TNF-α and IFN-γ in response to parasitic infection is well documented (Liew *et al.,* 1990; Titus *et al.,* 1984). Further investigation has implicated NO as the final mediator of parasitic defence. Nitric oxide-dependent mechanisms have been identified in the killing of intracellular *Trypanosoma* (Munoz-Fernández *et al.,* 1992), *Mycobacterium, Schistosoma* and *Leishmania* (Green *et al.,* 1990) organisms. The importance of macrophage NO production in tumoricidal activity has also been described using in vitro models (Klostergaard *et al.,* 1991; Jiang *et al.,* 1992). The precise mechanism of NO-mediated killing of these organisms is unknown; however, these observations suggest that macrophage-derived NO contributes significantly to nonspecific immunity.

1.5 Endotoxin receptors on mammalian cells

During the past twenty years, remarkable progress has been made in understanding the cellular basis for the diverse immunostimulatory and immunopathological activities of

bacterial lipopolysaccharides. There exists now abundant experimental evidence that LPS can interact non-cytotoxically with a variety of host immune and inflammatory mediator cells and that the consequences of these interactions are the synthesis and secretion of cytokines and other proinflammatory mediators by these cells. Perhaps one of the key cells important to the host response to LPS is the macrophage, unequivocally documented to play a pivotal role in mediating endotoxin lethality (Freudenberg *et al.*, 1986).

There is therefore considerable interest in understanding at the biochemical level the precise mechanism by which LPS triggers the activation of macrophages leading to secretion of cytokines. Within the framework of the LPS molecule itself, it has been recognised for many years that the lipid A component of LPS is sufficient for activation of many of the macrophage responses attributed to LPS. However, other studies have also implicated a potential role for inner core oligosaccharides (Haeffner-Cavaillon *et al.*, 1982) ; (Lüderitz *et al.*, 1989).

Fundamental to an understanding of LPS/lipid A macrophage interaction at the molecular biochemical level is the identification and detailed characterisation of specific membrane-localised receptors. While remarkably little detailed information was available concerning LPS receptors as little as ten years ago, recent studies from several laboratories have provided strong experimental evidence for the existence of several molecules on the surface of macrophages that can bind LPS and may serve as candidate LPS receptors. Since recognition of this major constituent of Gram-negative bacterial cell envelopes may be important to host defence within a number of contexts, there may well be multiple recognition systems available at the surface of macrophages and other host cells for LPS binding. The available evidence would in fact support the concept that multiple recognition pathways exist for LPS on macrophages.

1.5.1 Direct recognition of lipopolysaccharides

1.5.1.1 CD18 molecules

Many strains of *Escherichia coli* are recognised by macrophages without the intervention of the classic opsonins IgG and complement. Several lines of evidence indicate that this recognition event is due to the presence of CD18 antigens on the surface of the macrophage binding LPS in the outer leaflet of the bacterium. Firstly, CD18 molecules can be down-modulated from the apical surface of macrophages by allowing the cells to spread on substrates coated with anti-CD18 monoclonal antibodies (mAbs). Such treatment prevents macrophages from binding *E. coli* (Wright and Jong, 1986). Therefore CD18 molecules are necessary for recognition of bacteria. Secondly, spreading of cells on substrates coated with purified LPS (to deplete LPS receptors) depletes the capacity of macrophages to bind *E. coli.* Spreading on LPS also down-modulates CD18 molecules (Wright and Jong, 1986). Therefore, the CD18 receptors on macrophages that recognise *E. coli* are among the receptors that bind LPS. Finally, binding of LPS to macrophages may be directly assayed by incorporating LPS into the outer leaflet of the membrane of erythrocytes. The binding by macrophages of these LPS-coated erythrocytes directly correlates to the dose of LPS in the erythrocytes, and the binding of LPS-coated erythrocytes is inhibited by down-modulating CD18 molecules (Wright and Jong, 1986). Taken together, these studies indicate that CD18 molecules recognise surface-bound LPS.

The CD18 antigens comprise a family of three closely related, dimeric cell-surface glycoproteins (Detmers and Wright, 1988). Each is composed of a unique α-chain of approximately 180 kD (the CD11 antigen) non-covalently linked to an invariant β-chain of 95 kD (the CD18 antigen). Lymphocyte function-associated antigen, LFA-1 (CD11a/CD18), is present on all leucocytes, complement receptor type 3 (CR 3) (CD11b/CD18, also known as Mac-1 or the iC3b receptor), is present principally on phagocytes, and p150,95 (CD11c/CD18) is abundant on mononuclear phagocytes and is expressed at low levels on polymorphonuclear neutrophils. The role of the CD18 antigens

in the recognition of LPS has been confirmed by investigation of cells from patients with a genetic deficiency in CD18 (the common β-chain) (Todd and Fryer, 1988). Because of the absence of the β-chain, mature α-β dimers are not expressed on leucocytes. Monocytes and macrophages from these patients are unable to recognise unopsonised *E. coli* and unable to recognise LPS-coated erythrocytes (Wright *et al.*, 1990a).

Additional studies suggest that each of the three members of the CD18 family are capable of binding LPS. Down modulation of any one member or even any two members of the CD18 family by specific mAbs attached to the substrate has little effect on binding *E. coli* or LPS-coated erythrocytes to macrophages, but down modulation of all three members with a cocktail of antibodies against the three α-chains in the CD18 family completely inhibits binding (Wright and Jong, 1986).

The portion of the LPS molecule recognised by CD18 resides in the lipid A region because a truncated LPS with no O-antigen and incomplete core (Re) is recognised (Wright and Jong, 1986). In addition, a biosynthetic precursor of lipid A, lipid IVa is also recognised (Wright *et al.*, 1990a). Because LPS resides in a lipid bilayer, it is likely that the exposed glucosamine phosphates and not the hydrophobic chains of LPS buried within the bilayer, constitute the site that is recognised by CD18. This view is strengthened by the observation that CD18 appears to recognise additional phosphosugars in other microorganisms. The pathogenic yeast *Histoplasma capsulatum* has a cell wall rich in sugar phosphates and is recognised by each of the three CD18 molecules (Bullock and Wright, 1987). The parasite *Leishmania mexicana* expresses a complex glycolipid, lipophosphoglycan (LPG) rich in phosphosugars and two of the CD18 molecules (CR 3 and p195,95) have also been shown to recognise purified LPG (Taiamas-Rohana *et al.*, 1990). The hydrophilic sugar phosphate structure of LPG can be enzymatically released and purified away from the hydrophobic portion of the molecule. This soluble phosphosugar competitively blocks binding of LPG and LPS-coated particles to macrophages (Taiamas-Rohana *et al.*, 1990).

Recognition of LPS on whole *E. coli* by CD18 molecules on phagocytes results in adhesion, subsequent phagocytic engulfment and delivery to a degradative compartment. Recognition of LPS by CD18 may play an important role in killing and clearing of organisms from the circulation. In contrast, a role for CD18 molecules in the secretory responses to LPS is less certain.

Circumstantial evidence does suggest that CD18 molecules might participate in synthetic responses. Spreading of macrophages on protein-coated substrates requires CD18 molecules (Todd and Fryer, 1988) and has long been known to cause synthesis of cytokines such as IL-1 (Fuhlbrigge *et al.*, 1987). Moreover, anti-CD11b/CD18 antibodies have been shown to prevent spreading-induced synthesis of IL-1 in murine macrophages (Labadia *et al.*, 1988), and surface-bound anti-CD18 antibodies have been found to stimulate the synthesis of cell-associated IL-1 (Couturier *et al.*, 1989). However, only membrane IL-1 is induced by either spreading or anti-CD18 mAbs, and neither IL-1 nor TNF secretion is observed. Furthermore, the anti-CD11b antibody that blocks spreading induced synthesis of IL-1 has no effect on LPS induced synthesis of IL-1 (Couturier *et al.*, 1989).

More direct experiments have shown that a full secretory response to LPS can be observed in the complete absence of CD18 antigens. Mononuclear cells from CD18-deficient patients show normal or exaggerated synthesis of TNF-α and IL-1 in response to LPS (Wright *et al.*, 1990a). Also treatment of normal mononuclear cells with anti-CD 18 antibodies does not interfere with secretion of TNF (Wright *et al.*, 1990b). These data indicate that CD18 molecules are not necessary for secretory response of leucocytes to LPS. The CD 18 molecules might be important in the removal of LPS/ Gram-negative bacteria from the body but their role of cytokine production is unclear.

1.5.1.2 Scavenger receptor

Other investigators have sought LPS receptors through different means. The binding behaviour of sonicated dispersions of radiolabelled lipid IVa, a lipid A precursor has been

studied. This probe which closely resembles lipid A but is chemically synthesised, may be labelled to very high specific activity and forms small stable vesicles in the presence of albumin at physiological pH. Radioactive lipid IVa showed specific, saturable binding to intact murine macrophage-like tumour cells (Hampton *et al.,* 1988). Further studies, using western blots, showed that the radioactive lipid IVa binds to a 95 kD membrane protein derived from macrophages and therefore implicating an individual protein in this specific binding (Hampton *et al.,* 1988).

While the molecular weight of the lipid IVa-binding protein is identical to the molecular weight of the CD18 β-chain (both 95 kD), Golenbock and co-workers (1990) have shown that these two species are not identical: binding of LPS to CD18 requires divalent cations while binding of lipid IVa does not; simultaneous staining for CD18 and lipid IVa binding in a single gel lane shows a slight but clear difference in mobility of the two activities; and in the most telling experiments, normal binding of radiolabelled lipid IVa is observed using CD18 deficient leucocytes (Golenbock *et al.,* 1990). Therefore, the receptor molecule identified by binding of vesicular LPS differs from that identified by binding of LPS on the surface of *E. coli*. It is possible that the size of the structure bearing LPS may dictate the choice of receptors. Whole bacteria (size approximately 1μm) are recognised by CD18 molecules but vesicles (size approximately 0.01μm) are recognised by different receptors.

More recent studies have identified the acetyl-low-density lipoprotein (acetyl-LDL) or scavenger receptor as the molecule that binds radiolabelled lipid IVa (Hampton *et al.,* 1991). Acetylated LDL and other ligands of the scavenger receptor competitively block the binding of lipid IVa to the RAW macrophage-like cell line. and lipid IVa blocks binding of labelled acetylated LDL.

As with CD18, it appears that the interaction of lipid IVa with the scavenger receptor does not stimulate secretory responses. Acetyl-LDL binds RAW cells but does not initiate TNF synthesis. Moreover, blockade of lipid IVa binding to RAW cells by acetyl-LDL does not block the normal induction of TNF synthesis by LPS ((Hampton *et al.,* 1991).

The scavenger receptor is a trimeric transmembrane glycoprotein composed of cysteine-rich carboxy-terminal exoplasmic domains connected to a trans-membrane domain by a collagen-like stalk (Kodama *et al.,* 1990). It recognises a wide range of anionic ligands including maleylated serum albumin, polyvinyl sulphate, dextran sulphate and polyinosine. The receptor is capable of rapidly carrying these substances to lysosomal compartment. The scavenger receptor is found in large amounts in the liver (Kupffer cells and sinusoidal endothelial cells), and is well placed to remove endotoxin entering from the gut.

1.5.1.3 Additional receptors for LPS

A cross-linking approach was used to identify another potential LPS receptor. Purified

LPS was derivatised with an iodinated, photo-activatable probe and then incubated with mouse splenocytes. After photolysis, a single species of 80 kD and pI of 6.5 was labelled (Lei and Morrison, 1988a). Several lines of evidence suggest that this labelled species may be an LPS receptor. Firstly, the molecule is found in a membrane fraction of splenocytes. Secondly, cross-linking plateaus at high concentration of LPS suggests saturation of binding sites. Finally, cross-linking of labelled LPS to the 80 kD species is partially inhibited by unlabelled LPS preparations (Lei and Morrison, 1988b). In more recent studies, the 80 kD murine protein was partially purified and used to immunise hamsters. An IgM monoclonal antibody (mAb5D3) was obtained (Bright *et al.,* 1990). Addition of mAb5D3 to cultures of murine bone marrow-derived macrophages activated these cells to become tumouricidal for mastocytoma cells in vitro and the results of several experiments established that the observed LPS-like activity of mAb5D3 was not due to contaminating endotoxin (Chen *et al.,* 1990). These results when considered with those of Bright and co-workers (1990) and the previous work of Lei and Morrison (1988a &b), provide a support for the concept that the 80 kD LPS- binding protein might serve as a specific functional receptor for LPS on murine macrophages.

The ability of mAb5D3 to mimic the effects of LPS in activating murine bone marrow-derived macrophages for tumour cell killing in the absence of secondary stimuli, like LPS, suggests that the 80 kD membrane LPS-binding protein recognised by mAb5D3 may be at least one of the entities through which activation for tumour killing is regulated (Chen *et al.*, 1990).

1.5.2 Recognition of LPS complexed to lipopolysaccharide binding protein

Recent studies suggest that leucocytes may recognise LPS by a novel mechanism in which a serum protein first binds to LPS and the LPS-protein complex then associates with specific receptors on the cell surface. A serum protein has been described with the ability to bind endotoxin (Tobias *et al.*, 1986). This protein, lipopolysaccharide binding protein (LBP), forms high-affinity stoichiometric complexes with LPS from a number of bacterial species (Tobias *et al.*, 1989). LBP is a 60 kD protein synthesised in the liver as an acute phase reactant (Shumann *et al.*, 1990). The concentration of LBP is roughly estimated to be 200 $ng.ml^{-1}$ in resting serum and greater than 50 $\mu g.ml^{-1}$ in acute phase serum of rabbits. The ability of LBP to bind LPS enables it to function as an opsonin (Wright *et al.*, 1989). It binds stably to LPS-bearing particles such as living Gram-negative bacteria or LPS-coated erythrocytes, and strongly enhances the attachment of these particles to macrophage. LBP bridges LPS-coated particles to macrophage by first binding to LPS, then binding to the macrophage. Pretreatment of LPS-coated erythrocytes with LBP enables binding to the macrophage, but pretreatment of the macrophage has no effect. Moreover, macrophages do not recognise erythrocytes coated with LBP unless LPS is added, suggesting that macrophages recognise complexes of LBP with LPS but not LBP alone (Wright *et al.*, 1989).

Binding of particles coated with LPS-LBP complexes is mediated by receptors found on blood monocytes and macrophages but not on lymphocytes or umbilical vein endothelium (Wright *et al.*, 1989). The receptors are mobile in the plane of the membrane as binding activity of macrophages is down-modulated upon spreading of cells on surfaces coated with LPS-LBP complexes.

Studies have shown that the receptor that recognises LPS-LBP complexes is identical with CD14, a differentiation antigen of monocytes and macrophages (Wright *et al.*, 1990b). Down-modulation of CD14 by any of several surface bound anti-CD14 mAbs prevent binding of erythrocytes coated with LPS-LBP complexes to macrophages. Furthermore, addition of some (but not all) anti-CD14 mAbs in solution blocks binding of erythrocytes coated with LPS-LBP complexes: Finally purified CD14 adsorbed to non-blocking mAbs retains the capacity to bind LPS-LBP complexes. These observations indicate that LPS may interact with a soluble binding protein and subsequently become cell associated through interaction with the membrane glycoprotein, CD14.

CD14 is a 55kD cell surface glycoprotein which has long been recognised as a marker for monocytes and macrophages. It has been cloned and sequenced from both mouse and man (Setoguchi *et al.*, 1989).

Like CD18 and the scavenger receptor, LBP and CD14 appear to be capable of delivering LPS to a digestive compartment. In contrast with CD18 and the scavenger receptor, interaction of LPS-LBP complexes with CD14 on macrophages appears to be coupled to the secretory responses of leukocytes. Fig.1.7 summarises the role of multiple receptors in binding and response to LPS.

1.6 Septic shock therapy

The immediate approach to management of the patient in septic shock is institution of corrective measures that are designed, firstly, to confirm and characterise the condition and to correct rapidly any potential reversible factors, and secondly, to begin specific therapy of the underlying cause.

It is essential to ensure adequate oxygenation and to establish suitable means to monitor the haemodynamic status. Modern management practice in shock emphasises that the administration of fluid, inotropes and vasopressors must be tailored to the needs of each patient and will usually need frequent modification as the condition evolves (Shoemaker

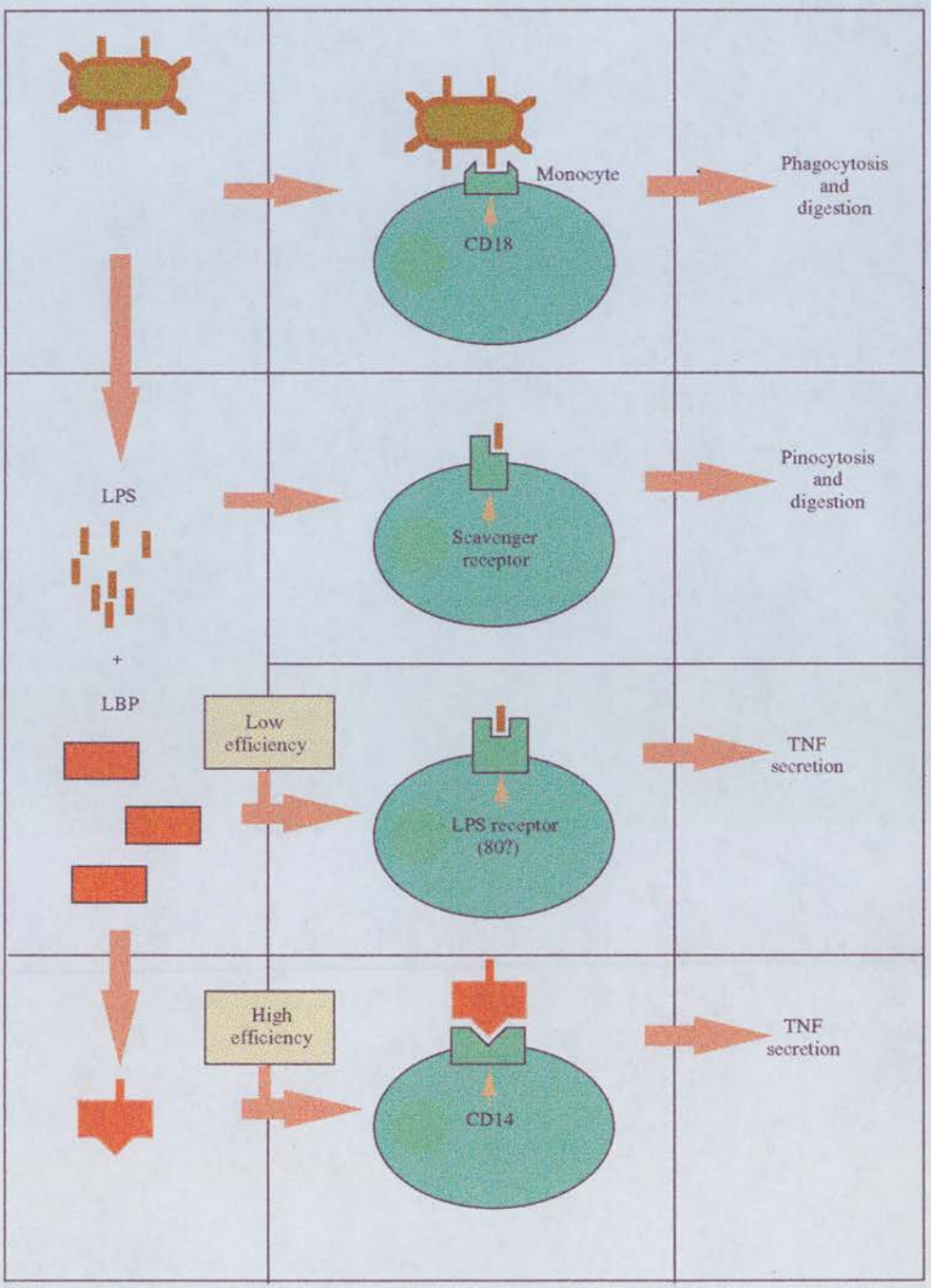

Figure 1.7 Role of multiple receptors in the description and response to lipopolysaccharide. (Reproduced from Wright, 1991)

et al., 1990). Metabolic abnormalities such as hypoxaemia or severe acidosis need to be identified and treated. Every effort must be made to identify the source of sepsis in order to drain abscesses and choose the most appropriate empirical antimicrobial therapy. Although it will seldom be possible to use an antibiotic directed against just one bacterial species, outcome is improved by choosing a regimen that proves to be active against the infecting organism (Kreger *et al.,* 1980b).

1.6.1 High dose steroids

Corticosteroids are anti-inflammatory and antipyretic. They induce a feeling of well-being in the patient which can give a subjective sense of improvement. Furthermore, they have profound effects on many of the mediator systems implicated in the pathogenesis of septic shock, and it is not difficult to understand why many doctors felt strongly that high-dose steroids given early in shock were beneficial (Sheagren 1985). Although there were experimental data that tended to confirm this impression, clinical findings were confused. Most early trials were flawed in design, but in the last ten years three major studies have overcome many of the early difficulties.

The effects of methylprednisolone (30 mg/kg), dexamethasone (6 mg/kg, one or two doses), and no steroids in patients with severe established shock were compared (Sprung *et al.,* 1984). Steroids delayed death but did not reduce overall mortality. In addition, patients given dexamethasone had an increased incidence of bacterial superinfections. Subsequently, two further studies have evaluated the effect of early intervention with high-dose methylprednisolone in septic shock (Hinshaw *et al.,* 1987) and (Bone *et al.,* 1987).

Both studies were placebo-controlled, and both were careful to control for the many variables that can influence outcome. Importantly, both trials insisted that patients only be enrolled if they could be treated within 2 h of shock being recognised. Results of the two trials were strikingly similar. Neither found any evidence of benefit from steroids, and steroid recipients had significantly more secondary bacterial infections.

Taken together, these studies provide no support for the routine use of high-dose steroids in septic shock.

1.6.2 Natural antibodies to endotoxin

1.6.2.1 Antibodies to O-specific side chain

The virulence of Gram-negative bacteria is not solely attributable to infectivity of pathogens, but is also determined by the host's susceptibility to endotoxin. In many cases endotoxaemia, presumably with endotoxin derived from gut commensals, occurs without bacteraemia (van Deventer *et al.,* 1988). It has often been difficult to distinguish between the distinct roles of anti-endotoxin antibodies in their effects on infectivity or endotoxicity. Antibodies to O-specific chains offer specific protection against infectionby Gram-negative pathogens. The phenomenon of acquired tolerance to endotoxin has been known since late last century, but it has only lately been recognised that late-phase tolerance is O-specific and is mediated by serotype-specific antibodies following an immune resonse to the O-polysaccharide of administered endotoxin (Johnston and Greisman, 1984).

1.6.2.2 Antibodies to core and lipid A

A number of studies have confirmed that circulating natural antibodies to the LPS core and lipid A are common in healthy humans (Barclay and Scott, 1987; Barclay *et al.,* 1989).

With an LPS-polymyxin ELISA system, it has been found that there is an epitope in the inner core of LPS (Re-LPS) to which antibodies are found in many healthy individuals. There is another epitope in the inner core expressed on Rc-LPS but not on Re-LPS to which many other healthy individuals have antibodies (Barclay and Scott, 1987). It appears that different individuals have different dominant patterns of immunity to common epitopes in the LPS core. It is not known how these patterns are engendered, but

they may arise from early formative encounters with endotoxin and be boosted through continuous exposure (Barclay, 1990).

1.6.3 Antibodies to endotoxin in Gram-negative septic shock

1.6.3.1 Antibodies to O-specific side chain

Treatment using antibodies directed against the O-polysaccharide component of endotoxin has produced antibacterial effects (complement-dependent bactericidal and opsonophagocytic activities) and increased survival (Sagawa *et al.*, 1990). Because the O-polysaccharide antigen is structurally different for each strain of Gram-negative bacteria, antibodies directed at this antigen are serotype-specific and therefore, only protect against infection from a homologous strain of bacteria. Consequently, the future success of this therapy would probably require faster identification of the infecting organism and a stockpile of antibodies against potentially pathogenic organisms. Alternatively, patients could be given a combination of effective serotype-specific monoclonal antibodies before identification of the organism to protect against the most likely infectious organisms (Baumgartner, 1990). Partly because of these problems, researchers have not actively pursued O-antibody therapy, and the researchers efforts concentrated on " cross-reactive" antibodies directed against core and lipid A regions of endotoxin.

1.6.3.2 Antibodies to core and lipid A

Antibodies directed against core and lipid A antigens were developed because they might possibly neutralise the toxic effects of or increase clearance of endotoxin (Ziegler *et al.*, 1982). Unlike the O-polysaccharide antigen, core and lipid A antigens are more phylogenetically conserved and similar among all Gram-negative bacteria. Consequently, a core or lipid A antibody that protectes against infection from one strain of Gram-negative bacteria might also cross-protect against a different strain or species of Gram-negative bacteria (Baumgartner *et al.*, 1987). This cross-protective feature has led researchers to investigate whether core or lipid A antibody therapies might improve the survival of patients with diverse Gram-negative bacteria infection.

1.6.3.2.1 Polyclonal antibodies to core and lipid A

In the early 1980s, investigators produced human antiserum directed against core structures of endotoxin. Serum was extracted from human volunteers immunised with heat-killed *E. coli* J5, a rough (Rc-like) mutant bacterium (Ziegler *et al.*, 1982). Therefore, the endotoxin from this bacteria contains only inner core and lipid A antigens. This therapy was tested in a multicentre trial of patients with presumed Gram-negative infection. In the double-blind study, researchers compared the effects of serum obtained after *E. coli* J5 immunisation (J5 antiserum) with those of serum obtained before J5 immunisation (control serum). Treatment with J5 antiserum was associated with decreased mortality in patients with Gram-negative bacteraemia (22% vs. 39%) and in patients who had been in shock before randomisation (44% vs. 77%) (Ziegler *et al.*, 1982). These results could not be explained by the investigators, who found no significant relationship between anti-J5 antibody levels and improved outcome. After the study was published, other scientists questioned whether the extremely high mortality rate in the control group with shock (77%) indicates an imbalance of pretreatment randomisation such that sicker patients were more frequently assigned to the control group (Baumgartner, 1991). Therefore it is not known whether factors other than the cross-protective effects of endotoxin antibodies contributed to the differences between treatment groups.

In another double-blind trial, investigators examined whether prophylactic J5 immune plasma decreases infection rates in high-risk surgical patients (Baumgartner *et al.*, 1985). Results from this study revealed that patients given J5 immune plasma as compared with controls did not have lowered rates of Gram-negative infections, but showed a nonsignificant trend toward decreased rates of Gram-negative septic shock (5% vs. 11%) and death from Gram-negative septic shock (2% vs. 7%).

Three subsequent studies using polyclonal antibodies directed at core and lipid A structures have shown no beneficial effect for prophylactic treatment of Gram-negative infections (Girardin *et al.*, 1992; Calandra *et al.*, 1988 and Cometta *et al.*, 1992). Cometta and coworkers (1992) compared standard intravenous immune globulin, core

endotoxin immune globulin (a preparation of standard immune globulin with more anti-core endotoxin antibodies) and human serum albumin (controls) as possible therapies to prevent infection in high-risk surgical patients. In a large collaborative study Cometta and colleagues (1992), found that patients receiving standard immune globulin had fewer infections and pneumonias and shorter hospital stay than patients receiving control therapy. Interestingly, patients receiving core endotoxin immune globulin therapy, as compared with controls, had no improved outcome. Thus, the core endotoxin immune globulin did not improve outcome above standard immune globulin. In fact, patients who received core endotoxin immune globulin did not do as well as those who received standard immune globulin. The reason for this unexpected finding is not fully explained by the data.

The use of monoclonal antibodies directed against core structures of endotoxin is probably a better option than polyclonal antiserum therapy. Moreover, such monoclonal antibody therapy circumvents problems in the production and use of human polyclonal J5 antiserum such as the toxicity produced during vaccination of serum donors, the variable antibody content of antiserum preparations, and transmission of infection from serum donors to patients (Smith *et al.,* 1992).

1.6.3.2.2 Monoclonal antibodies to lipidA

In animal studies investigators found that a murine monoclonal antibody directed against lipid A (E5) diminished the effects of endotoxin challenge in sheep and protected mice challenged with live bacteria (Wheeler *et al.,* 1990).

Other researchers have reported that a human monoclonal antibody (HA-1A) directed against lipid A decreased mortality of mice challenged with bacteria and diminished the dermal Shwartzman reaction in rabbits challenged with endotoxin (Teng *et al.,* 1985). However, independent investigators have not been able to confirm these results (Baumgartner *et al.,* 1990).

In a placebo-controlled trial, the murine antibody E5 was tested in patients with presumed Gram-negative sepsis (Greenman *et al.*, 1991). In this trial, 486 patients (316 with Gram-negative infections) were enrolled. Researchers found that E5 did not improve survival in patients with Gram-negative infection or in the subgroup of patients with Gram-negative infection and shock. However, E5 significantly increased survival (70% vs. 57%) and improved organ function in a subgroup of patients with Gram-negative infection who were not in refractory shock.

The monoclonal antibody HA-1A has also been tested in a randomised placebo -controlled trial of patients with presumed Gram-negative sepsis (Ziegler *et al.*, 1991). In a study of 543 patients, investigators found that HA-1A had no effect on patients with sepsis or patients with only Gram-negative infection. However, the investigators reported that HA-1A significantly increased the survival of a subgroup of patients with Gram-negative infection and bacteremia. Based on these results, HA-1A was approved for use in patients with presumed Gram-negative sepsis in several European countries.

In the United states, results from this study have caused considerable debate among the scientific community (Warren *et al.*, 1992). These concerns were heightened when the results of a blinded, controlled study of HA-1A using a canine model of septic shock were published (Quezado *et al.*, 1993). The original purpose of this study was to confirm a therapeutic benefit of HA-1A in a large animal model and to discover what mechanisms were associated with its reportedly beneficial effects. All animals were infected with an intraperitoneal clot containing *E. coli* O111:B4, the nonmutant form of the *E. coli* J5 used to generate the heteromyloma cells that synthesise HA-1A. Unexpectedly, results from the study showed HA-1A treatment significantly decreased survival. Only 2 of 13 animals in the HA-1A group, compared with 8 of 14 animals in the control group, survived. Animals in the HA -1A group also had more severe cardiovascular dysfunction and organ injury. Furthermore, HA-1A had no effect on bacteremia or endotoxemia.

After consideration of the available clinical and animal data, HA-1A did not seem to benefit patients with presumed Gram-negative sepsis or septic shock. Consequently, HA-

1A was withdrawn from the European market and is not being considered for approval by the FDA for patients with Gram-negative sepsis.

1.6.4 Anti-TNF monoclonal antibodies

Murine monoclonal antibodies raised against human recombinant TNF, prepared as the F(ab')$_2$ fragments, have been shown to neutralise baboon serum TNF bioactivity in the L929 cell cytotoxicity assay (Tracey *et al.*, 1987). In the same study baboons were given lethal doses (LD_{100}) of live *Escherichia coli* via intra-aortic infusion. Experimental animals were passively immunised with anti-TNF F(ab' .)$_2$ one hour before bacterial infusion, whereas control animals received carrier vehicle at a similar time.

Pretreatment with anti-TNF F(ab')$_2$ abrogated the serum levels of immunoreactive TNF (Tracey *et al.*, 1987). Control animals not receiving anti-TNF antibodies uniformly succumbed to septic shock, pulmonary oedema and multiple organ failure within hours (13.3 ± 3.5). Anti-TNF F(ab')$_2$ administered one hour before bacteria prevented the development of hypotension and shock, but did not prevent later organ injury and death. The investigators reasoned that adequate tissue penetration of the antibodies had not occurred in only one hour, and that the production of nonneutralised TNF mediated the development of subsequent toxicity, tissue injury and death (Tracey *et al.*, 1987). Therefore a second group of animals was immunised 2 hours before bacteraemia and the results were dramatic. The baboons were completely protected against the development of shock and tissue injury, and did not succumb to the LD_{100} dose of bacteria. Complete neutralisation of TNF in the protected animals prevented the appearance of elevated serum levels of catabolic stress hormones (Tracey *et al.*, 1987).

Other investigators using anti-TNF antibody (TN3-19.12) have confirmed the importance of this cytokine in animal models of endotoxic shock. Silva and co-workers (1990) neutralised the TNF-α produced in mice during lethal endotoxaemia caused by *Escherichia coli* OIII. TN3-19.12 prevented death in mice receiving an LD_{90} dose of *E. coli* if given 1.5 h before or 30 min after the challenge. Mice treated prophylactically with

TN3-19.12 showed 97% survival at 24 h compared with 47% of control mice (received irrelevant antibody). At the end of the experiment (72 h), 75% of the TN3-19.12-treatd mice survived compared to 6% of the control mice. In the same study two groups of mice were treated therapeutically. Mice given TN3-19.12 0.5 h after bacterial challenge had 79% at 72 h compared with 12% of control mice. In the second group where TN3-19.12 was administered 2.5 h after bacterial challenge, only 25% of TN3-19.12-treated mice survived compared to 12% of control mice. Similar survival improvements in *E. coli* endotoxaemic baboon model have been observed following immunisation against TNF-α (Emerson *et al.*, 1992).

1.6.5 TNF antagonism

Since TNF is a proximal mediator of septic shock, there are efforts to neutralise its toxic effects. Because antibody therapies in humans has its limitations, attention has been focused on the use of soluble receptors for TNF. These are naturally-occurring proteins which represent the extracellular domains of the two TNF receptors. They are thought to act as natural TNF antagonists. The administration of soluble TNF receptors has prevented *E. coli* induced shock in baboons (van Zee *et al.*, 1992) and death in mice (Lesslauer *et al.*, 1991).

1.6.6 TNF processing inhibitors

TNF is initially synthesised as a 26 kD type II transmembrane propeptide which is proteolytically cleaved at the cell surface, releasing the mature 17 kD cytokine assembled as a homotrimer (Kriegler *et al.*, 1988). Although both cell surface and secreted forms of TNF appear to be biologically active, it is soluble TNF released into the circulation which is of primary importance in deleterious physiological responses, such as cachexia or septic shock (Perez *et al.*, 1990). Blocking the cleavage of the cell surface TNF could be of a therapeutic benefit in septic shock. In support of this concept, a potent and selective metalloprotease inhibitor which blocks cleavage of cell surface TNF in activated

macrophages and T cells has been shown to reduce serum TNF levels and increase survival in mice challenged with lethal doses of endotoxin (Mohler *et al.*, 1994).

1.6.7 IL-1 antagonism

The kinetics of TNF and IL-1 stimulation during sepsis suggests that any attempt to treat sepsis by modulating TNF production may have to occur soon after onset of shock. At least in principle, there are hours in which to block the activities of IL-1. In support of this hypothesis lies in the results of a study in which IL-1 receptor antagonist (IL-1 RA) reduced the mortality rates in rabbit model of septic shock (Wakabayashi *et al.*, 1991). IL-1 RA is a naturally-occurring protein which binds to the human IL-1 receptor but has no agonist activity (Eisenburg*et al.*, 1990; Hannum *et al.*, 1990). The administration of IL-1 RA even a few hours after the onset of shock might be beneficial.

1.7 Aims of the study

More knowledge is needed about the interaction of LPS with host cells and the consequent production of cytokines and secondary mediators. Such knowledge might be helpful in developing new strategies for septic shock treatment.

The primary aim of this study was to investigate:

-the direct interaction of different forms of LPS (pure LPS, LPS attached to bacterial outer membranes, and LPS attached to intact bacterial cells) with host leucocytes.

-Cytokines and other mediators released from host leucocytes upon stimulation with different forms of LPS. Measurement of the release of these molecules in response to various forms of LPS affords a different approach to study LPS-host cell interaction.

Chapter 2

General materials and methods

2.1 Buffers

All chemicals were analytical grade obtained from BDH Chemicals Ltd. (Poole, Dorset, UK) unless otherwise stated.

2.1.1 Dulbecco's phosphate-buffered saline (PBS)

PBS was prepared by dissolving one PBS tablet (Oxoid, Basingstoke, Hampshire, UK) in 100 ml distilled water.

2.1.2 Phosphate buffered saline, solution B

Solution B was prepared by dissolving 2.09 g $CaCl_2$ and 2.09 g $MgCl_2$ in 100 ml distilled water (solution B). Phosphate buffered saline + B (PBS + B) was prepared by adding 0.5 ml of solution B to 100 ml PBS.

2.2 Culture media

2.2.1 10% RPMI

RPMI 1640 (Gibco, Paisley, UK) was supplemented with 10% foetal calf serum, 100 U ml^{-1} penicillin G, 100 µg ml^{-1} streptomycin sulphate (Sigma, Poole, Dorset) and 1 mM L-glutamine (Gibco, Paisley, UK).

2.2.2 10% DMEM

DMEM (Sigma, poole, Dorset) was supplemented with 10% FCS, 100 U ml^{-1} penicillin G, 100 µg ml^{-1} streptomycin sulphate (Sigma, Poole, Dorset) and 1mM L-glutamine (Gibco, Paisley, UK).

2.3 Cells

C3H male mice (Transgenic Unit, Medical School, Edinburgh University,UK) which

are conventionally used in the field of lipopolysaccaride studies were chosen as the major source of macrophages in this study. Other mouse strains obtained from the same source were also used. A human monocyte/macrophage cell line, THP-1, (European Collection of Animal Cell Culture, Salisbury, UK) and human peripheral blood monocytes were also used.

2.3.1 Collection of mouse peritoneal macrophages

Mice were killed by cervical dislocation and the peritoneal cavity was washed twice with PBS containing 10 U ml^{-1} heparin (Sigma, Poole, UK). Each mouse was injected with 5 ml PBS and the peritoneum was massaged to release the macrophages into the PBS. Using a 0.8 mm gauge needle the PBS containing the macrophages was collected and placed in a sterile plastic universal. The cells were centrifuged at 300 g for 10 min, the supernatant was discarded and the cell pellet suspended in a known volume of PBS, PBS + B or 10% DMEM. A 50 µl sample was diluted in 450 µl white cell diluting fluid (0.1% gentian violet in 1% acetic acid) and counted using a haemocytometer. Cells were then adjusted to the required number.

2.3.2 Collection of THP-1 cells

THP-1 cells were grown routinely in 10% RPMI 1640 at 37° C in a humidified 5% CO_2 containing incubator in tissue culture flasks. THP-1 cells were harvested by centrifugation at 300 g for 10 min and resuspended either in PBS, 10% RPMI 1640 or 10% DMEM, counted and adjusted as mouse peritoneal macrophages.

2.3.3 Collection of human peripheral blood monocytes

Freshly drawn heparinised blood (from healthy volunteers) was diluted 1: 2 using sterile PBS. The diluted blood (15 ml) was then layered carefully on histopaque (5 ml) (Sigma, Poole, UK) in sterile plastic centrifuge tubes and centrifuged for 25 min at 300 g. Mononuclear leucocytes were collected from the interface, washed once with pyrogen free PBS + B or 10% DMEM and adjusted to the desired count.

2.4 Bacterial strains

All bacteria strains used in this work and their growth conditions are listed in table 2.1. *Salmonella minnesota* Ra-R 60 a rough mutant with complete core was the main organism used in this study.

2.4.1 Standardisation of bacterial count

Bacterial concentrations were calibrated by comparison of numbers (several dilutions) detected by counting chamber (Thoma) with spectrophotometric reading at 525 nm. A standard curve was prepared from the OD versus total count of bacterial dilutions and used for further determination of the bacteria concentrations.

2.5 Extraction of pure LPS

Pure LPS was extracted by the aqueous phenol, chloroform and petroleum spirit method of Galanos *et al*, (1969) as described by Hancock and Poxton (1988) from *Salmonella minnesota* Ra-R 60.

2.5.1 Deionization of LPS

S. minnesota Ra LPS was deionized by the method of Galanos and Lüderitz (1975) using a two cell apparatus developed by ISCO for the electrophoretic concentration of proteins as described by Hancock and Poxton (1988).

The LPS solution/suspension (5-10 mg ml^{-1}) in distilled water was placed in the cell and distilled water placed in the two electrode chambers. The apparatus is ideally kept cool (below 10° C) in a cold room. A voltage of up to 500 V is maintained across the cell. The pH in the cathodic chamber rose; when it reached 9-10 the contents were replaced with fresh distilled water. This was repeated several times over 3-4 h. The free acid form of the LPS (deionized) precipitated from solution and was deposited as a gel on the membrane at the anodic end of the cell. It was recovered from the cell by draining off the excess water

Table 2.1: Bacterial strains

Species	Source	Growth media	Incubation conditions
Salmonella minnesota Ra	MPRL 1300	Nutrient broth	Aerobically at 37° C in an orbital incubator for 18 h
E. coli K 12	Departmental teaching collection	Nutrient broth	Aerobically at 37° C in an orbital incubator for 18 h
Neisseria meningitidis	I&I A14 C:2b:P1.2	MNYC	Aerobically at 37° C for 48 h
Staph. epidermidis	MPRL 7944	Blood agar	Aerobically at 37° C for 18 h
Bacteroids fragilis	NCTC 9343	PPY broth	Anaerobically at 37° C for 48 h

MPRL (Microbial Pathogenicity Research Laboratory, Department of Medical Microbiology, University of Edinburgh, UK). NCTC (National Collection of Type Culture, London, UK). I&I (Infection and Immunity Laboratory, Department of Medical Microbiology, University of Edinburgh, UK).

from the chamber, dismantling the apparatus, gently scraping off the gel and suspending it in pyrogen free water.

2.6 Extraction of bacterial outer membranes (OM)

Outer membrane fraction was extracted from *S. minnesota* Ra. Log phase bacteria (2 litres) were harvested by centrifugation at 16 000 g for 10 min and washed once in 0.01 M Hepes buffer, pH 7.4, containing 10 mM $MgCl_2$ then suspended in 40 ml Hepes containing 10 mM Mg Cl_2. Bacterial cells were broken by passage through a French pressure cell (Aminco, Silver Springs, MD, USA) at 6000-7000 p.s.i. Unbroken cells were removed by 2 cycles of centrifugation at 6000 g for 10 min.

Sarkosyl treatment was by a modification of the method of Filip *et al.* (1973). Sarkosyl (30% w/v solution of sodium *N*-lauryl sarcosinate) (Sigma) was added to the broken cell suspension to a final concentration of 0.7% (w/v) sodium *N*-lauryl sarcosinate. The suspension was then centrifuged at 50 000 g for 1 h, and the pellet of OM was washed once in water and finally suspended in 2 ml of pyrogen free water.

2.6.1 Determination of LPS content in bacterial outer membrane fraction (OM)

An assay for 3-deoxy-D-manno-octulosonic acid (KDO) was used to determine the amount of LPS in the bacterial OM fraction. This assay determined the amount of KDO present in LPS. By comparing the amount of KDO in a known concentration of pure LPS with the amount of KDO in an OM preparation, it was possible to estimate the amount of LPS in the OM preparations. The thiobarbituric acid KDO assay was performed as described by Hancock and Poxton (1988).

LPS samples containing from 1-20 µg of KDO were mixed with 1 ml of 0.125 M sulphuric acid and heated in a boiling water bath for 8 min. If insoluble material is present, it is removed by centrifugation. Samples (0.5 ml) of the acid hydrolysate were mixed with 0.25 ml of periodic acid reagent (25 mmol periodic acid in 62.5 mmol H_2SO_4) and incubated at

37° C for 30 min. The samples were cooled and 0.25 ml of sodium arsenate solution (2% w/v sodium arsenate in 0.5 M HCl) was added to each sample, mixing until the brown colour of iodine disappeared. Thiobarbituric acid (0.25 ml) (0.6% thiobarbituric acid adjusted to pH 9 with sodium hydroxide) was added per sample and the samples were heated at 100° C for 7.5 min. While the reaction mixture was still hot, dimethyl sulphoxide (1 ml) was added. The samples were then cooled and the absorption was measured against a reagent blank at 548 nm.

2.7 Determination of LPS content in whole bacteria

The content of LPS in whole *S. minnesota* Ra bacteria was estimated considering that R-LPS constitutes 1-2% of bacterial dry weight (personal communication, Dr. Poxton, Medical Microbiology Dept., Medical School, University of Edinburgh, UK). Bacteria grown overnight were washed three times in PBS, resuspended in a known volume of PBS and counted then the total number of bacterial cells in the sample was calculated. The sample was centrifuged at 1000 g for 20 min and the PBS was discarded. The bacterial pellet was freeze dried overnight and the lyophilized bacteria were weighed. The amount of LPS in the lyophilized bacteria was estimated and related to the total count of bacteria previously calculated.

Chapter 3

Binding of LPS to host cells

3.1 Introduction

Mononuclear phagocytes and neutrophils provide a defence against microbial invasion. Neutrophils, in general, are more efficient phagocytes, except when the particle is larger in relation to them or when the particle load is greater (Cohn 1968). Under these circumstances mononuclear phagocytes are more effective than neutrophils. Macrophages, therefore, represent a major defence against invasion of the host by a wide variety of micro-organisms. Macrophages move toward the microbial particles guided by a gradient of chemotactic molecules. Engulfment then begins with the macrophage advancing pseudopodia over regions of the micro-organism that are coated with opsonins which bind to specific sites on both micro-organisms and macrophages. Opsonins are of various types, but the most studied are IgG and fragments of the third component of complement (C3). Macrophages possess surface receptors which bind specifically to the Fc domain of various subclasses of IgG and to several catabolites of C3 (Adams and Hamilton, 1984). Many micro-organisms have the ability to activate the complement system generating complement fragments that opsonise the organism. The macrophage is an important source of complement components and macrophage-derived complement factors can opsonise micro-organisms for their subsequent destruction in the absence of other sources. The process of internalisation of micro-organisms is initiated after binding with the appropriate ligand (Nathan *et al.*, 1980). Attachment can also occur in the absence of opsonins if the micro-organism possess surface determinants that can be recognised directly by the macrophage such as the interaction of macrophage mannose-fucose receptor with carbohydrate residues (Sung *et al.*, 1983) and "lectin-like" receptors with glucose and galactose (Freimer *et al.*, 1978).

Macrophages secrete large amounts of oxygen and arachidonic acid metabolites when phagocytosis is mediated by Fc receptors (Rouzer *et al.*, 1980). In contrast,

ligation of C3 receptors fails to release either arachidonic acid or oxygen metabolites from mononuclear phagocytes (Aderem *et al.,* 1985 ; Wright and Silverstein, 1983). Macrophages also have receptors for the anaphylatoxin C5a. Occupancy of this receptor induces the secretion of IL-1 and initiates chemotactic phenomena (Goodman *et al.,* 1982). The ingestion of particles by mononuclear phagocytes may occur over a broad pH range and is accompanied by enhanced glucose oxidation similar to that which occurs when phagocytosis is mediated by neutrophils (Cline *et al.,* 1978).

Many micro-organisms are phagocytosed and destroyed by macrophages, but there are certain pathogens that can replicate within macrophages and parasitise them. When the macrophage is activated, these intracellular pathogens may be inhibited or destroyed. *Salmonella*, *Brucella*, *Listeria*, *Chlamydia*, *Mycobacterium*, *Rickettsia*, *Toxoplasma, Leishmania* and *Trypanosoma* species and *Legionella pneumophilia* have the capability of invading and inhabiting nonactivated macrophages (Nathan *et al.,* 1980). *Mycobacterium tuberculosis* may release sulpholipids that interfere with the fusion of primary lysosomes with phagosomes and therefore avoid exposure to the lysosomal enzymes of the macrophage (Shurin and Stossel, 1978). *Mycobacterium lepraemurium* and *Leishmania* survive within secondary lysosomes despite exposure to lysosomal enzymes, this might be because of the resistance of the microbial cell wall to the macrophages' degradative enzymes (Nathan *et al.,* 1980).

The aim of this chapter was to investigate the direct interaction of LPS from *S. minnesota* Ra with host macrophages by answering the following questions:

Does LPS play a role in mediating the attachment of Gram-negative bacteria to host macrophages in the absence of serum opsonins ?

Do different forms of LPS (Purified LPS, LPS attached to bacterial outer membranes, and LPS attached to intact bacterial cells) bind to the same receptor/s on host macrophages ?

Does LPS from different organisms bind to the same receptor/s on host macrophages ?

Do host macrophage receptors recognise a common ligand on different LPS types ?

3.2 Materials and methods

3.2.1 Buffered paraformaldehyde

1% paraformaldehyde was prepared by dissolving 10g of sodium cacodylate in one litre distilled water and the pH adjusted to 7.2 with HCl, then 10g of paraformaldehyde were added and dissolved by boiling the solution.

3.2.2 Serum-free Eagles medium

Serum-free Eagles medium was prepared by dissolving 1 g of Eagles medium powder (Gibco BRL, Paisly, UK) in 100 ml distilled water, then 1 ml 4.4% sodium bicarbonate and 0.5 ml 1M Hepes were added. The medium was incubated at 37° C in 5% CO_2 for 30 min to equilibrate the pH.

3.2.3 Labelling bacteria with FITC

This method is a modification of that described by Wright and Jong (1986). A heavy suspension of bacteria in PBS was prepared from bacteria grown overnight in nutrient broth by centrifugation at 1000 g for 20 min. Bacteria were washed three times in PBS by centrifugation at 1000 g for 20 min. Four hundred micrograms of fluorescein isothiocyanate (FITC) (Sigma, Poole, UK) were dissolved in 1 ml coupling buffer (0.05 M sodium carbonate + 0.1 M sodium chloride, pH 9.2) and added to the bacterial pellet. The mixture was incubated at 37° C for 20 min. Free FITC was removed by washing three times with PBS at 1000 g for 20 min. In some experiments bacteria were treated as above but the FITC was omitted.

3.2.4 Activation of mouse peritoneal macrophages

Three different preparations were used to activate mouse peritoneal macrophages and were prepared as follow: Glycogen was dissolved in distilled water to a final concentration of 1mg ml^{-1}. Thioglycollate (Difco, Surrey - UK) was dissolved in distilled water to a final concentration of 10% (w/v). *C. parvum* (Wellcome Biotechnology Limited, Beckenham- England) was diluted in normal saline to a final cocnentration of 1mg ml^{-1}.

Mice were injected intraperitoneally (ip) with 1 ml glycogen or thioglycollate or with 0.25 ml *C. parvum*. Four days after the injection of the above preparations mouse peritoneal cells were collected in PBS + B as described in section (2.3.1).

3.2.5 Binding assay for flow cytometry

Salmonella minnesota strain R 60 directly conjugated with FITC was used in this assay. After labelling, the bacterial count was adjusted to give the required ratios of bacteria to cells in the final mixture. 100 µl of cell suspension was mixed with 100 µl of each dilution of bacteria or PBS + B (control) in 15 ml centrifuge tubes and incubated under the desired incubating conditions. The macrophages were washed three times with PBS + B by centrifugation at 300 g for 10 min and fixed by final resuspension in 1% (w/v) buffered paraformaldehyde. Experiments were performed to study the effect of bacterial dose response, time course effect, temperature effect, divalent cation requirement, pH effect and macrophage activation state effect on the binding of *S. minnesota* Ra to macrophages. The pH effect was assessed by adjustment of the PBS to the desired pH by addition of NaOH or HCl. Divalent cation requirement was assessed by using PBS containing both 0.5 mM $CaCl_2$ and 0.5 mM $MgCl_2$, PBS containing 0.5 mM $CaCl_2$, PBS containing 0.5 mM $MgCl_2$ or PBS containing 10 mM EDTA. All concentrations stated here are final concentrations.

3.2.6 Analysis of cells by flow cytometer

Analysis of binding was performed on an EPICS 'C' flow cytometer (Coulter Electronics, Luton, UK) equipped with a 5 watt laser using a power output of 300 mw at 488 nm. The cells were selected from a display of forward angle light scatter versus 90° light scatter by means of a bit map (Fig. 3.1a). More than 1000 cells were analysed from each sample. The percentage of cells showing fluorescence greater than the background level was recorded on a one parameter histogram, measuring fluorescence on a logarithmic scale. The mean fluorescence channel values for the positive cells were acquired from a one parameter histogram, measuring fluorescence on a linear scale. The results were analysed by the immuno analysis program (Coulter).

In some experiments results were expressed as an attachment index obtained by multiplication of the percentage of positive cells by the mean fluorescence. This value gives an indication of the total fluorescence in a cell sample.

3.2.7 Inhibition assay for flow cytometry

Bacteria and macrophages were adjusted to give the desired ratio of bacteria per cell. Macrophges were incubated with different forms of LPS (free LPS, LPS attached to bacterial outer membranes and whole bacteria) for 30 min at 4° C. FITC-labelled bacteria were added to macrophages and incubated for a further 30 min at 4 °C. The macrophages were washed three times with PBS + B by centrifugation at 300 g for 10 min and fixed in 1% (w/v) buffered paraformaldehyde. Results were expressed as percentage inhibition of attachment compared to cells incubated in PBS + bacteria as measured by using flow cytometry.

3.2.8 Binding assay for microscopy

Glass circular cover slips (Chance Propper Ltd, Warly, UK) were soaked in detergent

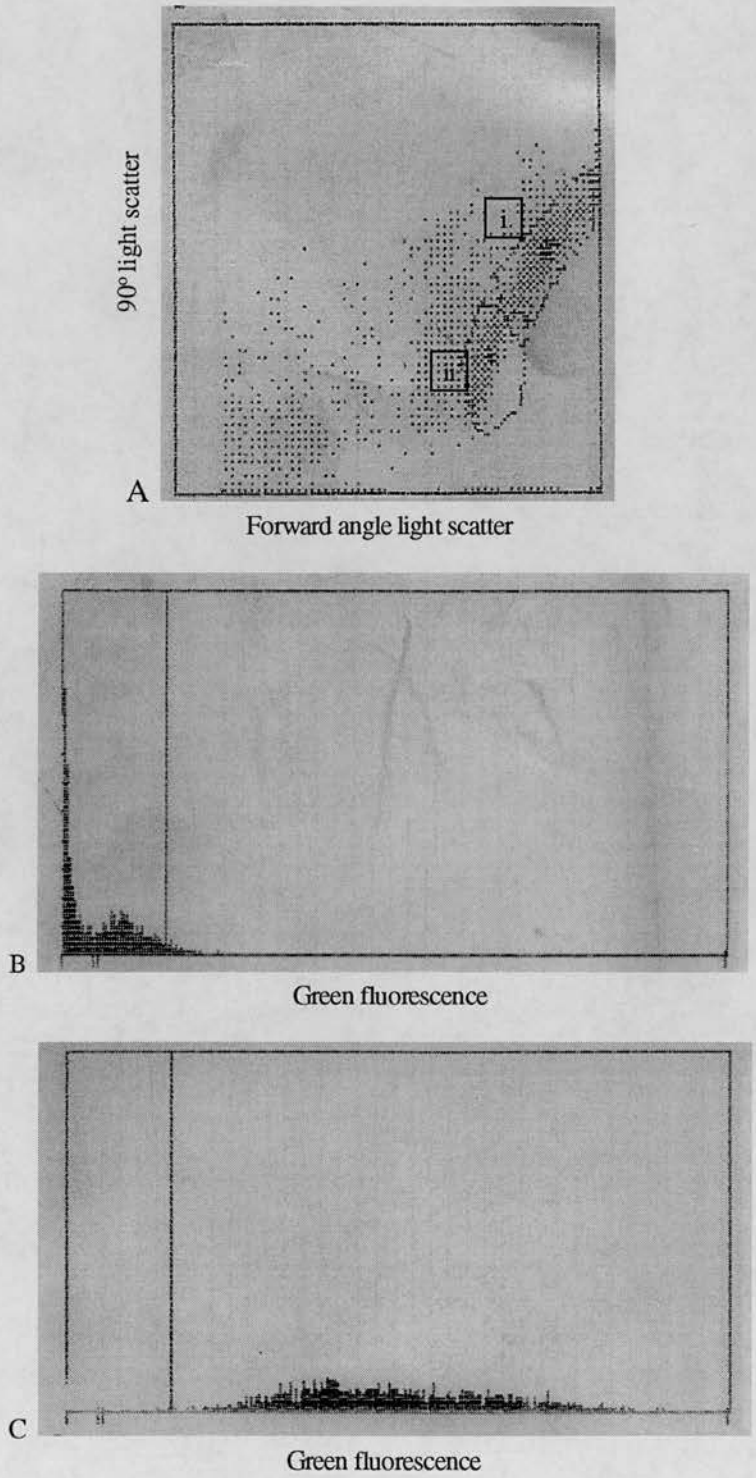

Figure 3.1a: (A) bit map of macrophages (i) and lymphocytes (ii), (B) histogram of negative control (macrophages without FITC-labelled bacteria) and (C) histogram of positive macrophages (cells with FITC-labelled bacteria attached to them) of a typical binding experiment.

and washed thoroughly with distilled water. Water was then drained and the cover slips were then immersed in 70% ethanol until used. Cover slips were flamed to dry and placed in 24 well tissue culture plates (Greiner Labortechnik Ltd, Dursley, Gloucestershire, UK). Peritoneal cell suspension in serum-free Eagles medium (1 ml at 4 x 10^5 cells/ ml) were placed in each well and incubated in a humidified 37° C incubator containing 5% CO_2 for 45-60 min to allow macrophages to adhere to the glass coverslips. Non-adherent cells were removed by washing three times with PBS+B. FITC-labelled and unlabelled bacteria (originally from the same preparation of bacteria) were suspended in PBS + B and their concentrations adjusted to give four ratios of bacteria to cells (30:1, 100:1, 300:1 and 1000:1). Bacteria were added to macrophage monolayers and incubated for 90 min at 4° C in the presence of divalent cations. Both preparations were treated identically in the binding assay. Duplicate coverslips from each bacteria dose of both preparations were prepared. Coverslips were then washed ten times with PBS, shaking the plates until hearing the rattling of the coverslips each time. Coverslips were dried out of the plates (on a pad of tissues), and replaced in a new plate and fixed with methanol for at least 20 min and dried. The coverslips were then stained with May Grünwald stain for three min, washed with tap water and stained for further three min with Giemsa stain. The coverslips were allowed to dry and finally mounted by inversion with DPX onto microscope slides and examined under the microscope. Results were expressed as percentage of macrophages with bacteria attached at two or more discrete points. At least 200 macrophages were counted per coverslip.

3.2.9 Inhibition assay for microscopy

Free LPS was added at different concentrations to macrophage monolayers and incubated for 30 min at 4° C. Free excess LPS was removed by washing with PBS, bacteria were added to macrophage monolayers and incubated for 60 min at 4° C. Unattached bacteria were removed by washing, and the monolayers fixed, stained

and mounted as described in section 3.2.9. Results were expressed as percentage inhibition of binding compared with untreated controls.

3.2.10 Statistics

The student's t-test was used for all statistical analysis.

The percentage inhibition of binding was calculated by using the formula:

a) for flow cytometry results:

[(Attachment index of control - attachment index of test)/ attachment index of control] x 100.

b) for visual assay results:

[(% binding of control - % binding of test)/ % binding of control] x 100.

3.3 Results

3.3.1 Optimisation of binding conditions

3.3.1.1 Bacterial dose response

The effect of varying the bacterial concentration on the attachment of FITC-labelled *S. minnesota* Ra to mouse peritoneal macrophages was studied by using four ratios of bacteria to macrophages at a temperature of 4° C and an incubation time of 30 minutes. The mean attachment index of three experiments is shown in Fig. 3.1. The attachment increased slightly and steadily when the bacterial to cell ratio was increased from 30 : 1 to 300 : 1. There was a sharp increase in the attachment when bacterial dose was increased to 1000 : 1. The percentages of macrophages binding bacteria at 1000: 1, 300: 1, 100: 1 and 30: 1 were 84%, 67%, 47% and 20% respectively. At a dose of 300 : 1 the attachment was significant and was at a point which allowed detection of increases or decreases in attachment. Therefore the dose of 300 : 1 was used in further attachment experiments.

3.3.1.2 Time course

The effect of incubation time on the attachment of *S. minnesota* Ra to mouse peritoneal macrophages was studied by using five incubation times at a temperature of 4° C and a bacterial dose of 300 : 1. The mean attachment index of three experiments is shown in Fig. 3.2. There was an initial rapid increase in the binding between 5 and 15 min, then the rate of binding slowed down until 60 min. After 60 min there was a rapid increase in the attachment which had not dropped or slowed down by 120 min incubation time, when the experiment was terminated. The percentages of macrophages binding bacteria at 30, 60 and 120 min were 72%, 72% and 77% respectively. Incubation for 30 min gave a significant attachment of bacteria to macrophages and was in the middle of the initial increase of the

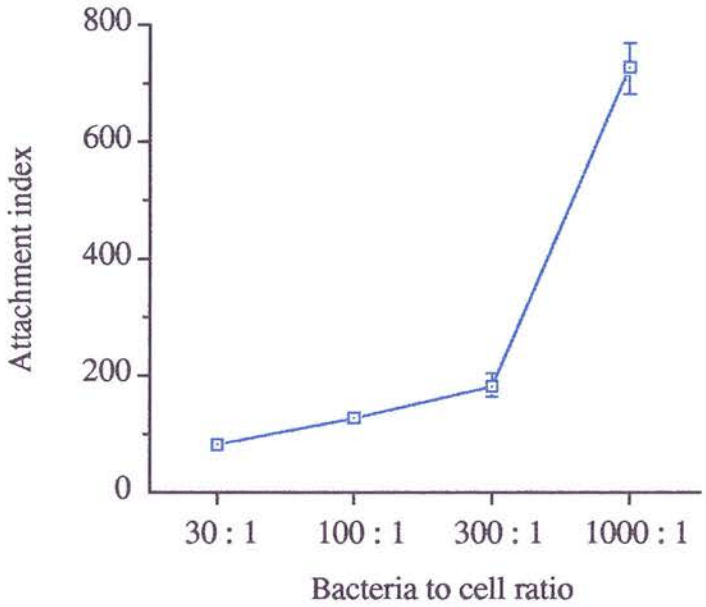

Figure 3.1: The effect of bacterial dose on the attachment of *S. minnesota* Ra to mouse peritoneal macrophages at 4° C and 30 min incubation time. Each point represents the mean attachment index of three experiments ± SEM

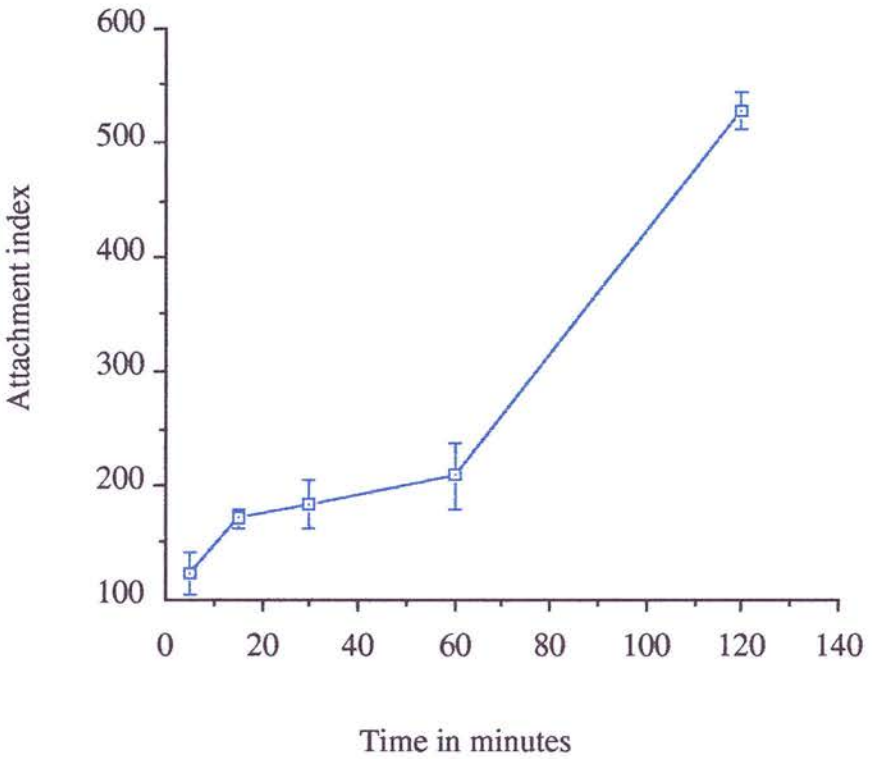

Figure 3.2 The effect of incubation time on the attachment of *S. minnesota* Ra to mouse peritoneal macrophages at 4° C by using bacterial dose of 300 : 1 macrophage. Each point represents the mean attachment index of three experiments ± SEM

attachment. With incubation times greater than 30 min, the number of cells recovered decreased. Therefore 30 min incubation time was selected for further attachment experiments.

3.3.1.3 Effect of temperature

The effect of temperature on binding of *S. minnesota* Ra to mouse peritoneal macrophages was studied by using three incubation temperatures and a bacteria to macrophage dose of 300 : 1 when incubated for 30 min. The mean attachment index of three experiments is shown in Fig. 3.3. The number of bacteria binding to cells rose as the incubation temperature was increased. The percentages of macrophages binding bacteria at 37° C , 20° C and 4° C were very similar, 66%, 65% and 63% respectively. The mean fluorescence level was higher at high temperatures, therefore the degree of binding was higher at higher temperatures than that at low temperatures. Although the lowest level of binding was seen at 4° C, the attachment at this temperature was significant and at a levels where increases or decreases of attachment can easily be detected. At this temperature phagocytosis would not occur and therefore the fluorescence signal would not be quenched by internalisation. Therefore 4° C was continued to be used in all further experiments.

3.3.1.4 Requirement for divalent cations

The role of divalent cations in the binding of *S. minnesota* Ra to mouse peritoneal macrophages was studied by using PBS (control) and PBS + EDTA (to deplete cations from the buffer). Ca^{2+} and Mg^{2+} were added separately and in combination to PBS. The assay was carried at 4° C and by using a bacterial dose of 300 : 1 and an incubation time of 30 min. The mean attachment index of three experiments is shown in Fig. 3.4. The attachment increased significantly by addition of Ca^{2+} as compared to control and the increase was statistically significant ($P < 0.05$). Mg^{2+} increased the attachment to a small degree, but this increase was not statistically

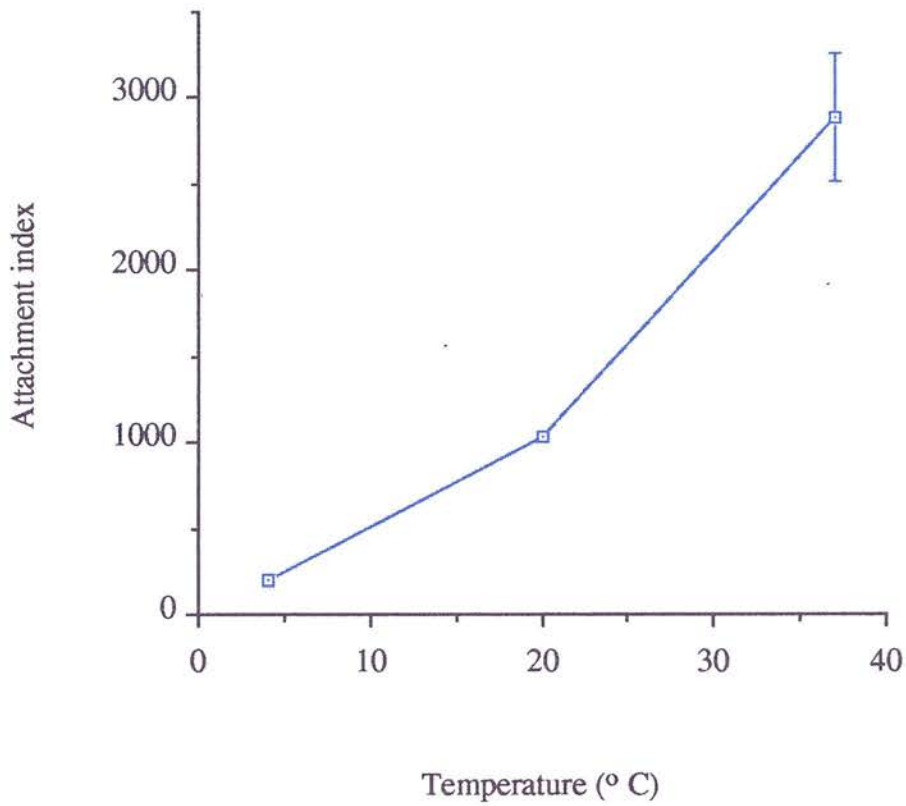

Figure 3.3: Temperature effect on the binding of *S. minnesota* Ra to mouse peritoneal macrophages at an incubation time of 30 minutes by using bacterial dose of 300 : 1 macrophage. Each point represents the mean attachment index of three experiments ± SEM

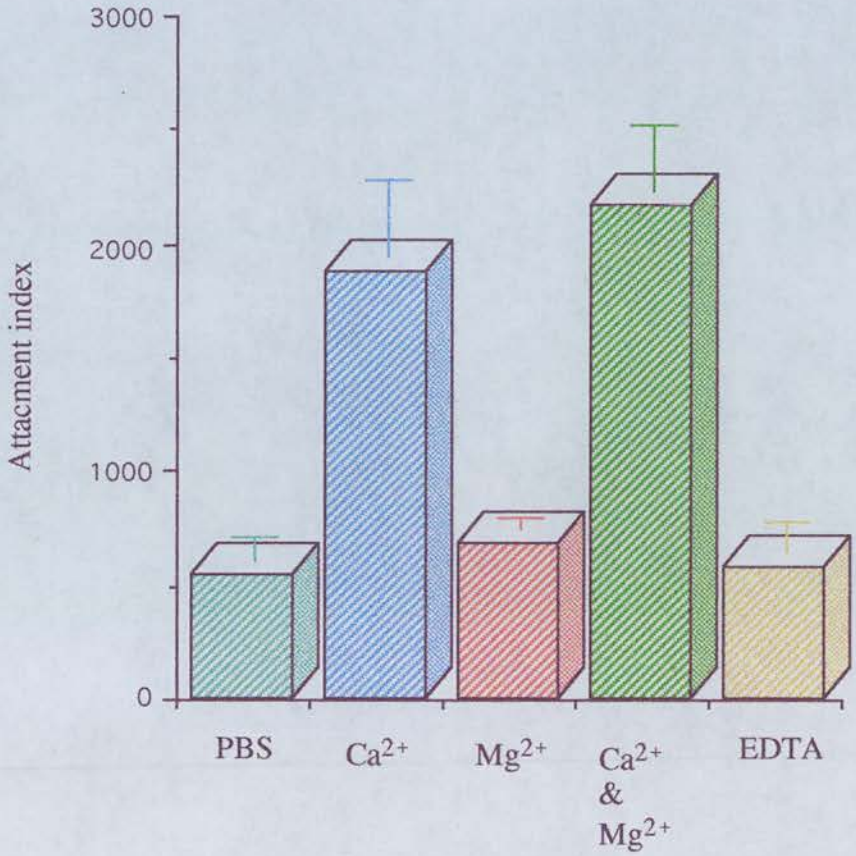

Figure 3.4: Effect of divalent cations on the binding of *S. minnesota* Ra to mouse peritoneal macrophages at 4° C and an incubation time of 30 min using a bacterial dose of 300 : 1 macrophage. Each column represents the mean attachment index of four experiments ± SEM

significant. The combination of both cations increased the attachment to a higher degree than Ca^{2+} did on its own and the increase was statistically significant ($P < 0.02$). EDTA gave similar results to the control suggesting that there were no cations available in control samples medium. Taking the above results together, divalent cations were used in all further experiments.

3.3.1.5 Effect of pH

The effect of pH was studied by using three pH values at 4° C and a bacterial dose of 300 : 1 and an incubation time of 30 min. The mean attachment index of three experiments is shown in Fig. 3.5. The attachment of bacteria to macrophages did not show a significant variation over the range used, therefore the physiological pH (7.2) was used in following experiments.

3.3.1.6 Effect of macrophage activation state on binding

Macrophages were elicited by ip injection of three different preparations in order to study the effect of state of mouse peritoneal macrophages activation on the binding of *S. minnesota* Ra. Bacterial dose of 300: 1 was used and an incubation time of 30 min at 4° C in the presence of divalent cations. The mean attachment index of three experiments is shown in Fig. 3.6. Glycogen and *Corynebacterium parvum* activation of macrophages increased the binding to a small degree and in both cases the increase was not statistically significant as compared to control macrophages (normal macrophages). Thioglycollate activation of macrophages increased the binding significantly and the increase was statistically significant as compared to control macrophages ($P < 0.02$). Administration of glycogen, *C. parvum* or thioglycollate resulted in a substantial increase in the number of macrophages obtained from the peritoneal cavity.

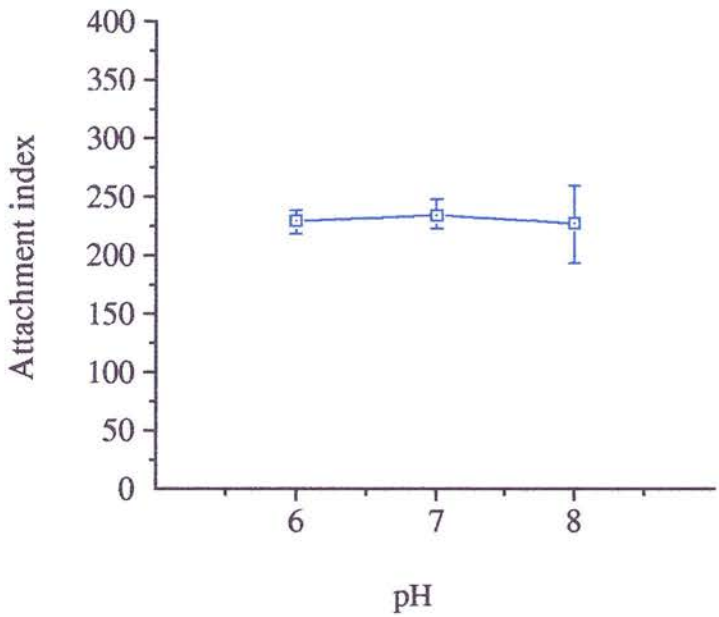

Figure 3.5: The effect of pH on the attachment *S.minnesota* Ra to mouse peritoneal macrophages at 4° C and an incubation time of 30 min by using a bacterial dose of 300 : 1 macrophage. Each point represents the mean attachment index of three experiments ± SEM

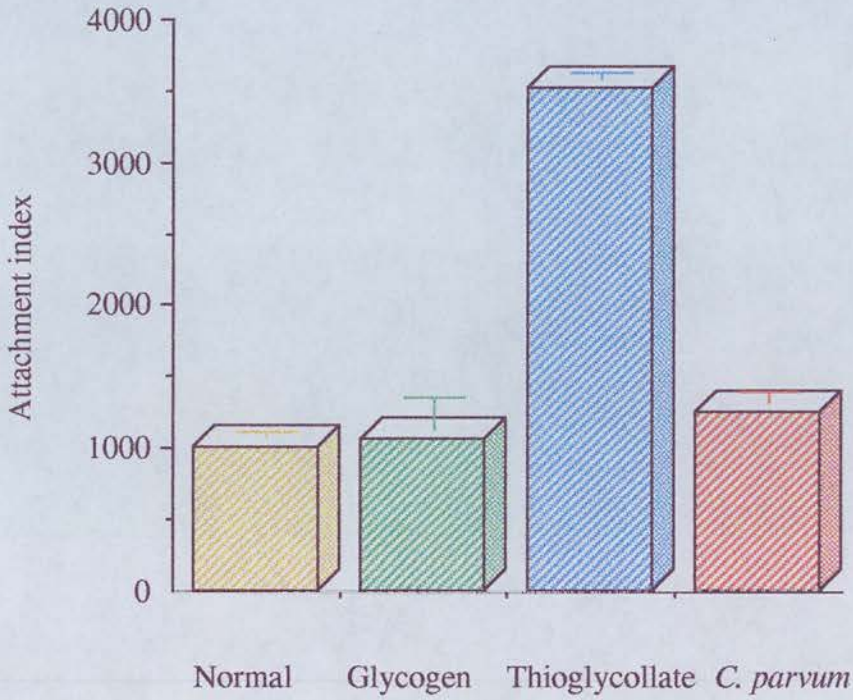

Figure 3.6: The effect of the state of macrophage activation on the attachment of *S. minnesota* Ra at 4° C and an incubation time of 30 min using bacterial dose of 300 : 1 macrophage. Each column represents the mean attachment index of three experiments ± SEM

3.3.1.7 Binding of *S. minnesota* Ra human macrophage cell line (THP-1)

THP-1 is a human immature macrophage cell line. The binding of *S. minnesota* Ra to THP-1 cells was investigated to find out if these cells display similar binding characteristics to those of mouse macrophages. The binding of *S. minnesota* Ra to THP-1 cells was tested by using four doses of bacteria at 4° C and an incubation time of 30 min. The mean attachment index of three experiments is shown in Fig. 3.7. The attachment of bacteria to THP-1 rose as the dose of bacteria was increased. There was a slight increase in the attachment between the doses 30: 1 and 300: 1 followed by a sharp increase in the attachment up to a dose of 1000: 1. The percentages of THP-1 binding bacteria at 1000: 1, 300: 1, 100: 1 and 30: 1 were 58%, 19%, 8% and 5% respectively.

3.3.1.8 Binding of *S. minnesota* Ra human peripheral blood monocytes and neutrophils

The binding of *S. minnesota* Ra to human peripheral blood monocytes and neutrophils was investigated in a few experiments. Mouse peritoneal macrophages were used for comparison. The binding of human monocytes and neutrophils and mouse macrophages were tested using four ratios of bacteria : cells at 4° C and incubation time of 30 min. The percentages binding and the mean fluorescence level of two experiments are shown in table 3.1. In experiment one the binding of human neutrophils was higher than the binding of human monocytes and mouse macrophages. The binding of human monocytes was slightly higher than that of mouse macrophages. In experiment two (sample from different individual) the binding of human monocytes was much higher than the binding of mouse macrophages. The percentage binding of human monocytes was similar to the percentage binding of human neutrophils, but the mean level fluorescence of the monocytes was higher than that of neutrophils.

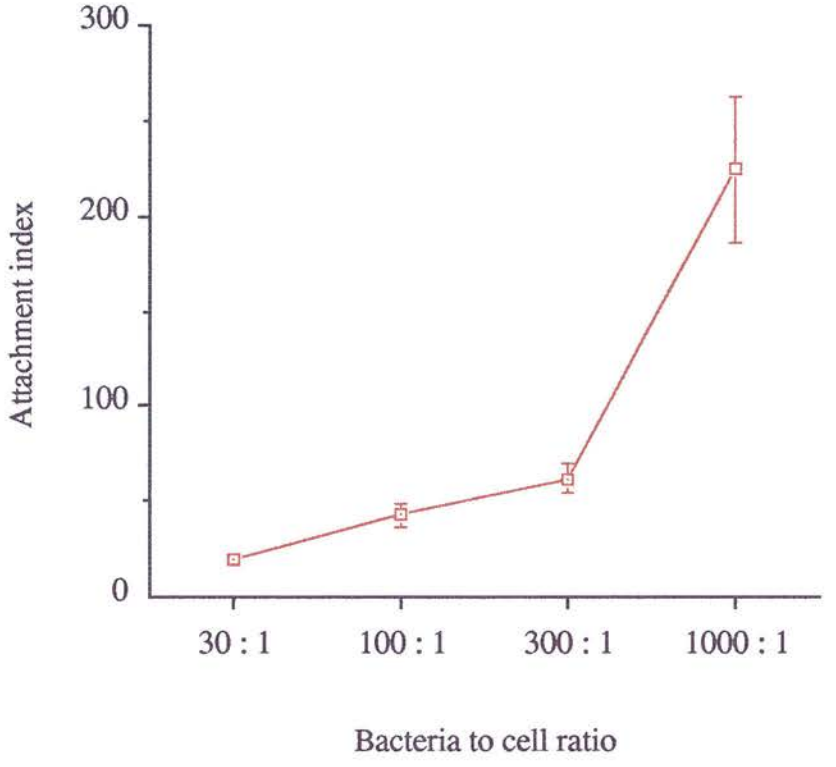

Figure 3.7: The binding of *S.minnesota* Ra to the human cell line, THP-1, by using four doses of bacteria to cells at an incubation time of 30 min. at 4° C. Each point represents the mean attachment index of three experiments ± SEM

Table 3.1: Binding of *S. minnesota* Ra to human peripheral blood monocytes and neutrophils.

Experiment 1

	Mouse peritoneal macrophages		Human peripheral blood monocytes		Human peripheral blood neutrophils	
bact.: cell	% binding	mean	% binding	mean	% binding	mean
1000:1	57	22	61	36	64	194
300:1	60	21	59	30	95	88
100:1	42	10	46	13	91	41
30:1	32	6	39	7	86	19

Experiment 2

	Mouse peritoneal macrophages		Human peripheral blood monocytes		Human peripheral blood neutrophils	
bact.: cell	% binding	mean	% binding	mean	% binding	mean
1000:1	78	17	91	225	96	78
300:1	56	12	85	145	91	56
100:1	34	7	73	84	79	27
30:1	19	6	51	36	58	12

3.3.2 Inhibition studies

3.3.2.1 Inhibition of binding of *S. minnesota* Ra to mouse peritoneal macrophages by using free LPS

Mouse peritoneal macrophages were incubated with various concentrations of purified *S. minnesota* Ra LPS then different numbers of bacteria were added to the macrophage suspension. The binding of LPS treated macrophages was compared to control macrophages (untreated macrophages). The results are expressed as percentage inhibition of attachment compared to control (see section 3.2.10). The mean percentage inhibition of binding of three LPS concentrations and two doses of bacteria per macrophage is shown in Fig. 3.8. All concentrations of LPS used gave significant inhibition of binding. At low dose of bacteria (100: 1 macrophage), the greatest level of inhibition (80%) was seen with the highest concentration of LPS (1mg ml^{-1}) and was statistically significant ($P < 0.005$). By using the same dose of bacteria and lower concentration of LPS (0.1mg ml^{-1}), the level of inhibition was decreased a little (61%) and was statistically significant ($P < 0.025$). Further decrease in LPS concentration (0.01mg ml^{-1}) resulted in more decrease in the level of inhibition (50%) that was still statistically significant ($P < 0.02$). At a higher dose of bacteria (300: 1 macrophage) the level of inhibition was 77% with LPS concentration of 1mg ml-1 ($P < 0.005$), 58% with LPS concentration of 0.1mg ml^{-1} ($P < 0.05$) and 35% with LPS concentration of 0.01mg ml-1 ($P < 0.02$).

Both the percentage of macrophages binding bacteria and the mean fluorescence level were notably decreased at higher concentrations of LPS. Lowering the concentration of LPS and increasing the bacterial dose led to a decrease in the level of inhibition. The percentages of macrophages binding bacteria rose gradually until they nearly matched the control. For example, in one experiment where a bacterial dose of 300: 1 macrophage was used, the percentage binding of the control

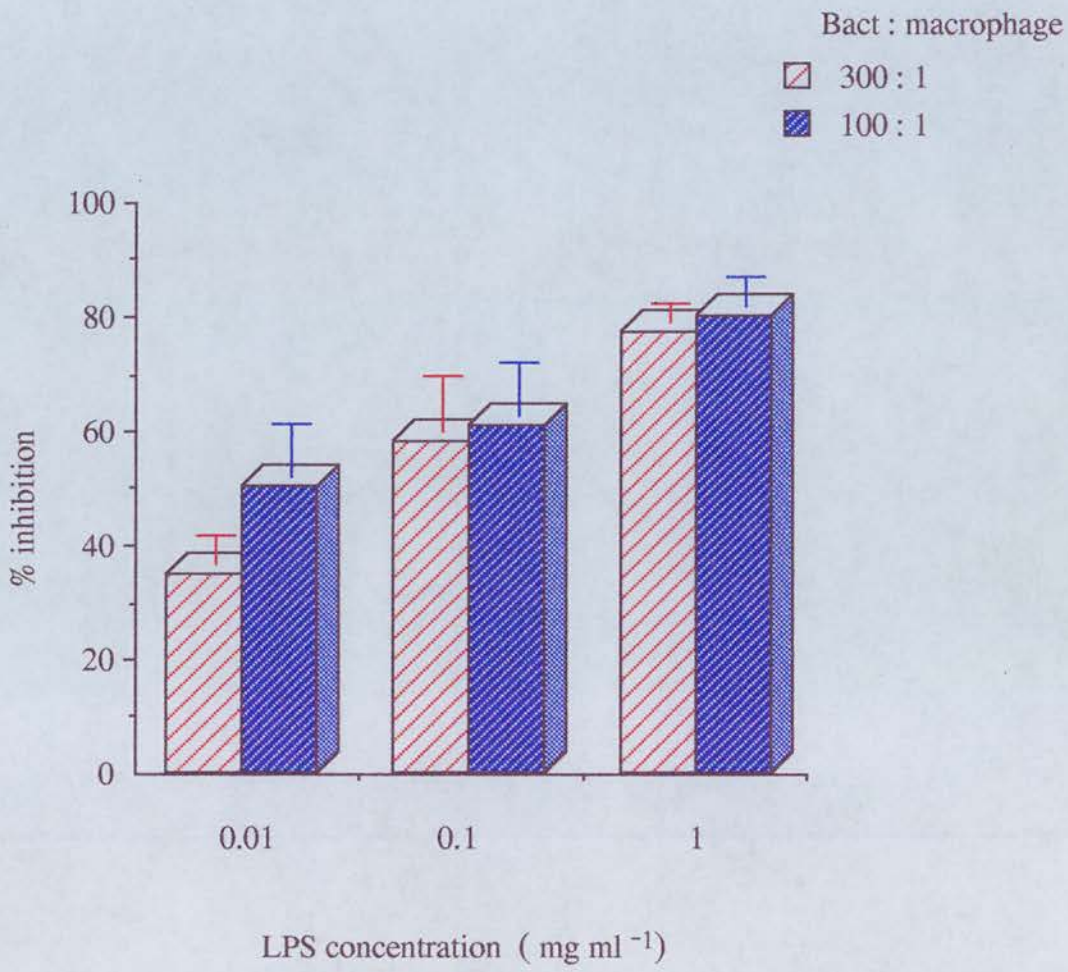

Figure 3.8: The inhibition of attachment of *S. minnesota* Ra to mouse peritoneal macrophages by purified LPS. Two bacterial doses were used and assay performed at 4° C. The attachment index of positive controls of three experiments at a ratio of 300 bacteria: cell were 1650, 1869 and 3933, and at a ratio of 100 bacteria: cell were 508, 540 and 1768. Each column represents the mean percentage inhibition of attachment of three experiments ± SEM

(untreated macrophages) was 74% and the percentages binding of LPS treated macrophages were; 41% by using 1mg ml^{-1} LPS, 58% by using 0.1mg ml^{-1} LPS and 65% by using 0.01mg ml^{-1} LPS. The mean fluorescence level increased when the concentration of LPS was lowered but never matched the mean fluorescence level of controls in any experiments.

3.3.2.2 Inhibition of binding of *S. minnesota* Ra to mouse peritoneal macrophages by using bacterial outer membranes (OM)

Mouse peritoneal macrophages were incubated with various concentrations of bacterial outer membranes prepared from *S. minnesota* Ra prior to incubation with FITC-labelled bacteria. The results were expressed as percentage inhibition of attachment (see section 3.2.10). Fig. 3.9 shows the mean percentage inhibition of binding using three outer membrane concentrations and a bacterial dose of 300 : 1. The LPS content of the outer membrane preparations was determined (see section 2.6.1) and the concentrations adjusted to give the same concentrations of LPS as in 3.3.2.1. Similar to free LPS, all outer membrane preparation concentrations significantly inhibited the binding. The greatest level of inhibition (64%) was seen with the highest concentration of OM (1mg ml^{-1}) and was statistically significant ($P < 0.02$). Lowering the OM concentration resulted in a decrease in the level of inhibition; 48% with OM concentration of 0.1mg ml^{-1} ($P < 0.05$) and 32% with OM concentration of 0.01mg ml^{-1} ($P < 0.01$). Both the percentage of macrophages binding bacteria and the mean fluorescence level were decreased at a high concentration of OM. The percentage of macrophages binding bacteria was increased as the OM concentration was decreased until it nearly matched the percentage binding of the control. In one experiment the percentage binding of the control was 70% and the percentage binding of OM treated macrophages were; 36 %, 58 % and 68 % by using OM concentrations of 1mg ml^{-1}, 0.1mg ml^{-1} and 0.01mg ml^{-1} respectively. Again, the mean level of fluorescence rose as the concentration of OM

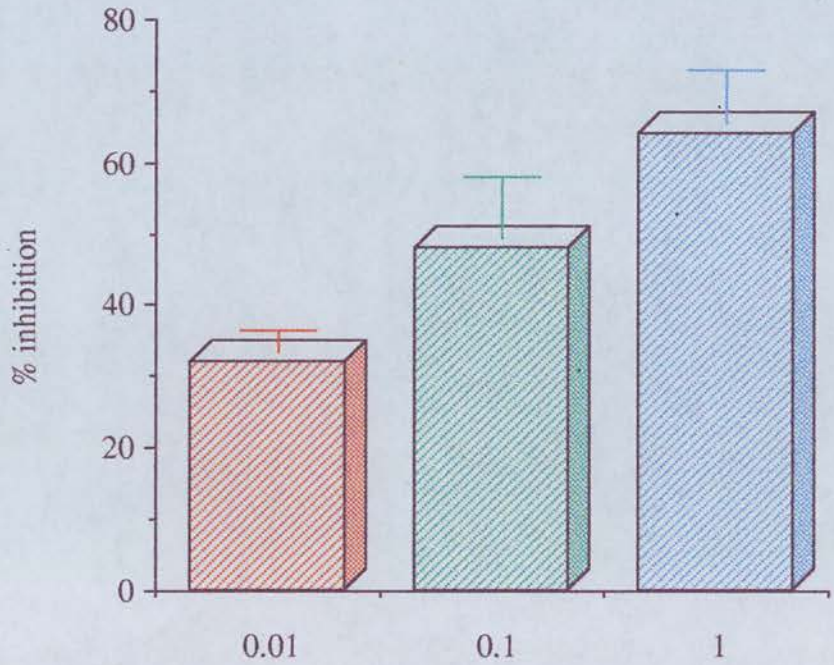

Figure 3.9: The inhibition of binding of *S. minnesota* Ra to mouse peritoneal macrophages by outer membrane preparations. Bacterial dose of 300 : 1 macrophage at 4° C. Each column represents the mean percentage inhibition of attachment of three experiments ± SEM

was decreased but did not match the mean fluorescence level of control even at the lowest OM concentration used in any experiments.

3.3.2.3 Inhibition of binding of *S. minnesota* Ra to TIIP-1 cells by free LPS

The binding of *S. minnesota* Ra to LPS treated THP-1 cells was compared to control cells (untreated cells) and the results expressed as percentage inhibition of attachment. The mean percentage inhibition by three LPS concentrations at two doses of bacteria per cell is shown in Fig. 3.10. Again the greatest inhibition of binding was observed with the highest LPS concentration and the lower ratio of bacteria to cells. At bacterial dose of 300: 1 THP-1 cell the percentage inhibition by using 1 mg ml^{-1} LPS was 44% ($p < 0.01$). The lowest inhibition of binding (10.7%) observed was at bacterial dose of 1000: 1 THP-1 cell and LPS concentration of 0.01 mg ml^{-1} ($p < 0.02$).

3.3.2.4 Inhibition by using whole bacteria

Whole unlabelled bacteria (*S. minnesota* Ra) were incubated with mouse peritoneal macrophages prior to the addition of FITC labelled bacteria. In eleven experiments whole unlabelled bacteria failed to inhibit the binding of labelled bacteria even when unlabelled bacteria were used at a dose of 10,000 : 1 macrophage (300 times excess unlabelled bacteria compared with labelled bacteria). This finding raised the question, has FITC labelling of bacteria enhanced the capacity of macrophages to bind bacteria ? To answer this question the effect of FITC labelling of bacteria on their attachment to macrophages was studied.

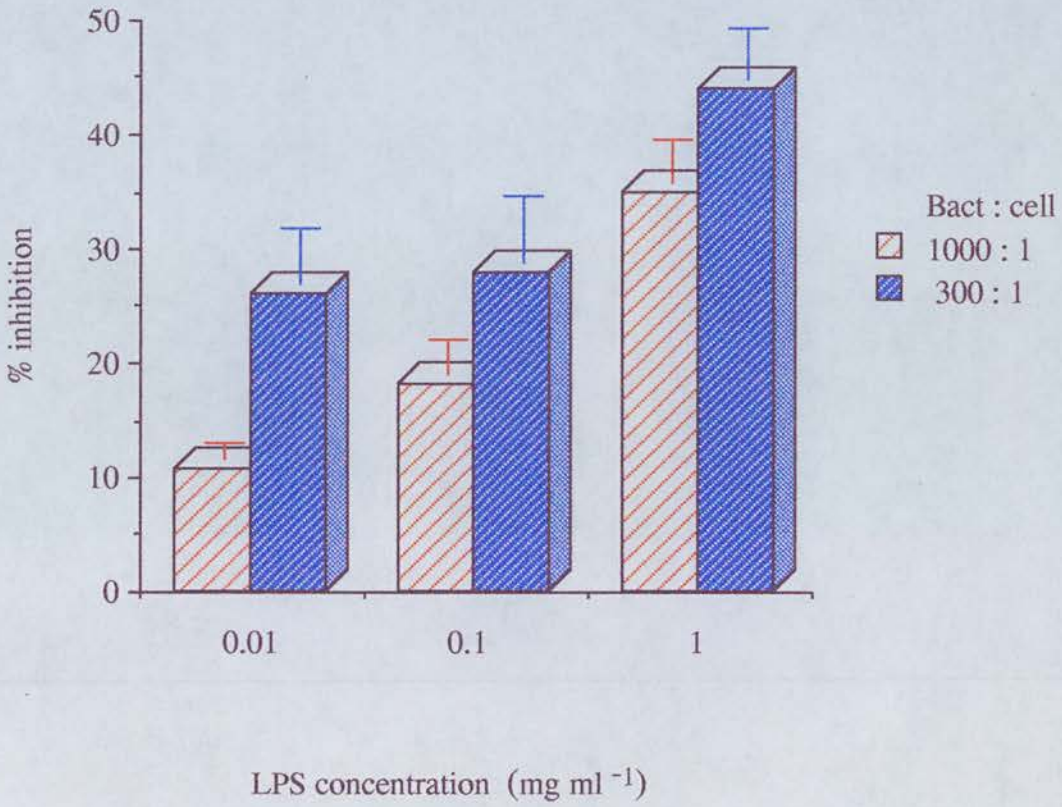

Figure 3.10: The percentage inhibition of binding of *S. minnesota* Ra to THP-1 cells by purified LPS at two bacterial concentrations, at 4° C. Each column represents the mean percentage inhibition of attachment of three experiments ± SEM

3.3.3 Effect of FITC labelling of bacteria on the binding to mouse peritoneal macrophages

To study the effect of FITC labelling on the binding, a method which would allow the detection of the binding of unlabelled bacteria was necessary. Transmitted light microscopy was selected for this part of the thesis.

3.3.3.1 Effect of FITC labelling of *S. minnesota* Ra on the binding to mouse peritoneal macrophages

Initially two preparations of bacteria were used, FITC-labelled and unlabelled bacteria.

FITC labelling of bacteria considerably enhanced the binding of bacteria to macrophages at all bacterial doses used (Fig. 3. 11). The increase in the attachment of all doses of FITC-labelled bacteria used was statistically significant as compared to the attachment of similar doses of unlabelled bacteria, at a dose of $3x10^9$ ml^{-1} ($p < 0.001$), at $3x10^8$ ml^{-1} ($p < 0.01$), at $3x10^7$ ml^{-1} ($p < 0.02$) and at $3x10^6$ ml^{-1} ($p < 0.01$). Equivalent binding of the two bacteria preparations was seen when the unlabelled bacteria were used at approximately 100-fold higher concentration than FITC-labelled bacteria. In one experiment the percentage binding of FITC labelled bacteria at dose of $3x10^7$ ml^{-1} was 27% and the percentage binding of unlabelled bacteria at a dose of $3x10^9$ ml^{-1} was 26%.

This finding was further examined to find out whether the enhancement of the binding was related to the FITC dye itself or was related to the high pH of FITC coupling buffer. Also the effect of FITC labelling on the binding of other bacterial species was examined.

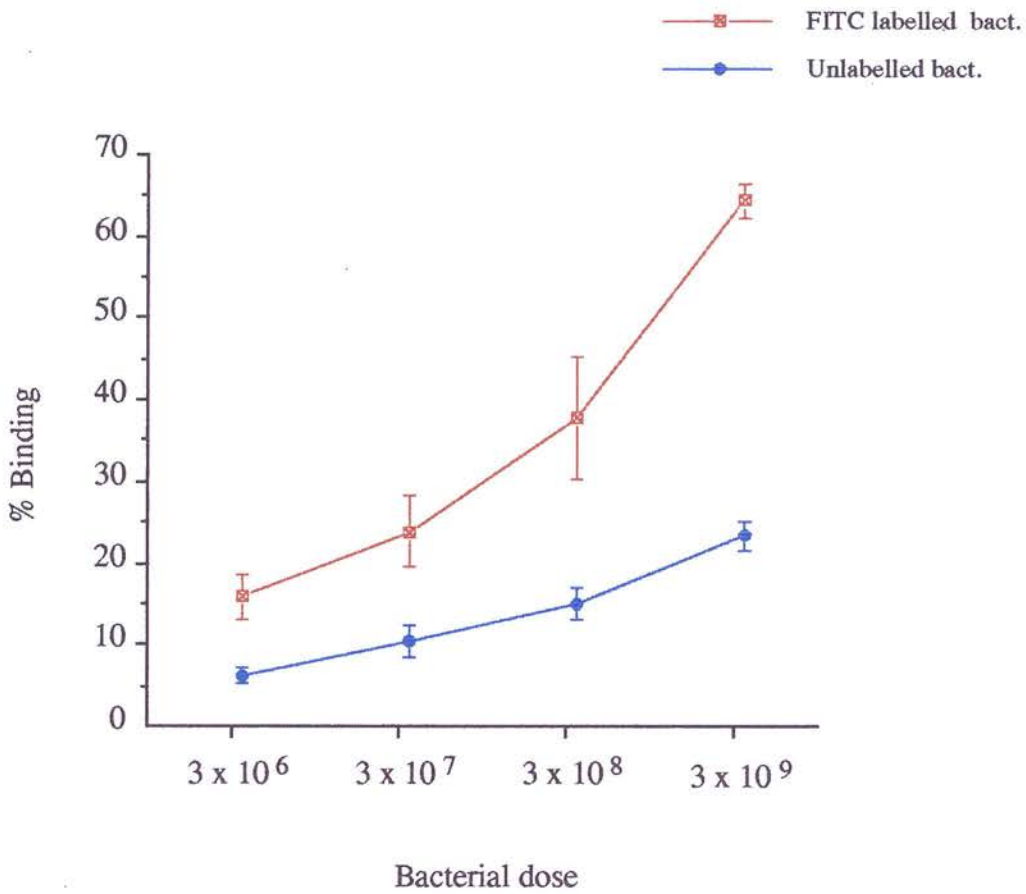

Figure 3.11: Comparison between the binding of FITC labelled and unlabelled *S. minnesota* Ra to mouse peritoneal macrophages at 4° C and an incubation time of 90 min in the presence of divalent cations. Each point represents the mean percentage binding of four experiments ± SEM

3.3.3.2 FITC effect on the binding of bacteria to mouse peritoneal macrophages

Bacteria harvested from the same preparation were split into three parts. One part was labelled with FITC as normal. The other two samples where treated as the first but without FITC. One of these samples was incubated in PBS and the other in FITC coupling buffer. The four different bacterial species used were treated in this way. A dose of 3 x 10^9 ml^{-1} was used for all bacterial species. Bacteria were added to monolayers of macrophages and incubated for 90 min at 4° C. Fig. 3.12 shows the binding of *S. minnesota* Ra, *E. coli* K 12, *N. meningitidis* and *Staph. epidermidis* to macrophage monolayers. FITC dissolving buffer treated bacteria showed no enhanced binding compared to PBS treated bacteria for all species used; however FITC labelling enhanced the binding of all species compared to PBS treated bacteria and the enhancement of binding of FITC labelled bacteria was statistically significant for*S. minnesota* Ra ($p < 0.05$), *E. coli* K 12 ($p < 0.05$), *N. meningitidis* ($p < 0.05$) and for *Staph. epidermidis* ($p < 0.05$).

3.3.3.3 Inhibition of binding of FITC-labelled and unlabelled *S. minnesota* Ra by purified LPS to mouse peritoneal macrophages

FITC-labelled bacteria at 3.0 x 10^7 ml^{-1} and unlabelled bacteria at 3.0 x 10^9 ml^{-1} were added to macrophage monolayers previously incubated with purified LPS for 30 min at 4° C. The bacteria were incubated with treated macrophage monolayers for 60 min at 4° C. The mean percentage inhibition of binding of both bacterial preparations is shown in Fig. 3.13. The inhibition of binding was similar between both preparations of bacteria at all LPS concentrations used. The more LPS used the greater the inhibition. The binding of unlabelled bacteria at a dose of 3.0 x 10^9 ml^{-1} was inhibited by all LPS concentrations used. At a high concentration of LPS (1mg ml^{-1}) the inhibition level was (33%) and was statistically significant ($P < 0.005$). By lowering LPS concentration (0.1mg ml^{-1}), the level of inhibition was

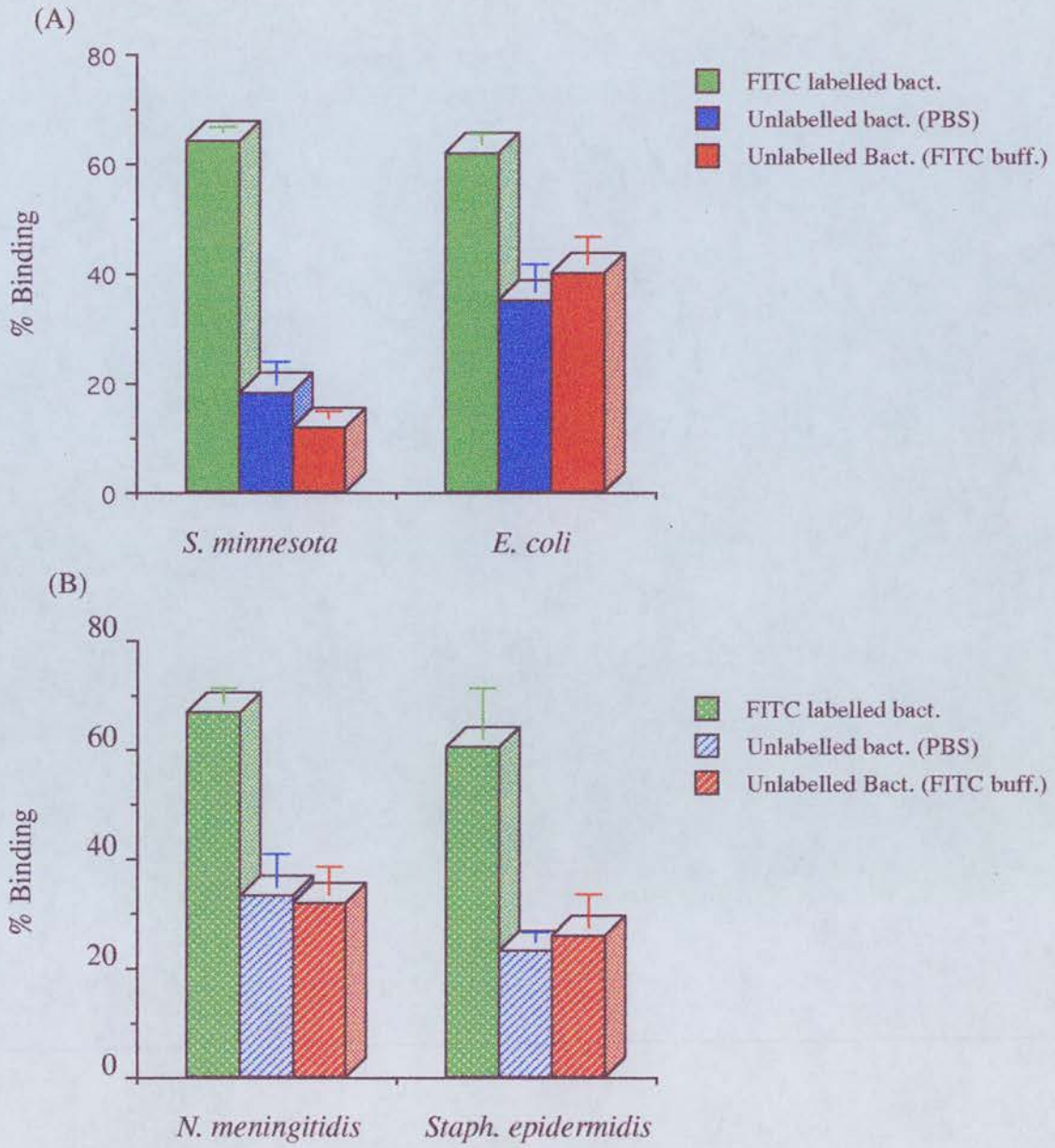

Figure 3.12: Effect of labelling bacteria with FITC on their binding to mouse peritoneal macrophages. (A) *Salmonella minnesota* Ra and *Escherichia coli* K 12. (B) *Neisseria meningitidis* and *Staphylococcus epidermidis.* Bacterial concentration of 3 x 10^9 ml^{-1} and macrophage concentration of 4 x 10^5 ml^{-1}. The assay was at 4° C and an incubation time of 90 min in the presence of divalent cations. Each column represents the mean percentage binding of four experiments ± SEM

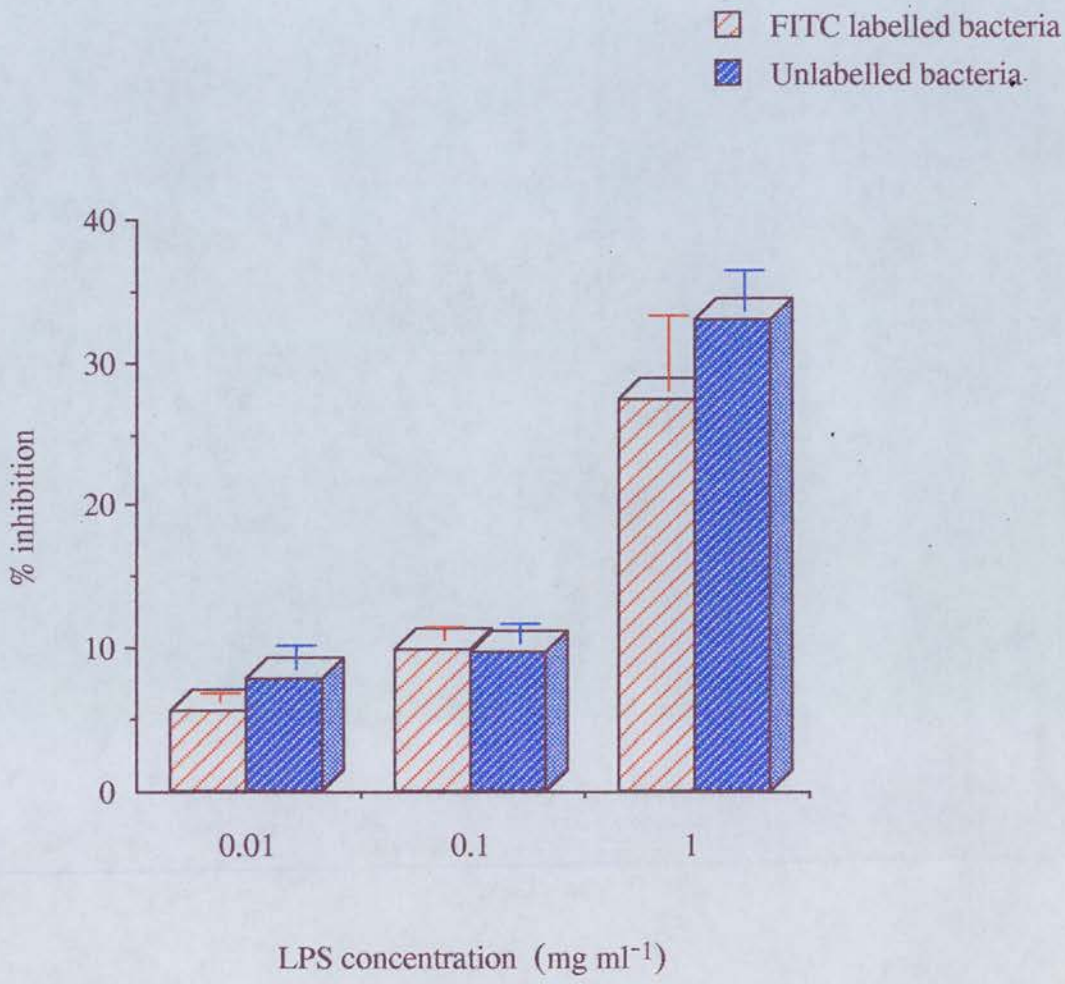

Figure 3.13: Comparison of the percentage inhibition of binding of FITC labelled *S. minnesota* Ra at a dose of 3.0 x 10^7 ml^{-1} and unlabelled *S. minnesota* Ra at a dose of 3.0 x 10^9 ml^{-1} to mouse peritoneal macrophages by purified LPS. Each column represents the mean percentage inhibition of four experiments ± SEM

decreased (9.7%) and was statistically significant ($P < 0.005$). Further decrease in LPS concentration (0.01mg ml^{-1}) resulted in a greater decrease in the level of inhibition (7.8%) which was still statistically significant ($P < 0.02$). By using FITC-labelled bacteria at a dose of 3.0×10^7 ml^{-1} the level of inhibition was 27.3% with LPS concentration of 1mg ml^{-1} ($P < 0.02$), 9.8% with LPS concentration of 0.1mg ml^{-1} ($P < 0.001$) and 5.6% with LPS concentration of 0.01mg ml^{-1} ($P < 0.005$).

3.3.3.4 Detection of the binding of unlabelled bacteria using monoclonal antibodies (mAb)

In one experiment the binding of unlabelled *S. minnesota* Ra at a dose of 1000:1 mouse peritoneal cells was detected using two concentrations of murine anti *Salmonella* LPS mAb (250 μg ml^{-1} and 25 μg ml^{-1}) and two concentrations of FITC conjugated anti-mouse immunoglobuln. The percentages binding and the mean level fluorescence of samples are shown in Table 3.1. The highest percentage of binding obtained using this method was 48% and the mean fluorescence level of this sample was 45. In the same experiment *S. minnesota* Ra (from the same bacterial preparation) directly labelled with FITC at a dose of 1000:1 macrophages was used for comparison. The percentage binding of FITC labelled *S. minnesota* Ra to macrophages was 92% and the mean fluorescence level was 294.

Table 3.2: Detection of the binding of *S. minnesota* Ra to mouse peritoneal macrophages as detected by mAb.

Anti *Salmonella* LPS mAb concentration	% Positive	
	FITC conjugated antibody concentration	
	1/50	1/200
250 µg/ml	42 (54)	34 (22)
25 µg/ml	48 (45)	30 (21)
FITC-labelled bacteria	92 (294)	

Bacteria were used at a ratio of 1000: 1 macrophage. Numbers in brackets represent the mean fluorescence of the samples

3.4 Discussion

This work aimed to investigate the role of LPS in mediating Gram-negative bacteria attachment to leucocytes. The First stage was to measure direct interaction of Gram-negative bacteria with mononuclear phagocytes; therefore a flow cytometric assay to measure attachment of *Salmonella minnesota* Ra to mouse peritoneal macrophages was used. C3H mouse strain, conventionally used in the field of LPS studies, was mainly used in this work. Collection of mouse peritoneal macrophages was a more convenient source of macrophages than human peripheral blood monocytes. This species also allowed *in-vivo* modulation of the macrophage population. Therefore mouse macrophages were selected for this work.

The use of flow cytometry in studying phagocyte functions was described by Bjerknes and co-workers (1989). Other investigators have used flow cytometry to study bacterial attachment to different cell types. Raza and co-workers (1993) reported that the infection of human epithelial cell line (HEp-2) with respiratory syncytial virus (RSV) significantly enhanced the binding of three strains of *Neisseria meningitidis* and two strains of *Haemophilus influenzae* using flow cytometry. Others (Raybourne and Bunning, 1994) used flow cytometry to study phagocytosis by macrophage cell line (J774A.1) and short-term survival of *Salmonella typhimurium* and *Listeria monocytogenes.*

Flow cytometry was used to determine the attachment of bacteria to macrophages. By using this method the percentages of cells binding bacteria as well as the mean level of binding were obtained. This method apparently overcame some of the difficulties associated with studying the binding of bacteria to the cells. First, unlike counting bacteria bound to cells by light microscopy, attachment assessed by the flow cytometer is objective. Second, large number of cells (> 1200 cells in each sample) can be analysed in a short period of time.

The initial work in this study was to optimise the conditions for investigating the binding of *S. minnesota* Ra to mouse peritoneal macrophages.

Under the experimental conditions used, attachment of bacteria to macrophages was increased when the ratio of bacteria to macrophage was increased. Similar observation using a visual assay had been reported by Weir and co-workers (1981). By increasing incubation time the level of attachment was increased due to allowing more time for the attachment to occur. The attachment was greater at physiological temperatures than that at lower temperatures, and this result was in agreement with the work of Wright and Jong, (1986). They reported the presence of receptors on human macrophages that bind *E. coli* by recognising LPS. The presence of Ca^{2+} and Mg^{2+} ions plays a role in part of the interaction between bacteria and macrophages. This might indicate the involvement of lectin-like interaction, in which the presence of Ca^{2+} is known to be required (Cook and Bugg 1975), and this result was in agreement with the work of Wright and Jong, (1986) who reported the requirement of divalent cations for the binding of *E. coli* to human macrophages. Over the pH range tested, attachment of bacteria to macrophages did not show significant variation.

Activated macrophages differ from resting macrophages morphologically, biochemically and functionally. They attach more rapidly to glass and plastic and also exhibit increased rate and speed of phagocytosis, probably due to increased expression of receptors. They are able to destroy microorganisms intracellularly and large parasites or tumour cells extracellularly.

The possibility of using activated macrophages in this study was investigated. Following in vivo activation there are increased number of macrophages present in the peritoneal cavity which means fewer mice are required for experimental work. Activation of macrophages with glycogen and *C. parvum* increased attachment to a

small degree but this increase was not statistically significant. Activation of macrophages with thioglycollate increased the attachment to a high degree and was statistically significant compared to control macrophages. These results were in disagreement with other investigators (Glass *et al.*, 1983) who were investigating membrane changes in murine macrophages after *in-vivo* stimulation or activation. They reported that activated mouse peritoneal macrophages bound fewer unopsonised bacteria compared to normal macrophages. The increase in bacterial binding mediated by thioglycollate activated macrophages was of interest, but because the mechanism was not clear and because the increased binding of bacteria mediated by activated macrophages contrasted with the work of Glass and co-workers (1983), it was felt that it would be safer to use normal macrophages in further experiments.

The attachment of *S. minnesota* Ra to the human macrophage cell line THP-1 was tested under similar conditions to those used for mouse macrophages to find out if human cells have similar binding characteristics to mouse macrophages. THP-1 cells showed similar binding patterns to mouse macrophages although THP-1 cells bind bacteria to a lower extent. The low bacterial binding capacity of THP-1 cells as compared to mouse peritoneal macrophages might be because of their maturation state. THP-1 are an immature macrophage cell line and might express fewer receptors than mouse peritoneal macrophages.

Human peripheral blood monocytes and neutrophils were used in a few experiments to compare their binding characteristics with mouse peritoneal macrophages. Human monocytes and neutrophils from different individuals showed variable binding characteristics. Binding of human monocytes to *S. minnesota* Ra showed similar levels of binding to mouse peritoneal macrophages in a few experiments. Human peripheral blood neutrophils showed much higher levels of binding to *S. minnesota* Ra than that of mouse peritoneal macrophages in these experiments. In

other experiments human monocytes showed higher levels of binding than that of human neutrophils and mouse macrophages. There, therefore, appears to be differences in the binding ability of cells from different individuals. This could be due to genetic or environmental factors. There might be inherited differences in the degree of expression of receptors for bacteria and the levels of expression or affinity might be altered by environmental factors such as infection or exposure to certain stimuli.

After setting up the optimal experimental conditions for binding, the role of LPS in the attachment of bacteria to macrophages was investigated. Various forms of LPS (free LPS, LPS attached to bacterial outer membranes and LPS attached to whole bacteria) were used. The use of different forms of LPS was because of LPS might be present in the form of whole bacteria inside the host circulation or LPS might be found attached to bacterial outer membranes as a result of bacterial lysis. LPS might also shed free from bacteria as a result of bacterial growth and multiplication or translocate from the gut into the circulation. On reaching the host circulation free LPS is normally complexed immediately to host proteins and lipoproteins. Free LPS inhibited the binding of bacteria significantly. The inhibition of binding was greater when high concentrations of LPS and low ratios of bacteria to macrophages were used. LPS attached to bacterial outer membranes (at similar concentrations to free LPS as determined by KDO assay) inhibited the binding of bacteria to macrophages in a similar way to free LPS. These results indicated that LPS was involved in the interaction between bacteria and cells in the assay system used. Attachment of bacteria to THP-1 cells was also inhibited by using free LPS at a similar concentrations used to inhibit the binding of bacteria to mouse macrophages. Because of variation in day to day level of binding it was necessary to express results as percentage inhibition of binding. This standardised the data from day to day and allowed comparisons to be made.

As part of the inhibition study, whole unlabelled bacteria (as another form of LPS) were pre-incubated with mouse peritoneal macrophages before FITC-labelled bacteria were added. The results were unexpected, whole unlabelled bacteria failed to inhibit the binding of FITC-labelled bacteria. This finding raised a number of questions about the assay. Were there enough whole unlabelled bacteria to inhibit the binding of FITC-labelled bacteria ? This was investigated by increasing the dose of whole unlabelled bacteria to macrophages while keeping the ratio of FITC-labelled bacteria to macrophages unchanged. Whole unlabelled bacteria failed to inhibit the binding of FITC-labelled bacteria even when the ratio of whole unlabelled bacteria to macrophages was 300 times greater than the ratio of FITC-labelled bacteria to macrophages. This indicated that the failure of whole unlabelled bacteria to inhibit the binding of FITC-labelled bacteria was not due to shortage of whole unlabelled bacteria. Also previous studies using a visual assay (Glass *et al.*, 1983) with activated macrophages showed contrasting results to ours. These workers found that murine peritoneal macrophages activated by *C. parvum* and glycogen bound fewer bacteria than untreated controls. The present study found that murine peritoneal macrophages activated by *C. parvum* and glycogen bound bacteria to a similar level as untreated controls. Also in this study thioglycollate activated murine peritoneal macrophages bound much greater numbers of bacteria than untreated controls. At this point the ability of mouse peritoneal macrophages to bind to unlabelled *S. minnesota* Ra was questioned. Therefore another method which would enable the detection of unlabelled bacteria was sought. By using a murine monoclonal antibody directed against *S. minnesota* Ra LPS and FITC conjugated antimouse immunoglobulin it was possible to detect the binding of unlabelled *S. minnesota* Ra to mouse peritoneal macrophages by flow cytometry. The sensitivity of this method for detecting bacterial attachment was lower than that obtained by using bacteria directly labelled with FITC. This method gave positive results, and although it confirmed that unlabelled *S. minnesota* Ra were binding to macrophages

in the absence of serum, it was not practical to use it in further studies. Low level of binding detected was one problem. More importantly it was not possible to use this method for inhibition experiments. In these experiments different forms of LPS were used as an inhibitors of the binding of *S. minnesota* Ra to mouse peritoneal macrophages. Because the monoclonal antibody was directed against *S. minnesota* Ra LPS it was not possible to use it in inhibition experiments using various forms of *S. minnesota* Ra LPS.

Other questions concerning the specificity of the binding of FITC-labelled bacteria to macrophages were raised by the lack of inhibition by whole unlabelled bacteria. First, does the high pH (9.2) of FITC coupling buffer affect the binding of bacteria to the macrophages ? Second, does the FITC itself affect the binding of bacteria to macrophages ? These questions led to a study on the effect of FITC labelling of bacteria on the binding to macrophages. To study the effect of FITC labelling of bacteria on the binding, a visual assay was used. This method allowed direct comparison between the binding of FITC-labelled bacteria and unlabelled bacteria to macrophages. Visual assays were used in the past by other investigators to study bacterial binding to different cell types, mouse peritoneal macrophages (Freimer *et al.,* 1978; Glass *et al.,* 1983), buccal epithelial cells (Bagg *et al.,* 1982) and human macrophages (Wright and Jong, 1986).

By using the visual assay FITC-labelled bacteria were found to bind to macrophages to much higher levels compared to unlabelled bacteria. To obtain similar levels of binding of unlabelled and FITC-labelled bacteria to macrophages, unlabelled bacteria had to be used at concentrations 100 times higher than FITC-labelled bacteria. This observation was further investigated to find out whether the enhancement of the binding seen with FITC-labelled bacteria was specific to the FITC itself or to the FITC coupling buffer. The effect of FITC labelling on the binding of different bacterial species, including Gram-positive bacteria was also investigated. The

bacteria used to investigate the effect of FITC labelling of bacteria on the binding to macrophages were selected because they were previously used by other investigators who used FITC-labelled bacteria in their studies. *E. coli* K 12 was used by Wright and Jong (1986), *Neisseria meningitidis* has been used in a number of studies (Raza *et al.,* 1993; Zorgani *et al.,* 1994) and Saadi *et al.,* (1993) used *Staphylococcus aureus*. FITC labelling of all bacterial species used enhanced the binding of bacteria to macrophages as compared to the binding of control bacteria (bacteria suspended in PBS). FITC coupling buffer did not affect the binding of any bacterial species used as compared to the binding of control bacteria (bacteria suspended in PBS). These results indicated that the enhancement of the binding seen with FITC-labelled bacteria was specific to the FITC and not to the FITC coupling buffer. The enhancement of the binding was not specific to the strain of *S. minnesota* Ra used in this study, but also applied to other bacterial species.

The enhancement of binding shown by FITC-labelled *E. coli* K 12 in this work is in disagreement with previous studies (Wright and Jong, 1986) in which the investigators used fluorescence microscopy to detect the binding of FITC-labelled *E. coli* K 12 to human macrophages. This method allowed easy distinction between fluoresceinated bacteria and similarly sized macrophage organelles. They claimed that in control experiments in which transmitted-light microscopy was used to measure the binding of bacteria to macrophages after Wright-Giemsa staining of samples indicated that FITC did not alter the extent or specificity of binding. This data was not shown in their paper. Although the level of enhancement of binding of FITC-labelled *E. coli* K *12* in our work was the lowest among the bacteria used, it is still significant.

The enhancement of binding of FITC-labelled bacteria might be due to non-specific recognition of the FITC by the macrophages. Alternatively it might be because FITC is altering the structure of bacterial cell surface component/s or having some

kind of charge effect. It might also be changing the hydrophobicity of bacterial cells. The exact mechanism for the enhanced binding of FITC-labelled bacteria is not known; however, it was felt that this line of investigation could not be continued as the physiological significance of studying the interaction between host cells and FITC-labelled bacteria had been called in question.

The inhibition of binding of *S. minnesota* Ra to mouse peritoneal macrophages was investigated using the visual assay. The binding of *S. minnesota* Ra to macrophages was inhibited using different concentrations of free LPS. When similar concentrations of LPS were used to inhibit the binding of FITC-labelled and unlabelled bacteria, similar levels of inhibition were seen for both preparations of bacteria although unlabelled bacteria were used at a concentration 100 times higher than FITC-labelled bacteria. The level of inhibition was greater when higher concentrations of LPS were used.

The binding of *S. minnesota* Ra to macrophages in the absence of serum is shown in this chapter. The involvement of LPS in the interaction with host cells was also shown in that LPS inhibited the binding of *S. minnesota* Ra to macrophages.

Planned work could not be continued using flow cytometry because of the problems concerning FITC labelling of bacteria. Also it was not practical to continue the work using visual assay. The visual assay is time consuming and subjective to some extent; therefore, further investigations about the direct interaction of LPS with host cells such as the binding of different Gram-negative bacteria to host cells, the inhibition of binding of these Gram-negative bacteria to host cells using different forms and types of LPSs, and investigation of whether different types of LPSs bind to the same receptor/s on host cells could not be continued.

At this point the study moved on to investigate the release of cytokines and mediators from host cells as another form of their interaction with LPS.

Chapter 4

Production of tumour necrosis factor (TNF) and nitric oxide (NO) in response to various forms of LPS

4.1 Introduction

Upon stimulation with bacterial products, including LPS, mononuclear phagocytes release a variety of cytokines and mediators including tumour necrosis factor and nitric oxide.

TNF includes two structurally and functionally related proteins, TNF-α or cachectin (Beutler *et al.*, 1985b) which is mainly produced by monocytes and/or macrophages and TNF-β or lymphotoxin (Ruddle and Waksman, 1968) which is the product of lymphoid cells. The close relationship between these two proteins was not known until 1984 when cloning of the cDNAs for human TNF and lymphotoxin revealed that they are about 30% homologous at the amino acid level (Gray *et al.*, 1984; Pennica *et al.*, 1984). TNF-α and TNF-β bind to the same cell surface receptor (Aggarwal *et al.*, 1985) and they are very similar (but not identical) in the spectra of their activities. TNF-α and TNF-β (especially TNF-α), originally thought of as an anti-tumour agents, are now grouped among the major inflammatory cytokines, i.e. they are characteristically produced at the sites of inflammation by infiltrating mononuclear cells. They play a beneficial role as immunostimulants and important mediators of host resistance to many infectious agents and malignant tumours (Old, 1985; Havell, 1987). On the other hand TNF-α has been shown to be identical to cachectin and has been postulated to mediate wasting during chronic infections (Beutler *et al.*, 1985). There is increasing evidence that over production of TNF-α during infections leads to severe systemic toxicity and even death. TNF-α is a major factor in the development of septic shock following infection with Gram-negative bacteria (Tracy *et al.*, 1987). TNF-α also has been implicated in the pathogenesis of some autoimmune disorders (Pujol-Borrell *et al.*, 1987) and of graft-versus-host disease (Piguet *et al.*, 1987).

Nitric oxide (NO), synthesised from L-arginine, is a potent effector molecule produced by many mammalian cell types (Moncada *et al.*, 1991). Nitric oxide accounts for the biological activity of the endothelium-derived relaxing factor produced by endothelial

cells (Palmer *et al.*, 1987). Macrophage-derived NO functions also as a cytotoxic molecule active against invading microorganisms (Adams *et al.*, 1990: Adams *et al.*, 1991; Green *et al.*, 1990) as well as tumour cells (Drapier and Hibbs, 1988; Stuehr and Nathan, 1989). NO released from Kupffer cells and hepatocytes has inhibitory effects on the total protein synthesis of these cells (Curran *et al.*, 1989). Recent reports have suggested that NO synthesis in these cells may serve to reduce hepatic damage during acute murine endotoxaemia (Harbrecht *et al.*, 1992). Large amounts of NO formation might cause significant nitrosation of amines, which have been implicated in carcinogenesis (Kosaka *et al.*, 1989).

Regulatory mechanisms for NO oxide production have been studied intensively, and two types of NO synthases were characterised (Stuehr and Griffith, 1992). One is constitutive and Ca^{2+} and calmodulin dependent. The other is Ca^{2+} and calmodulin independent and inducible after immunological activation of macrophages, endothelial cells and some other cell types. Bacterial LPS can increase both blood NO concentration in vivo and production of NO by macrophages in vitro (Stuehr and Marletta, 1985). The production of inducible NO synthase by endothelial cells or vascular smooth muscle after exposure to LPS, TNF, IL-1 or IFN-γ leads to an over production of NO in the vasculature and profound vasodilation. This might be the cause of sepsis-related hypotension which is characterised by low vascular resistance and is often refractory to vasopressor therapy (Parker *et al.*, 1987).

All three types of interferon (IFN-α, β and γ) are able to enhance LPS-induced NO (Amber *et al.*, 1988 ; Ding *et al.*, 1988). IFN-γ in combination with IL-1α and/or TNF-α/β can interact synergistically to initiate NO production (Amber *et al.*, 1988 ; Ding *et al.*, 1988). Among many cytokines tested, including IL-1β, IL-2, IL-3, IL-4, IFNs, macrophage CSF, GM-CSF, TNF-α/β and transforming growth factor beta (TGF-β), only IFN-γ has been reported to be capable of inducing NO production by itself under some experimental conditions in certain murine macrophages (Drapier *et al.*, 1988 ; Stuehr and Marletta, 1987). Other cytokines such as IL-10, TGF-β1, TGF-β2, TGF-β3 and

macrophage deactivating factor have been shown to inhibit the induction of NO production (Ding *et al.*, 1990 ; Gazzinelli *et al.*, 1992). The anti-inflammatory reagents hydrocortisone and dexamethasone have also been shown to prevent NO formation (Di Rosa *et al.*, 1990 ; Knowles *et al.*, 1990). These studies indicate that inducible NO production is tightly regulated and that a fine functional balance between different microbial stimuli, host-derived cytokines and hormones in the microenvironment is very important for this regulation. Different regulatory pathways might exist for inducible NO production. First, different macrophage cell lines respond differentially to IFN-γ activation in terms of NO production (Stuehr and Marletta, 1987). Second, LPS-induced NO production has been observed with both CBA/N and nude mice. CBA/N mice lack mature B cells and nude mice lack functional T cells. This indicates that neither mature B cells nor functional T cells are required for the response that is initiated by LPS in vivo (Stuehr and Marletta, 1985).

Although the importance of NO synthesis in rodent macrophages is well established, the existence of similar pathway in human mononuclear phagocytes is not accepted by many investigators and is a subject of great controversy.

Because of the difficulties concerning the study of direct interaction of LPS with host cells (discussed in section 3.4), studying mediators released in response to various forms of LPS provided an indirect approach to study the interaction between LPS and host cells.

The aims of this chapter were:

1- to measure TNF-α production from THP-1 cells in response to various forms of LPS to determine which form is the most potent in stimulating host cells;

2- to investigate the involvement of CD14 in signalling host cells to release TNF-α after stimulation with various forms of LPS;

3- to determine if there are other pathways for LPS induced-signalling of host cells in addition to CD14;

4- to determine if all forms of LPS induce cell signalling through the same pathway;

5- to measure NO production from peritoneal macrophages from different mouse strains in response to various forms of LPS and different bacteria to determine if differences exist between different genetic backgrounds in their response to various forms of LPS and different organisms;

6- to determine whether human cells possess NOS activity as part of their antimicrobial armoury.

4.2 Materials and methods

4.2.1 Buffers

4.2.1.1 Tris- HCl buffer (stock soln.)

This solution was prepared by dissolving 0.6 g of Tris and 0.85 g of NaCl in 100 ml distilled water, then the pH of the buffer adjusted to 7.4 using HCl.

4.2.1.2 Tris-buffered saline (working soln.)

This buffer was prepared freshly before use by diluting Tris-HCl buffer (stock soln.) 1: 10 with normal saline and adding normal rabbit serum (SAPU, Carluke, UK) to a final concentration of 2% (v/v).

4.2.2 Bacterial strains

S. minnesota Ra and *E. coli* were grown in nutrient broth for 18 h, *N. meningitidis* was grown on MNYC agar for 48 h, *B. fragilis* was grown in PPY broth anaerobically for 48 h and *Staph. epidermidis* was grown on blood agar for 18 h. Gram-negative bacteria were harvested, washed three times with PBS, counted spectrophotometrically and subsequently fixed with 1% formaldehyde in saline at 37° C for 1 h. The killed bacteria were washed three times in RPMI 1640 (Gibco) or DMEM (Sigma) without antibiotics and resuspended in RPMI 1640 or DMEM containing 10% FCS without antibiotics. *Staph. epidermidis* were treated as above except 2% formaldehyde was used to kill them. Sterility was confirmed by incubation of killed *S. minnesota* Ra and *E. coli* on nutrient agar plates overnight, *N. meningitidis* on MNYC agar plate overnight, *B. fragilis* on blood agar plate anaerobically for 48 h and *Staph. epidermidis* on blood agar plate overnight. In some experiments all bacteria used were heat killed for 30 min at 60° C in PBS and then treated as above.

4.2.3 Stimulation of host cells

4.2.3.1 Stimulation of THP-1 cells to release TNF

THP-1 were harvested (as described in section 2.3.2), resuspended in 10% FCS RPMI 1640 without antibiotics and adjusted to $2.0x10^6$ ml^{-1}. Cells were placed in 96 well tissue culture plates (100 µl /well), then various forms of LPS (suspended in RPMI 1640 containing 10% FCS without antibiotics) at different concentrations, whole bacteria at different doses or diluent controls were added (100 µl/well) and incubated for the desired time in a 37° C in a humidified 5% CO_2 containing incubator. After incubation the plates were then centrifuged at 300 g for 10 min, supernatants were collected and used directly to estimate the amount of TNF. Control samples were treated the same way except that they received 100 µl of medium in place of stimuli. In some experiments THP-1 cells were incubated in 10% FCS RPMI 1640 with antibiotics and containing 0.1 mM 1, 25-dihydroxyvitamin D_3 (ICN, Biomedicals Ltd, Oxfordshire, UK) for 72 h to enhance the expression of CD14 prior to stimulation of the cells. In some other experiments THP-1 cells were incubated with three dilutions (1/50, 1/100 and 1/200) of anti-CD14 (SAPU) monoclonal antibody prior to stimulation of the cells.

4.2.3.2 Stimulation of mononuclear phagocytes to release nitric oxide (NO)

Murine peritoneal cells were harvested (as described in section 2.2.1) in pyrogen free PBS, washed once in DMEM without antibiotics and resuspended in DMEM containing 10% FCS without antibiotics at $2.0x10^6$ ml^{-1}. THP-1 cells were harvested, washed once in DMEM without antibiotics, resuspended in 10% FCS DMEM without antibiotics and adjusted to $2.0x10^6$ ml^{-1}. Human peripheral blood monocytes were obtained from two healthy individuals (as described in section 2.2.3). The cells were resuspended in 10% FCS DMEM without antibiotics at $1.0x10^7$ ml^{-1}. Cells were placed in 96 well tissue culture plates (100µl/ well), incubated for 1 h at 37° C in a humidified incubator containing 5% CO_2. After incubation, non-adherent cells were removed by washing gently with DMEM without antibiotics. Various forms of LPS (suspended in DMEM

containing 10% FCS without antibiotics) at different concentrations and whole bacteria at different doses were added (100µl/ well) to the mononuclear phagocytes and incubated for 24 h in a 37° C humidified 5% CO_2 containing incubator. Plates were then centrifuged at 300 g for 10 min. Supernatants were collected and used directly to estimate the amount of nitrite in each sample. Control samples were treated the same way except for that they received 100µl of medium in place of stimuli. In some experiments THP-1 cells were incubated for 72 h in the presence of 0.1 µM 1,25-dihydroxyvitamin D_3 to enhance the expression of CD14 prior to stimulation with various forms of LPS. In some other experiments various forms of LPS were incubated with monoclonal antibodies (SZ 27.19, Blood Transfusion Service, Edinburgh and WN1, gift from Dr. Ian Poxton) for 20 min before adding to cells. Both antibodies were directed against the core region of *Salmonella* and *E. coli* LPS.

4.2.4 TNF bioassay

L929 mouse fibroblast cell line obtained from the European collection of animal cell culture (ECACC, Salisbury, UK) was cultured in growth medium (10% FCS DMEM) and sub-cultured twice a week. L929 cells were dislodged by 0.1% trypsin (Gibco) to avoid cell clumping, washed, resuspended in growth medium and adjusted to $3.0x10^5$ ml^{-1}. Cells were placed in flat-bottomed 96 well tissue culture plate at 100 µl/ well and incubated in a 37° C humidified 5% CO_2 containing incubator for 24 h. The growth medium was discarded and replaced with 100 µl/well (triplicate wells) of test supernatants after being diluted 1: 1 in assay medium, DMEM containing 5% FCS, 1 mM L-glutamine and 2 µg ml^{-1} actinomycin D (Sigma). A standard of recombinant human TNF (NIBSC, Hertfordshire, UK) was diluted serially 1: 3 in assay medium containing 1 µg ml^{-1} actinomycin D at a starting concentration of 1000 IU/ml and 100 µl was added to triplicate wells. TNF standard was used in every plate. Control wells (6 wells) received 100 µl assay medium containing 1 µg ml^{-1} actinomycin D. Blanks were included in each plate (6 wells without L929 cells), and were treated identically to the test samples. Plates were then incubated in a 37° C humidified 5% CO_2 containing incubator for 24 h. The

supernatant in each well was removed and replaced with 50 µl crystal violet stain (0.5% crystal violet in 20% methanol in distilled water, filtered through Whatman no 1 filter paper) for 2 min to stain surviving cells. The plates were then washed vigorously with tap water and allowed to dry, then each well received 50 µl/ well 20% acetic acid to solubilise the stained cells. Any air bubbles were removed and plates were read at 570 nm using MR 700 microplate reader (Dynatech Laboratories). The amount of TNF in each sample was determined relative to a TNF standard curve and results were expressed in IU/ml.

4.2.5 Nitric oxide assay

Nitric oxide production was assessed by measuring nitrite, a stable metabolic product of NO, in mononuclear phagocytes' culture supernatants (as described by Stuehr and Marletta, 1987). In brief, Griess reagent, (1:1, v/v) mixture of 0.1% *N*-[1 naphthyl]ethylenediamine dihydrochloride [Sigma] in H_2O-1% sulfanilamide [Sigma] in 5% H_2PO_4) was added to 96 well tissue culture plates at 100µl/ well. An equal volume of mononuclear phagocytes culture supernatants was then mixed with the Griess reagent and incubated for 10 min at room temperature, then the absorbance was determined on a MR 700 microplate reader (Dynatech Laboratories) at 570 nm. The nitrite amount was then calculated from $NaNO_2$ standard curve. The amount of measured nitrite in µmol was related to the number of cells per well per 24 h This technique enabled the measurement of amounts of nitrite as little as 0.1 µmol.

4.2.6 Nitric oxide production enhancement by Interferon-gamma (IFN-γ)

Human recombinant IFN-γ (NIBSC, Hertfordshire, UK) was suspended in DMEM containing 10% FCS and used at a final concentration of 200 µg ml^{-1}.

4.2.7 Inhibition of TNF and NO production with polymyxin B sulphate

Polymyxin B sulphate (Sigma) was dissolved in RPMI 1640 containing 10% FCS or 10% FCS DMEM to the desired concentrations. Initially, polymyxin B at various

concentrations was incubated with THP-1 cells for 24 h. After incubation, the viability of cells was checked by using Trypan blue exclusion. Polymyxin B concentrations that permitted cell viability of more than 95% were used. Polymyxin B was added to free *S. minnesota* Ra LPS, outer membrane (OM) preparations or whole bacteria and incubated at room temperature for 25 min then added to cells. Two controls were used, cells plus medium only and cells plus medium containing the highest polymyxin B concentration used.

4.2.8 Detection of CD14 receptors on THP-1 cells

THP-1 cells were harvested and resuspended in Tris-buffered saline containing 2% normal rabbit serum, counted and adjusted to $2\text{-}3 \times 10^6$ ml^{-1}. The cell suspension (100 µl) was placed per well in a 96 well V bottomed microtiter plate. Normal rabbit serum was added at 25 µl per well and the plate was incubated for 20 min on ice. The plate was centrifuged at 300 g for 10 min and the supernatants were discarded by inverting the plate over a container. Cells were then resuspended in residual medium and 20 µl of mouse anti-CD14 monoclonal antibody (SAPU) at two concentrations (undilute and 1/10) were added to each well and incubated for 30-45 min on ice. The plate was washed three times by centrifugation at 300 g for 10 min using Tris-buffered saline containing 2% normal rabbit serum each time. The cells were then resuspended in residual medium and 50 µl of FITC conjugated $F(ab`)_2$ sheep anti-mouse IgG (Sigma) was added to each well and incubated for 30 min on ice. The plate was then washed three times by centrifugation at 300 g for 10 min using Tris-buffered saline containing 2% normal rabbit serum each time. After the last wash the cells were resuspended in residual medium and fixed with 150 µl 1% buffered paraformaldehyde and transferred to tubes using 100 µl PBS. Samples were then analysed using flow cytometry as described in section 3.2.6.

4.2.9 Statistics

The student's t-test was used for all statistical analysis. The significance of the effect is related to the amount of TNF or NO released from control cells (untreated cells).

S. minnesota Ra cells at a dose of 10^8 ml^{-1} were used as an internal standard in every single experiment because of the variation in day to day results. The amount of NO produced in response to this challenge was converted to 100%, then the amount of NO in every sample was converted to a percentage using the formula:

[Amount of NO in test sample (in µmol/10^6 cells/ day) /amount of NO in 10^8 ml^{-1} *S. minnesota* Ra(µmol/10^6 cells/ day)] x 100.

4.3 Results

4.3.1 TNF production after different stimulation times from THP-1 cells in response to different concentrations of free LPS

The level of TNF produced by THP-1 cells was measured at 2, 4, 6 and 24 h after stimulation with several concentrations of free *S. minnesota* Ra LPS. Results are shown in Figure 4.1. After 2 h of stimulation, THP-1 cells produced measurable amounts of TNF in response to the highest three concentrations of LPS (10, 30 and 100 ng ml^{-1}) but not to the two lower concentrations of LPS (1 and 3 ng ml^{-1}). TNF production peaked after 4 h of stimulation of THP-1 cells for all LPS concentrations used. Increasing the stimulation time to 6 h led to a drop of TNF level produced by THP-1 cells in response to all LPS concentrations used. Further increase in stimulation time to 24 h resulted in TNF levels produced by THP-1 cells in response to all LPS concentrations to drop back to base line (> 1 IU/ml). TNF production from THP-1 cells in response to free LPS was dose dependent. Stimulation of THP-1 cells for 6 h with free LPS induced the production of TNF amounts of: 8.3 IU/ml ($p < 0.02$) in response to a dose of 1 ng ml^{-1}, 14.3 IU/ml ($p < 0.05$) in response to a dose of 3 ng ml^{-1}, 38 IU/ml ($p < 0.02$) in response to a dose of 10 ng ml^{-1}, 80 IU/ml ($p < 0.01$) in response to a dose of 30 ng ml^{-1} and 108 IU/ml ($p < 0.025$) in response to a dose of 100 ng ml^{-1}. From this work 6 h stimulation time was selected for further work on TNF production.

4.3.2 TNF production from THP-1 cells in response to *S. minnesota* Ra OM preparations

TNF levels produced by THP-1 in response to several concentrations of OM preparations from *S. minnesota* Ra (OM preparation concentrations stated here represent the concentration of LPS attached to each OM preparation as determined by KDO assay) were investigated by stimulation of the cells for 6 h. Free *S. minnesota* Ra LPS at several

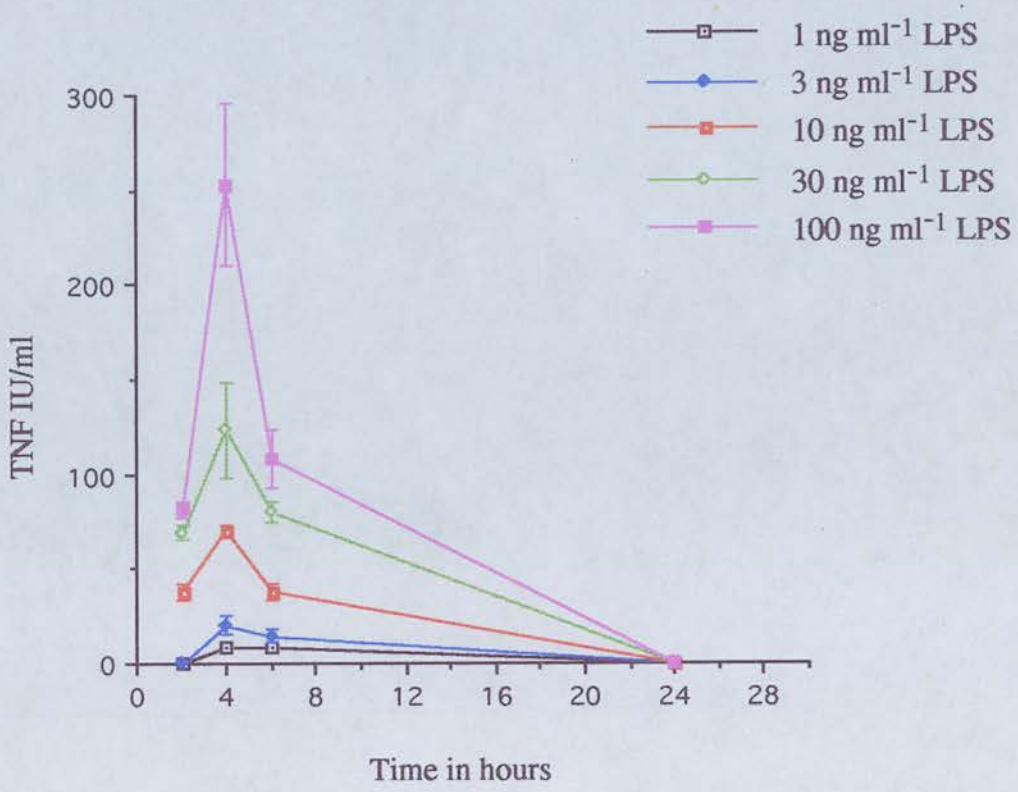

Figure 4.1: TNF production from THP-1 cells in response to several concentrations of free *S. minnesota* Ra LPS over 24 hours. Each point represent the mean TNF release of three experiments ± SEM

concentrations was used for comparison. THP-1 cells produced significant amounts of TNF in response to all OM preparations used except for 1ng ml^{-1} concentration and TNF levels produced were dose dependent (Figure 4.2). OM preparation stimulated THP-1 cells to produce TNF amounts of: 30 IU/ml ($p < 0.02$) in response to a dose of 3 ng ml^{-1}, 51 IU/ml ($p < 0.02$) in response to a dose of 10 ng ml^{-1}, 86 IU/ml ($p < 0.025$) in response to a dose of 30 ng ml^{-1}, 150 IU/ml ($p < 0.02$) in response to a dose of 100 ng ml^{-1}.

4.3.3 TNF production by THP-1 cells in response to whole *S. minnesota* Ra and whole *Staph. epidermidis*

Five concentrations of *S. minnesota* Ra and *Staph. epidermidis* were used to stimulate TNF production from THP-1 cells for 6 h. Results are shown in Figure 4.3. Whole *S. minnesota* Ra stimulated the production of significant amounts of TNF from THP-1 cells at all doses of bacteria used. Again as for the other forms of LPS, TNF production in response to whole *S. minnesota* Ra was dose dependent. At low doses (10, 10^3 and 10^5 ml-1) *Staph. epidermidis* did not stimulate THP-1 cells to produce TNF. *Staph. epidermidis* at a dose of 10^7 ml^{-1} stimulated THP-1 to produce small amounts of TNF; and only at a dose of 10^9 ml^{-1} did *Staph. epidermidis* stimulated the production of significant amounts of TNF (38 IU/ml) from THP-1 cells ($p < 0.02$).

4.3.4 TNF production from normal and CD14-enhanced THP-1 cells in response to various *S. minnesota* Ra LPS forms

To find out if CD14 receptors were involved in the induction process of TNF from THP-1 cells, CD14-enhanced THP-1 cells and normal THP-1 cells (without CD14-enhancement) were stimulated for 6 hours with various forms of *S. minnesota* Ra LPS. Normal and CD14-enhanced THP-1 cells produced significant amounts of TNF in response to all *S. minnesota* Ra LPS forms used. There were no significant differences in TNF levels produced from normal THP-1 cells as compared to CD14-enhanced THP-1 cells (Figure 4.4). The percentage of THP-1 cells which expressed CD14 was determined by flow cytometry. Results of three experiments are shown in Table 4.1. THP-1 cells incubated

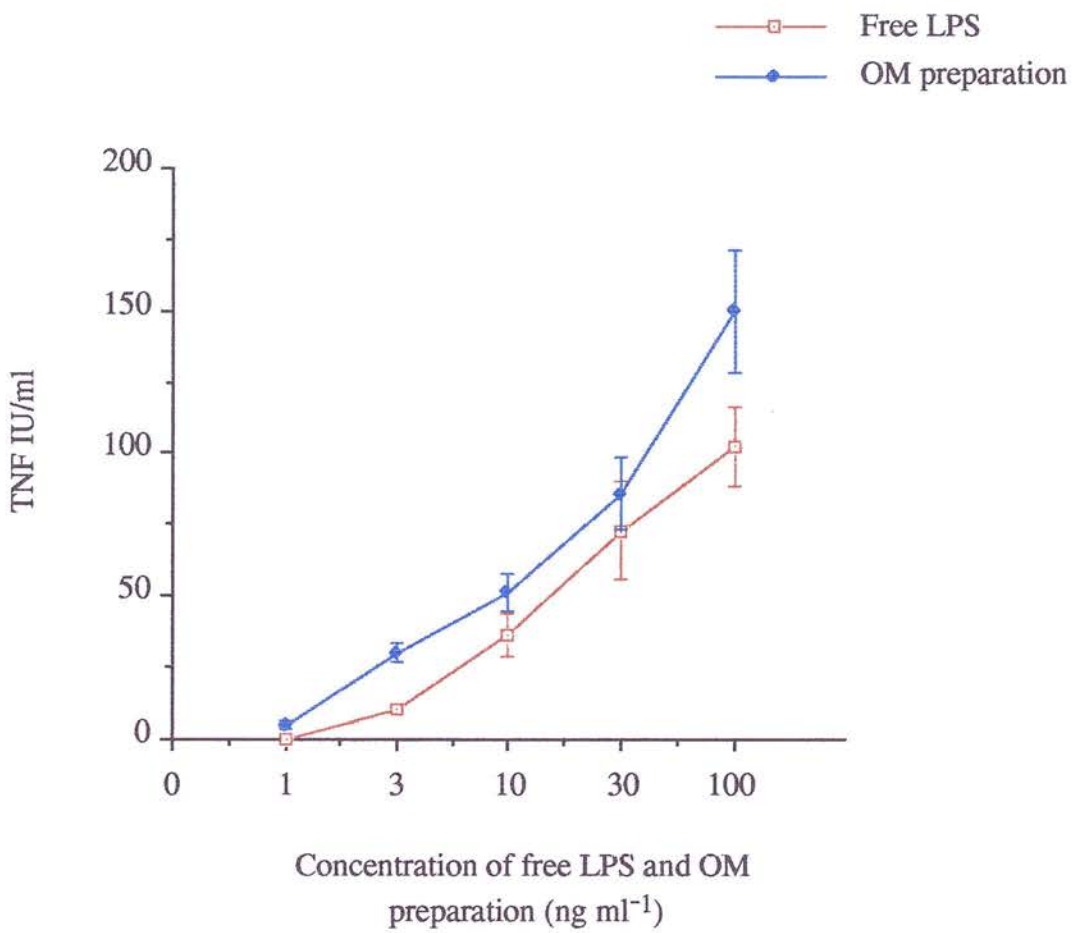

Figure 4.2: TNF production from THP-1 cells in response to several concentrartions of *S. minnesota* Ra outer membrane fraction (OM) and free LPS when stimulated for 6h. Each point represents the mean TNF release of three experiments ± SEM

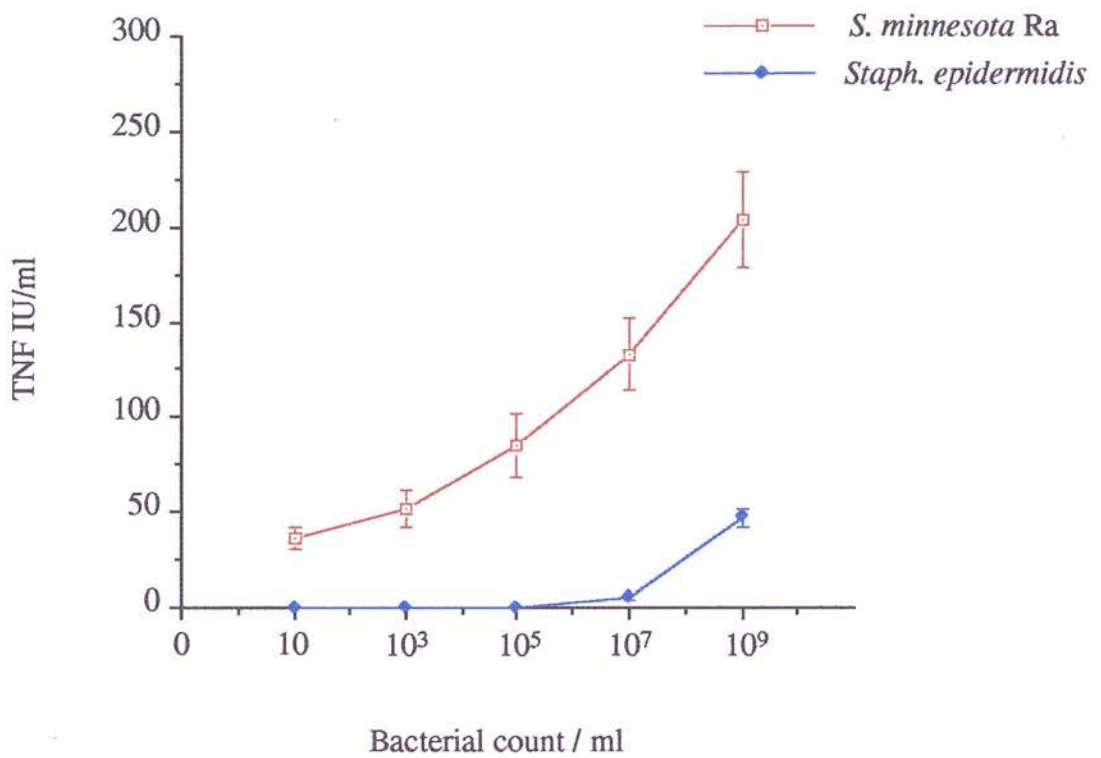

Figure 4.3: TNF production from THP-1 cells in response to several doses of whole *S. minnesota* Ra and *Staph. epidermidis* when stimulted for 6 h. Each point represents the mean TNF release of three experiments ± SEM

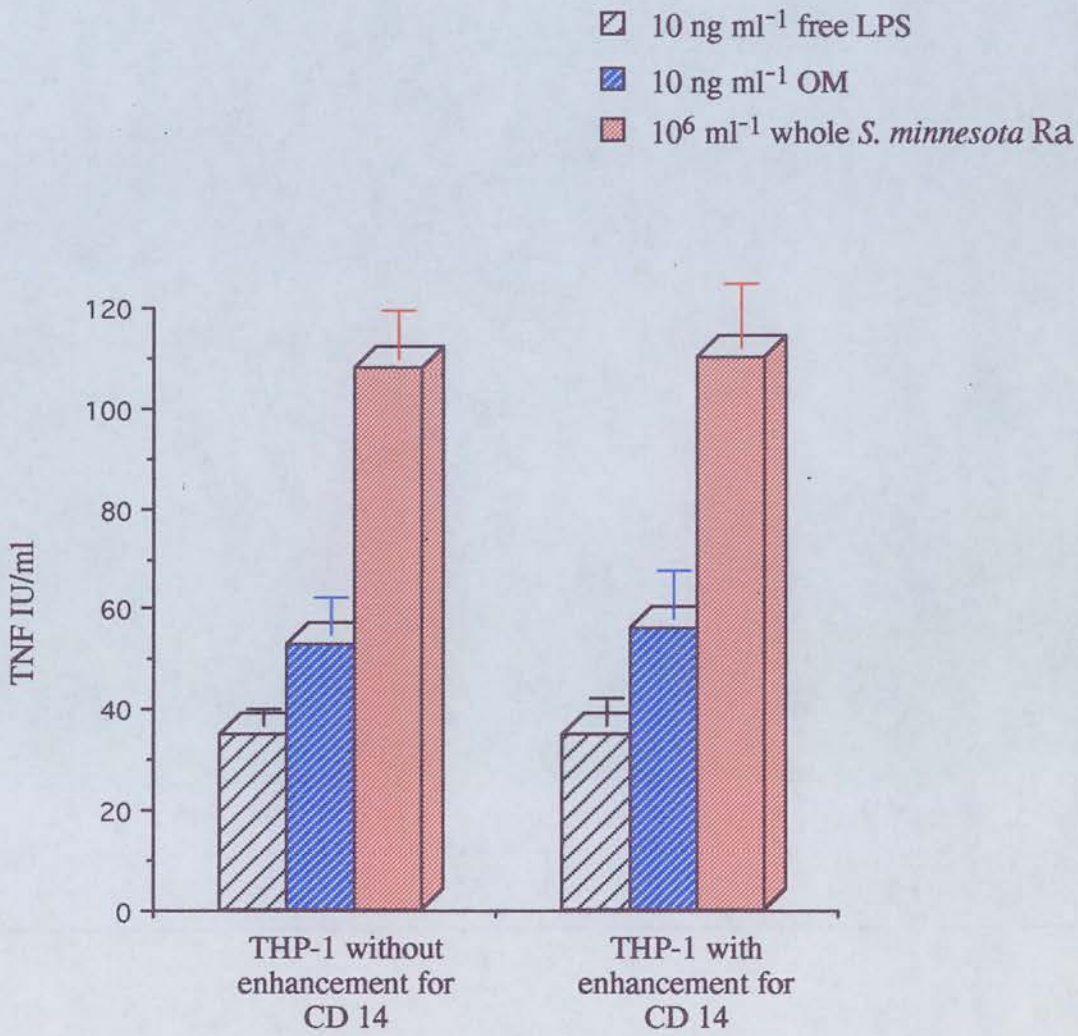

Figure 4.4: Comparison between TNF levels produced by CD14-enhanced THP-1 cells and normal (untreated) THP-1 cells in response to various forms of *S. minnesota* Ra LPS at 6 h incubation time. Each column represents the mean TNF release of three experiments ± SEM

(OM = outer membrane fraction of *S. minnesota* Ra).

Table 4.1: Expression of CD14 on THP-1 cells as detected by flow cytometry.

	THP-1 cells without enhancement for CD14 % positive cells	THP-1 cells with enhancement for CD14 % positive cells
Experiment 1	6 (314)	32 (374)
Experiment 2	4 (280)	20 (325)
Experiment 3	9 (319)	37 (386)

Figures in brackets represent the mean fluorescence level of samples.

with 1, 25-dihydroxyvitamin D_3 expressed more CD14 receptors than untreated cells both in terms of percentage positive and level of expression.

4.3.5 Inhibition of TNF production from THP-1 cells by anti-CD14 monoclonal antibody (mAb)

Anti-CD14 mAb was used at three dilutions (1/50, 1/100 and 1/200) to inhibit the production of TNF from THP-1 cells when stimulated for 6 h with a concentration of 30 ng ml^{-1} free *S. minnesota* Ra LPS (Figure 4.5). Anti-CD14 at all three dilutions used did not inhibit the production of TNF from THP-1 cells in response to free *S. minnesota* Ra LPS.

4.3.6 Inhibition of TNF production from THP-1 cells by polymyxin B sulphate

Polymyxin B sulphate was used at three concentrations to inhibit TNF production from THP-1 cells in response to various forms of *S. minnesota* Ra LPS (Figure 4.6). All three concentrations of polymyxin B used (0.1, 1, 10 μg ml^{-1}) blocked TNF production from THP-1 cells in response to a concentration of 10 ng ml^{-1} free LPS and the results were statistically significant ($P < 0.05$ for all polymyxin concentrations) as compared to controls (free LPS without polymyxin B treatment). Polymyxin B at concentrations of 0.1, 1, 10 μg ml^{-1} also blocked TNF production from THP-1 cells in response to a concentration of 10 ng ml^{-1} OM preparation and results were statistically significant ($p < 0.025$, $P < 0.025$ and $p < 0.02$ respectively) as compared to untreated controls . There was no inhibition of TNF production from THP-1 in response to a dose of 10^7 ml^{-1} whole *S. minnesota* Ra at any polymyxin B concentration used as compared to untreated control.

4.3.7 Nitric oxide release from peritoneal macrophages of CBA mouse

Peritoneal macrophages of CBA mice were stimulated with various forms of LPS and various bacteria including Gram-negative anaerobic and Gram-positive bacteria.

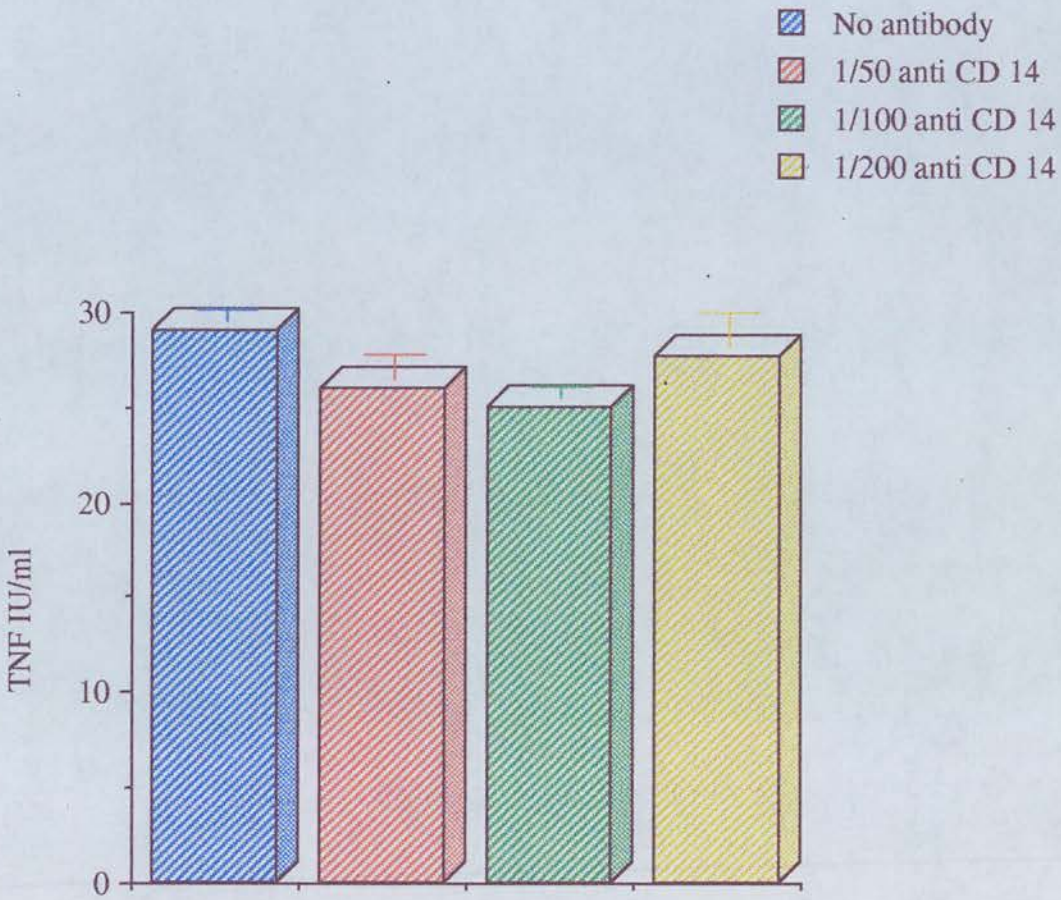

Figure 4.5: Inhibition of TNF production by anti CD14 antibody. THP-1 cells were stimulated by a concentration of 10 ng ml^{-1} free *S. minnesota* Ra LPS for 6 h. Three dilutions of anti CD14 mAb were used. Each column represents the mean TNF release of three experiments ± SEM

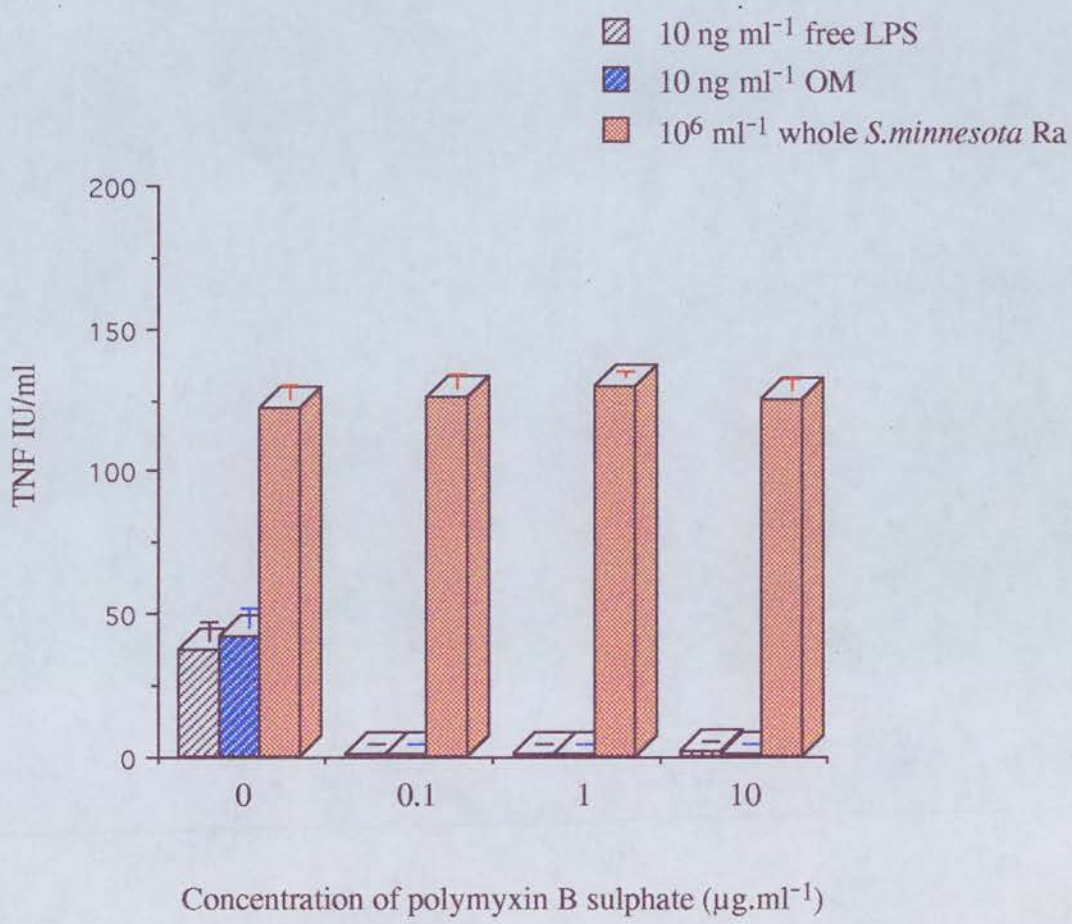

Figure 4.6: Inhibition of TNF production by polymyxin B. THP-1 cells were stimulated for 6 h with various forms of *S. minnesota* Ra LPS using three concentrations of polymyxin B sulphate. Each column represents the mean TNF release of three experiments ± SEM

(OM = outer membrane fraction of *S. minnesota* Ra).

4.3.7.1 Nitric oxide release from peritoneal macrophages of CBA mice in response to various forms of LPS

Peritoneal macrophages of CBA mice were stimulated with several concentrations of free LPS and LPS attached to outer membrane preparations extracted from *S. minnesota* Ra. At high concentration of free LPS and OM preparation (100 μg ml^{-1}) there was no production of NO and that was probably because these high concentrations were killing the cells. At a concentration of 10 μg ml^{-1} of free LPS, peritoneal macrophages of CBA mice produced small but measurable amounts of NO and when compared to non-stimulated controls results were statistically significant ($p < 0.02$) (Figure 4.7). At a concentration of 10 μg ml^{-1} of OM preparation peritoneal macrophages of CBA mice produced a slightly higher amount of NO than that obtained by using similar concentration of free LPS and results were statistically significant ($p < 0.05$) compared to non-stimulated controls. The response to whole bacteria is also shown in Fig. 4.7. At a dose of 10^8 ml^{-1} of whole *S. minnesota* Ra there was a substantial release of NO. An internal standard has to be used in every experiment because of day to day variations in the results. Whole *S. minnesota* Ra was used in this work as a standard at a dose of 10^8 ml^{-1}. The mean amount of NO produced in response to this dose was 14 μmol/ 10^6 cells/ day. Lower concentrations of free LPS and OM preparation (1 μg ml^{-1} and 0.1 μg ml^{-1}) failed to stimulate the cells to produce any measurable amounts of NO from peritoneal macrophages of CBA mice.

4.3.7.2 Nitric oxide release from peritoneal macrophages of CBA mice in response to three different Gram-negative aerobic bacteria

S. minnesota Ra, *N. meningitidis* and *E. coli* were used in these experiments. Three doses of each bacteria were used to stimulate peritoneal macrophages of CBA mice to produce NO. Figure 4.8 shows the relative release of NO from peritoneal macrophages of CBA mice in response to the different bacteria used. *S. minnesota* Ra at a dose of 10^8 ml^{-1} gave the highest amount of NO and again was used as internal standard. The mean amount of NO produced in this set of experiments in response to this dose was 16 μmol/

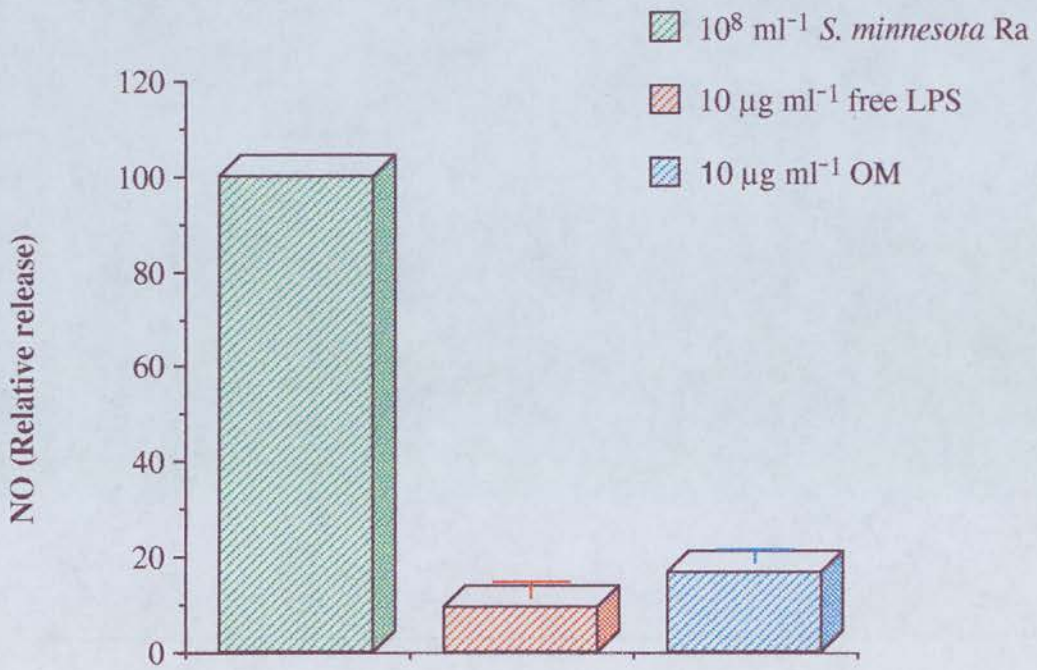

Figure 4.7: NO release from peritoneal macrophages of CBA mice in response to various forms of LPS from *S. minnesota* Ra. Each column represents the mean NO release ± SEM of four to seven experiments.
(OM= outer membrane fraction of *S. minnesota* Ra).

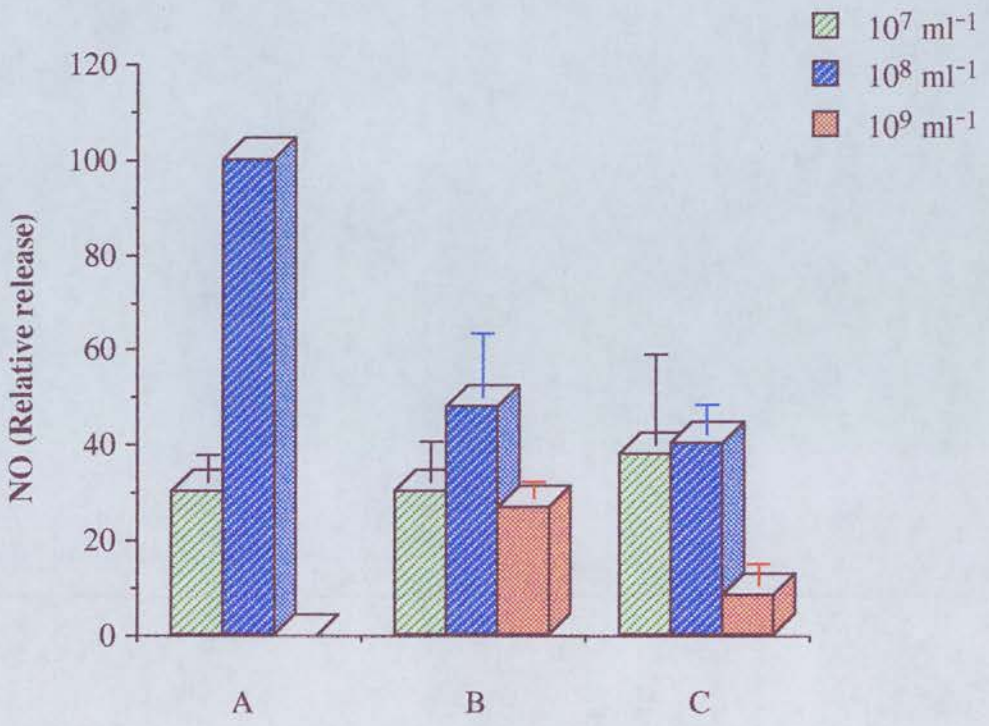

Figure 4.8: NO release from peritoneal macrophages of CBA mice in response to *S. minnesota* Ra (A), *N. meningitidis* (B) and *E. coli* (C). Each column represents the mean NO release ± SEM of four to seven experiments.

10^6 cells/ day. At a dose of 10^7 ml^{-1}, *S. minnesota* Ra stimulated peritoneal macrophages of CBA mice to produce measurable amount of NO and the results were statistically significant ($p < 0.005$). *S. minnesota* Ra at the highest dose (10^9 ml^{-1}) failed to stimulate the production of any measurable amounts of NO from macrophages and that was probably because the high dose of the bacteria was killing the cells. *N. meningitidis* stimulated peritoneal macrophages of CBA mice to produce measurable amounts of NO at all three doses used. The highest amount of NO was produced in response to a dose of 10^8 ml^{-1} and was statistically significant ($p < 0.05$). At a dose of 10^7 ml^{-1} *N. meningitidis* stimulated macrophages to produce significant amounts of NO and results were statistically significant ($p < 0.05$). At a dose of 10^9 ml^{-1} *N. meningitidis* stimulated the production of significant amounts of NO similar to those produced in response to a dose of 10^7 ml^{-1}, ($p < 0.005$). *E. coli* stimulated peritoneal macrophages of CBA mice to produce measurable amount of NO at all three doses used. Again the highest amount of NO was produced in response to a dose of 10^8 ml^{-1} and was statistically significant ($p < 0.01$). NO production in response to a dose of 10^7 ml^{-1} was comparable to that produced in response to a dose of 10^8 ml^{-1}. At a dose of 10^9 ml^{-1} the least amount of NO was produced and that was probably because excess bacteria killed most of the cells.

4.3.7.3 Nitric oxide release from peritoneal macrophages of CBA mice in response to Gram-negative anaerobic and Gram-positive bacteria

B. fragilis and *Staph. epidermidis* were used to stimulate peritoneal macrophages of CBA mice to produce NO. Figure 4.9 shows relative NO release in response to both microorganisms compared to the standard stimulus of 10^8 ml^{-1} *S. minnesota* Ra. *B. fragilis* at a dose of 10^7 ml^{-1} stimulated peritoneal macrophages of CBA mice to produce low levels of NO. The amount of NO produced in response to a higher dose (10^8 ml^{-1}) was slightly increased and was statistically significantly higher than controls ($p < 0.005$). *Staph. epidermidis.* at doses of 10^7 ml^{-1} and 10^8 ml^{-1} stimulated peritoneal macrophages of CBA mice to produce similar amounts of NO. The mean amount of NO produced in

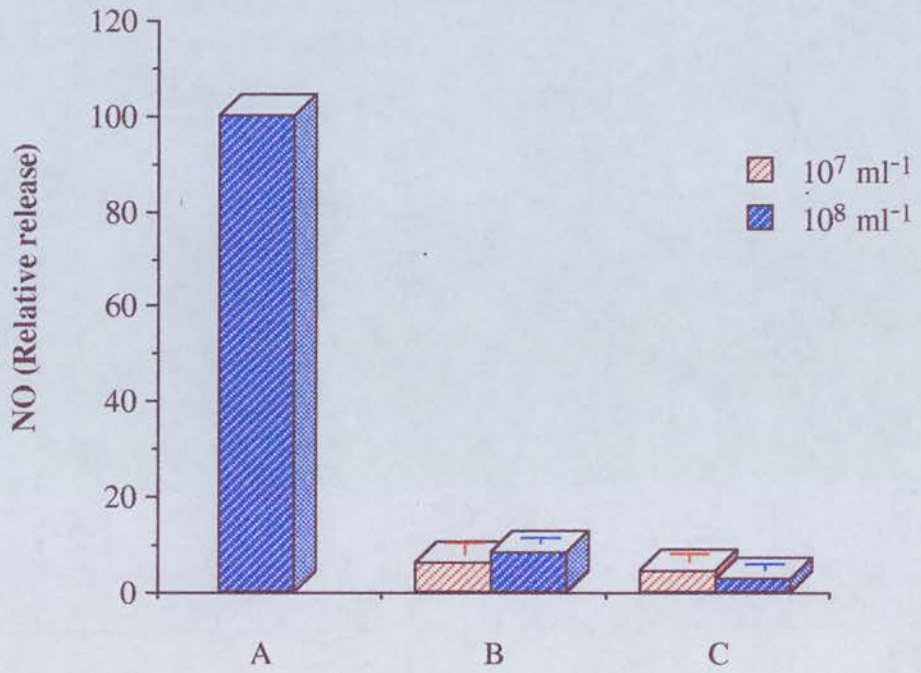

Figure 4.9: NO release from peritoneal macrophages of CBA mice in response to two doses of *B. fragilis* (B) and *Staph. epidermidis* (C). *S. minnesota* Ra (A) was used as a standard. Each column represents the mean NO release ± SEM of four to seven experiments.

this set of experiments by the internal standard (a dose of 10^8.ml^{-1} *S. minnesota* Ra) was 10 μmol/ 10^6 cells/ day.

4.3.8 Nitric oxide release from peritoneal macrophages of BALB/c mice

Several different bacteria, in addition to *S. minnesota* Ra free LPS, were used to stimulate NO production from peritoneal macrophages of BALB/c mice. Figure 4.10 compares NO release in response to a dose of 10^8 ml^{-1} of all bacteria used and a concentration of 10 μg ml^{-1} of free LPS. Peritoneal macrophages of BALB/c mice produced substantial amounts of NO in response to free LPS at a concentration of 10 μg ml^{-1} and the results were statistically significant ($p < 0.025$). Peritoneal macrophages of BALB/c mice produced the highest amount of NO in response to whole *S. minnesota* Ra at a dose of 10^8 ml^{-1}. This dose was used as an internal standard and the mean amount of NO produced in response to this dose was 22.4 μmol/10^6 cells/day. Both *N. meningitidis* and *E. coli* stimulated BALB/c mice peritoneal macrophages to produce similar substantial amounts of NO and results were statistically significant ($p < 0.02$ for both micro-organisms). Stimulation of BALB/c mice peritoneal macrophages with *B. fragilis* and *Staph. epidermidis*, resulted in the production of the lowest amounts of NO. Results were statistically significant ($p < 0.05$ for both microorganisms).

4.3.9 Nitric oxide release from macrophages of LPS hyporesponsive mice (C3H/HeJ)

The ability of various forms of *S. minnesota* Ra LPS and several other different bacteria to stimulate peritoneal macrophages of C3H/HeJ mice to produce NO was investigated. Figure 4.11 compares NO release from peritoneal macrophages of C3H/HeJ mice. In response to a concentration of 10 μg ml^{-1} free LPS, peritoneal macrophages of C3H/HeJ mice failed to produce any significant amounts of NO. In fact the amount of NO released in response to free LPS was less than 1 μmol/10^6 cells/day. OM preparation at a concentration of 10 μg ml^{-1} (this concentration represents the amount of LPS attached to the OM preparation) was more potent than free LPS in stimulating these

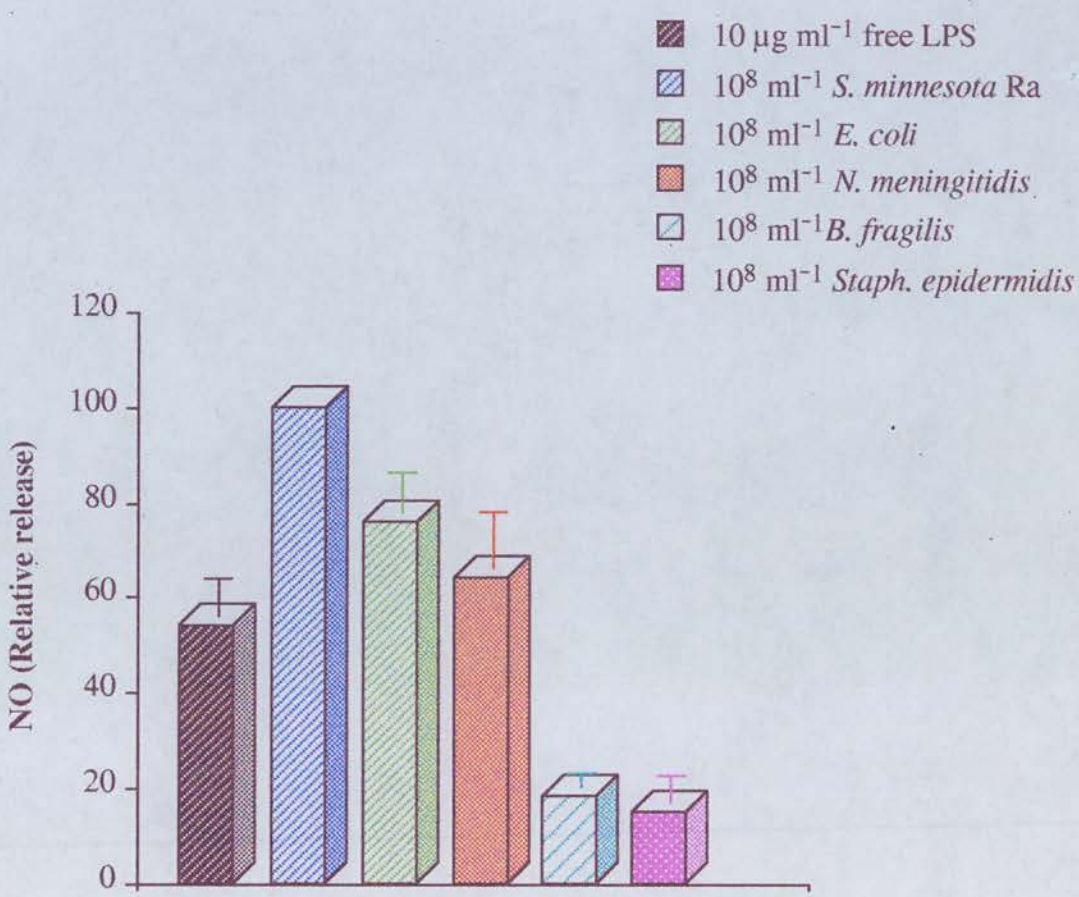

Figure 4.10: NO release from peritoneal macrophages of BALB/c mice in response to two forms of LPS from *S. minnesota* Ra (free LPS and whole organism) and different bacteria. Each column represents the mean NO release + SEM of four to six experiments.

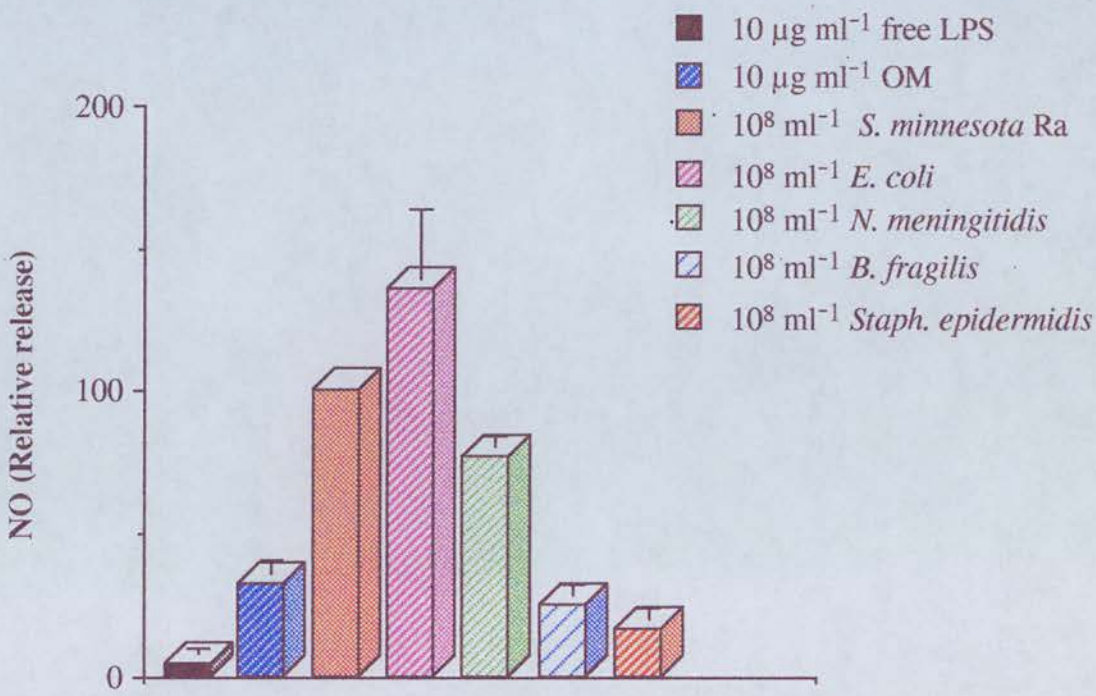

Figure 4.11: NO release from peritoneal macrophages of C3H/HeJ mice in response to various forms of *S. minnesota* Ra LPS and different bacteria. Each column represents the mean NO release ± SEM of four to eight experiments. (OM = outer membrane fraction of *S. minnesota* Ra).

peritoneal macrophages of C3H/HeJ mice to produce significant amounts of NO and results were statistically significant ($p < 0.01$). Whole *S. minnesota* Ra at a dose of 10^8 ml^{-1} was used as an internal standard, it was more potent than other forms of LPS in stimulating peritoneal macrophages of C3H/HeJ mice to produce NO. The mean amount of NO released in response to whole *S. minnesota* Ra was 9 $\mu mol/10^6$ cells/ day. *N. meningitidis* at a dose of 10^8 ml^{-1} stimulated peritoneal macrophages of C3H/HeJ mice to release significant amounts of NO and results were statistically significant ($p < 0.005$). *E. coli* at a dose of 10^8 ml^{-1} was the most potent of the tested organisms in stimulating peritoneal macrophages of C3H/HeJ mice to produce NO and results were statistically significant ($p < 0.05$). *B. fragilis* and *Staph. epidermidis* at a dose of 10^8 ml^{-1} stimulated peritoneal macrophages of C3H/HeJ mice to produce low but measurable amounts of NO. The results of both microorganisms were statistically significant above control levels ($p < 0.02$ for *B. fragilis* and $p < 0.005$ for *Staph. epidermidis*).

4.3.10 Nitric oxide release from a human macrophage cell line (THP-1)

The ability of the human macrophage cell line (THP-1) to produce NO in response to various forms of *S. minnesota* Ra LPS was investigated. Several other bacteria were also used to stimulate THP-1 cells to produce NO.

4.3.10.1 Nitric oxide release from THP-1 in response to various forms of *S. minnesota* Ra LPS

Different concentrations of free LPS, OM preparation and whole *S. minnesota* Ra were used to investigate the production of NO from THP-1 cells (Figure 4.12). All concentrations of free LPS used failed to stimulate THP-1 cells to release any significant amounts of NO. THP-1 cells released substantial amounts of NO in response to all three concentrations of OM preparation in a dose-dependent fashion. Results were statistically significant for all three concentrations of OM preparation used, for a concentration of 1 μg ml^{-1} ($p < 0.0\ 2$), for 10 μg ml^{-1} ($p < 0.01$) and for 100 μg ml^{-1} ($p < 0.01$). THP-1 cells also released substantial amounts of NO in response to whole *S. minnesota* Ra at all

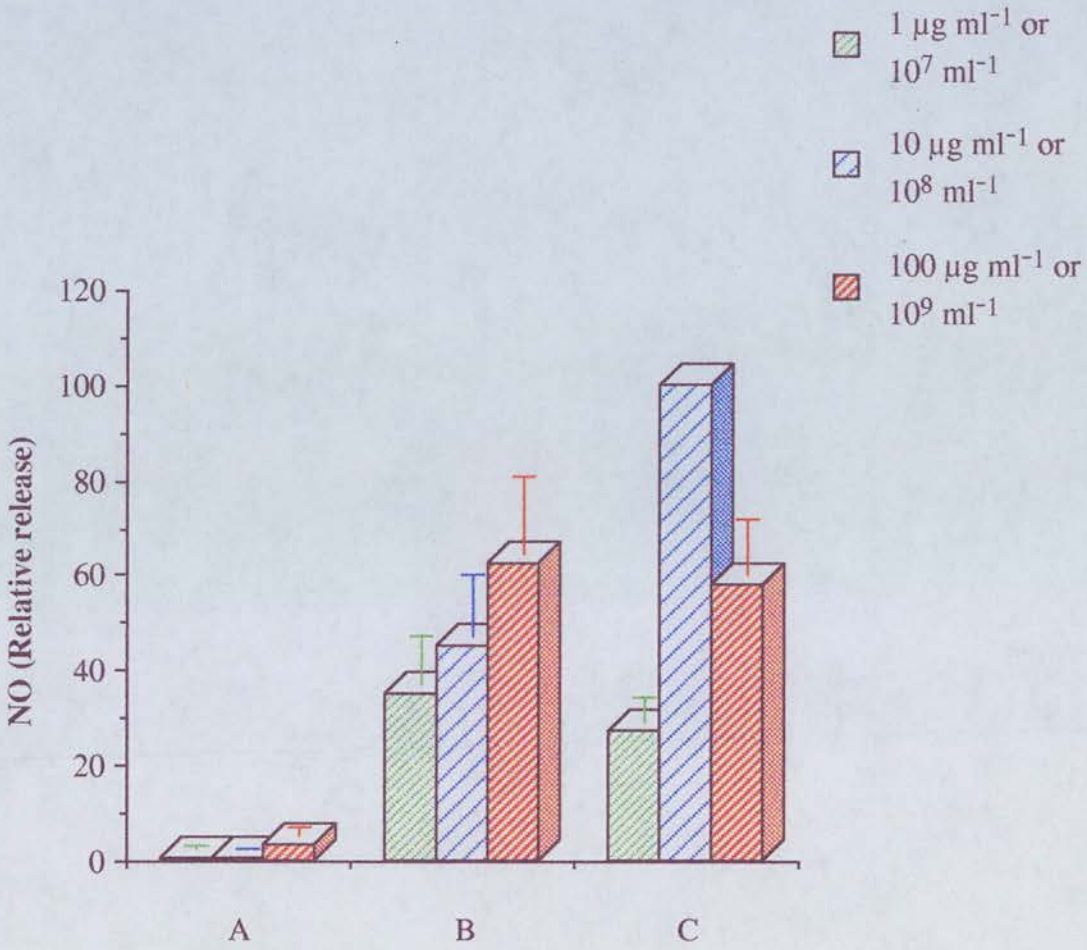

Figure 4.12: NO release from THP-1 cells in response to three different concentrations of various forms of *S. minnesota* Ra LPS: free LPS (A), outer membrane fraction (B) and whole organism (C). Each column represents the mean NO release ± SEM of four to fifteen experiments.

three doses used. The highest release of NO from THP-1 cells was seen in response to whole *S. minnesota* Ra at a dose of 10^8 ml^{-1} which was used as an internal standard. The mean amount of NO released in response to this dose was 19.2 $\mu mol/10^6$ cells/ day. The amount of NO released in response to a dose of 10^7 ml^{-1} ($p < 0.001$) was lower than that released in response to a dose of 10^9 ml^{-1} ($p < 0.001$).

4.3.10.2 Nitric oxide release from THP-1 cells in response to different bacteria

The ability of different Gram-negative bacteria, including an anaerobe and one Gram -positive bacterium to stimulate THP-1 cells to produce NO was investigated. Figure 4.13 shows release of NO from THP-1 in response to various bacteria. *S. minnesota* Ra was used as standard at a dose of 10^8 ml^{-1}. All bacteria were used at the same dose as *S. minnesota* Ra. The mean amount of NO released from THP-1 in response to *S. minnesota* Ra was 22 $\mu mol/10^6$ cells/ day. THP-1 cells released low levels of NO in response to *N. meningitidis*, *B. fragilis* and *Staph. epidermidis* and results were statistically significantly different from the background release, $p < 0.05$, $p < 0.02$ and $p < 0.02$ respectively. *E. coli* was more potent in stimulating THP-1 cells to release substantial amounts of NO ($p < 0.005$) compared with the other microorganisms used except for *S. minnesota* Ra.

4.3.11 Nitric oxide release from human peripheral blood monocytes

Human peripheral blood monocytes were stimulated with different concentrations of various forms of *S. minnesota* Ra LPS. Relative release of NO from human peripheral blood monocytes is shown in Figure 4.14. In response to three concentrations of free LPS, human peripheral blood monocytes failed to release any significant amounts of NO. Results for all three LPS concentrations were less than 1 $\mu mol/10^6$ cells/day. OM preparations at a dose of 1 μg ml^{-1} stimulated human peripheral blood monocytes to produce small but significant amounts of NO ($p < 0.02$). Increasing the concentration of OM preparation to 100 μg ml^{-1} resulted in an increase in the amount of NO released by human peripheral blood monocytes ($p < 0.02$). Human peripheral blood monocytes

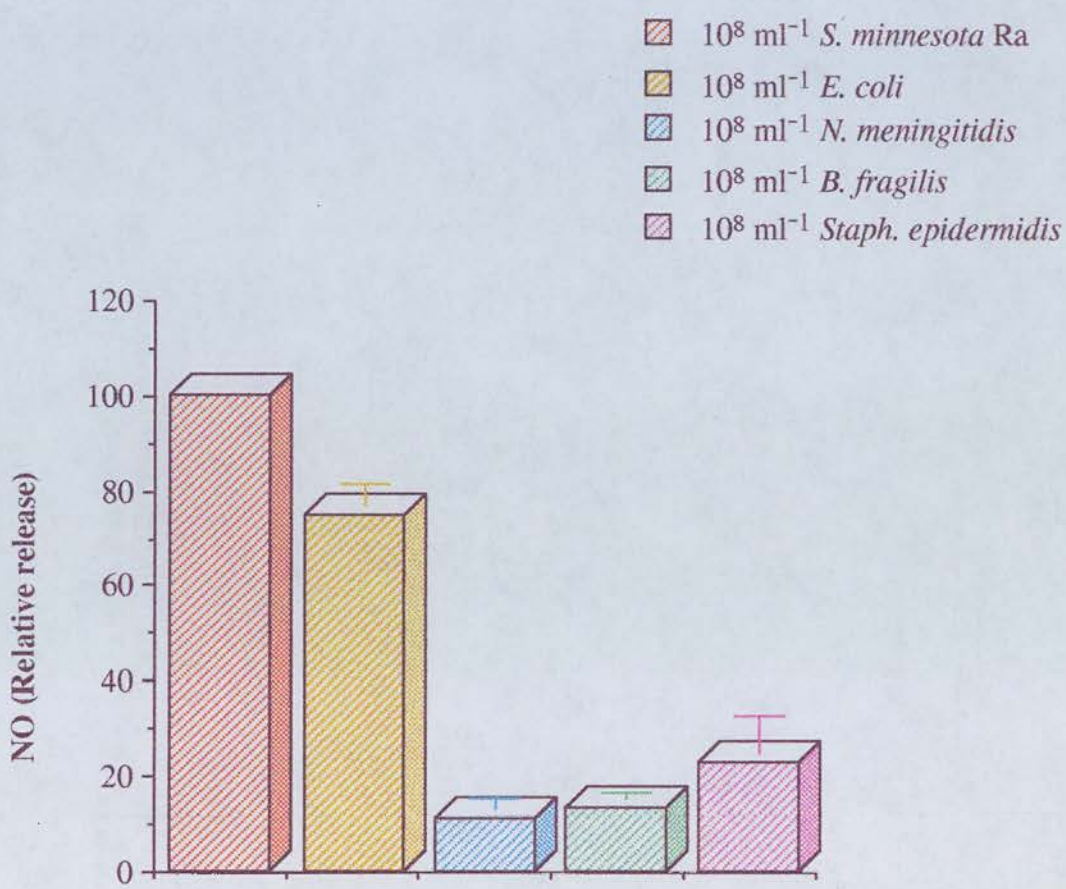

Figure 4.13: NO release from THP-1 cells in response to different bacteria at a dose of 10^8 ml^{-1}. Each column represents the mean NO release ± SEM of four to fifteen experiments.

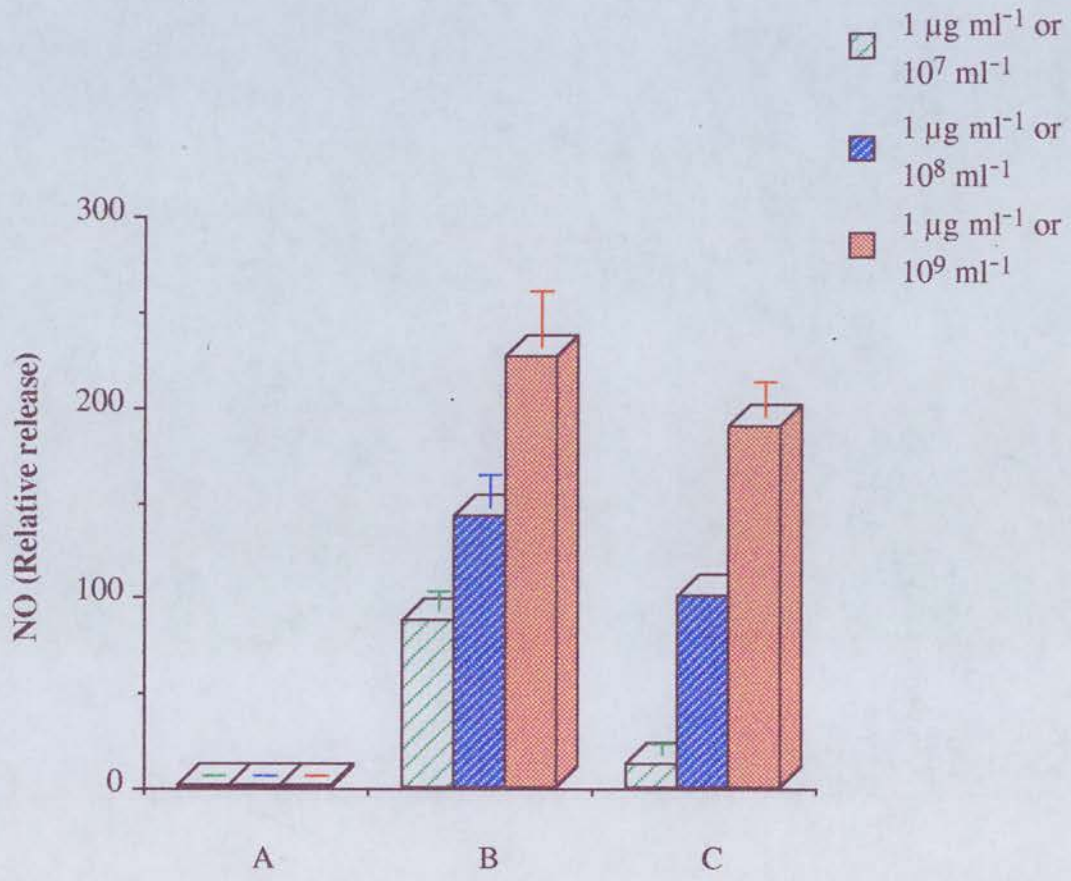

Figure 4.14: NO release from human peripheral blood monocytes in response to different concentrations of various forms of *S. minnesota* Ra LPS: free LPS (A), outer membrane fraction (B) and whole organism (C). Each column represents the mean NO release of three experiments ± SEM

released small amounts of NO in response to a dose of 10^7 ml^{-1} of whole *S. minnesota* Ra. Again the dose of 10^8 ml^{-1} *S. minnesota* was used as an internal standard and the amount of NO released in response to this dose was 3.1 $\mu mol/10^6$ cells/day. Increasing the dose of *S. minnesota* Ra to 10^9 ml^{-1} resulted in an increase in the amount of NO released from human peripheral blood monocytes ($p < 0.01$).

4.3.12 Effect of the method of killing of bacteria on NO release from THP-1 cells

All previous work on NO release in response to whole bacteria was performed using formaldehyde killed bacteria. Heat is another method of killing of bacteria. To investigate the effect of both methods of killing of bacteria on NO production from cells, *S. minnesota* Ra and *Staph. epidermidis* killed using both methods were used at similar doses to stimulate THP-1 cells to release NO. Results of all doses of both preparations (heat- killed and formaldehyde-killed) of the two bacterial species used are shown in Figure 4.15. Formaldehyde-killed *S. minnesota* Ra at a dose of 10^8 ml^{-1} was used as an internal standard and NO released in response to this dose was 6.7 $\mu mol/10^6$ cells/day. Although the amounts of NO released in response to heat-killed *S. minnesota* Ra and *Staph. epidermidis* were slightly higher than those released in response to formaldehyde-killed bacteria, there were no significant differences between the amounts of NO released in response to both preparations at all bacterial doses used.

4.3.13 Nitric oxide release from THP-1 and human peripheral blood monocytes in response to stimulation with recombinant interferon-gamma (rIFN-γ) by itself and in combination with various LPS forms

Human peripheral blood monocytes and THP-1 cells were stimulated with human recombinant interferon-gamma (rIFN-γ) with or without a second stimulus (various forms of LPS). Results of a representative experiments are summarised in Table 4.2. Neither THP-1 cells nor human peripheral blood monocytes produced any measurable amounts of NO in response to stimulation with rIFN-γ by itself or in combination with free LPS.

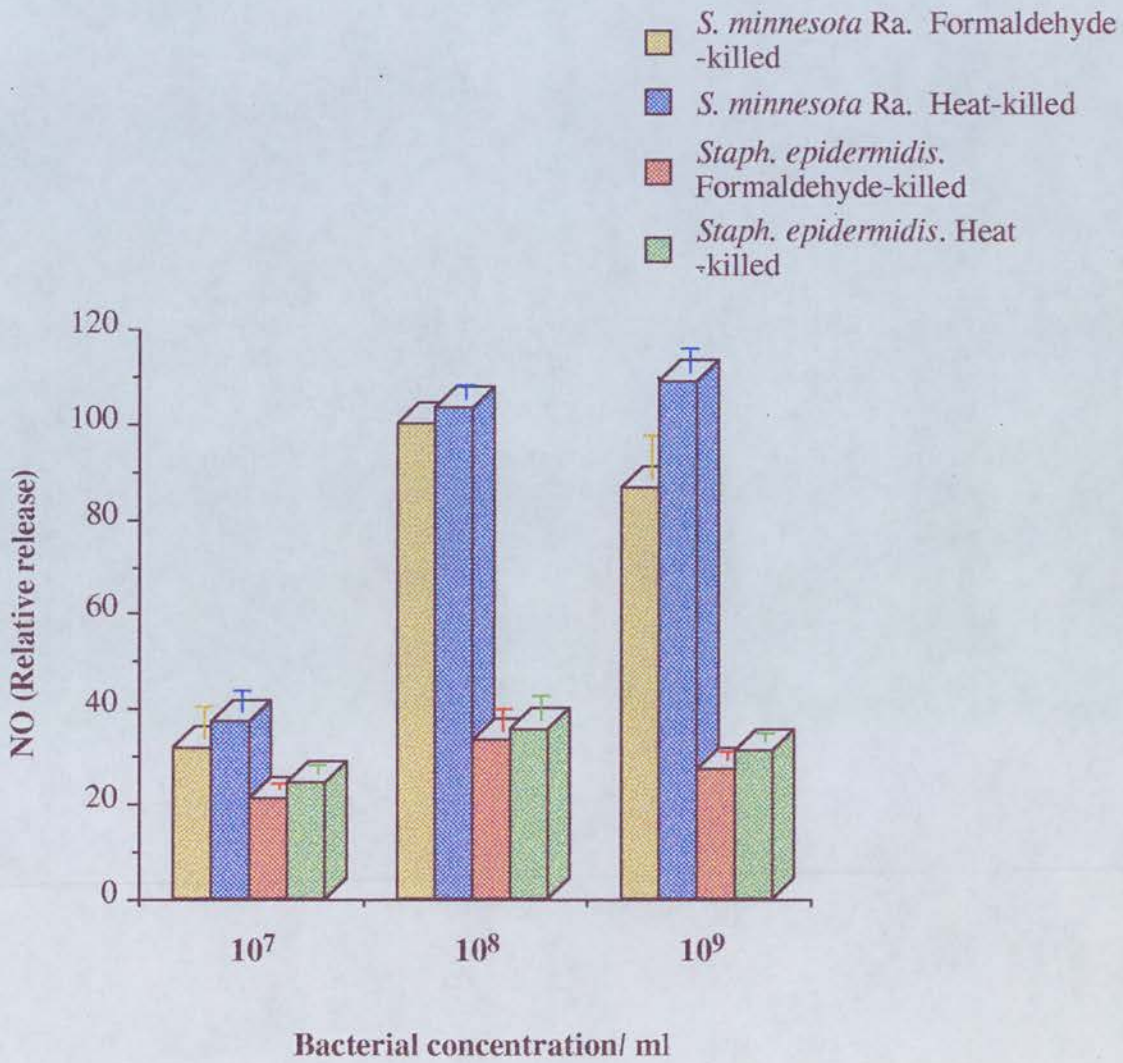

Figure 4.15: Comparison between NO release in response to formaldehyde-killed bacteria and heat-killed bacteria from THP-1 cells using different doses of Gram-negative and Gram-positive bacteria. Each column represents the mean NO release of four experiments ± SEM

Table 4.2: NO release from IFN-γ activated THP-1 and human blood monocytes.

Cells	Pre-treatment for 24 h.	NO (μmol/10^6 cells/day)
Human blood monocytes	None	< 1
	LPS	< 1
	OM	3.8
	Whole *S. minnesota* Ra	2.4
	rIFN-γ	< 1
	rIFN-γ + LPS	< 1
	rIFN-γ + OM	3.5
	rIFN-γ + Whole *S. minnesota* Ra	2.1
THP-1 cells	None	< 1
	LPS	< 1
	OM	5.3
	Whole *S. minnesota* Ra	12.4
	rIFN-γ	< 1
	rIFN-γ + LPS	< 1
	rIFN-γ + OM	5.1
	rIFN-γ + Whole *S. minnesota* Ra	11.3

rIFN-γ - human recombinant interferon-γ (200 ng ml^{-1})

LPS - *S. minnesota* Ra LPS (10 μg ml^{-1})

OM - outer membranes of *S. minnesota* Ra (10 μg ml^{-1})

Whole *S. minnesota* Ra (10^8 ml^{-1})

rIFN-γ did not enhance the ability of either OM preparation or whole bacteria to stimulate either THP-1 cells or human peripheral blood monocytes to release NO.

4.3.14 Nitric oxide release from CD14-enhanced THP-1 cells in response to various LPS forms

To study the role of CD14 receptors in NO production, the expression of CD14 on THP-1 cells was enhanced by treating the cells with 1,25-dihydroxyvitamin D_3, the cells were then stimulated with various forms of LPS and the amount of NO produced measured. Figure 4.16 shows relative release of NO from both CD14-enhanced and normal THP-1 cells. Enhancement of the expression of CD 14 receptors on THP-1 cells did not gave the cells the ability to produce NO in response to free LPS. There was no significant difference between NO production in response to a concentration of 10 μg ml^{-1} of OM preparation or a dose of 10^8 ml^{-1} whole *S. minnesota* Ra from CD14-enhanced THP-1 and from normal THP-1 (without enhancement for CD14). THP-1 cells treated with 1,25-dihydroxyvitamin D3 were found to express more CD14 receptors than normal THP-1 cells (see section 4.3.4).

4.3.15 Inhibition of release of NO from THP-1 cells by polymyxin B sulphate.

Polymyxin B sulphate was used at several concentrations to inhibit the production of NO from THP-1 cells when stimulated with OM preparation or whole *S. minnesota* Ra. Figure 4.17 shows NO relative release in response to a concentration of 10 μg ml^{-1} of OM preparation and a dose of 10^8 ml^{-1} whole *S. minnesota* Ra. There was no inhibition of NO release from THP-1 cells in response to whole *S. minnesota* Ra even with the highest concentration of polymyxin used as compared to control (whole *S. minnesota* Ra and without polymyxin treatment). The amount of NO released from THP-1 in response to whole *S. minnesota* Ra was 9.8 μmol/10^6 cells/ day. At a concentration of 0.1 μg ml^{-1}, polymyxin B inhibited the release of NO from THP-1 in response to OM preparation, but this inhibition in NO release was not significant. Increasing the concentration of polymyxin B to 1 μg ml^{-1} significantly inhibited ($p < 0.01$) NO release from THP-1 cells

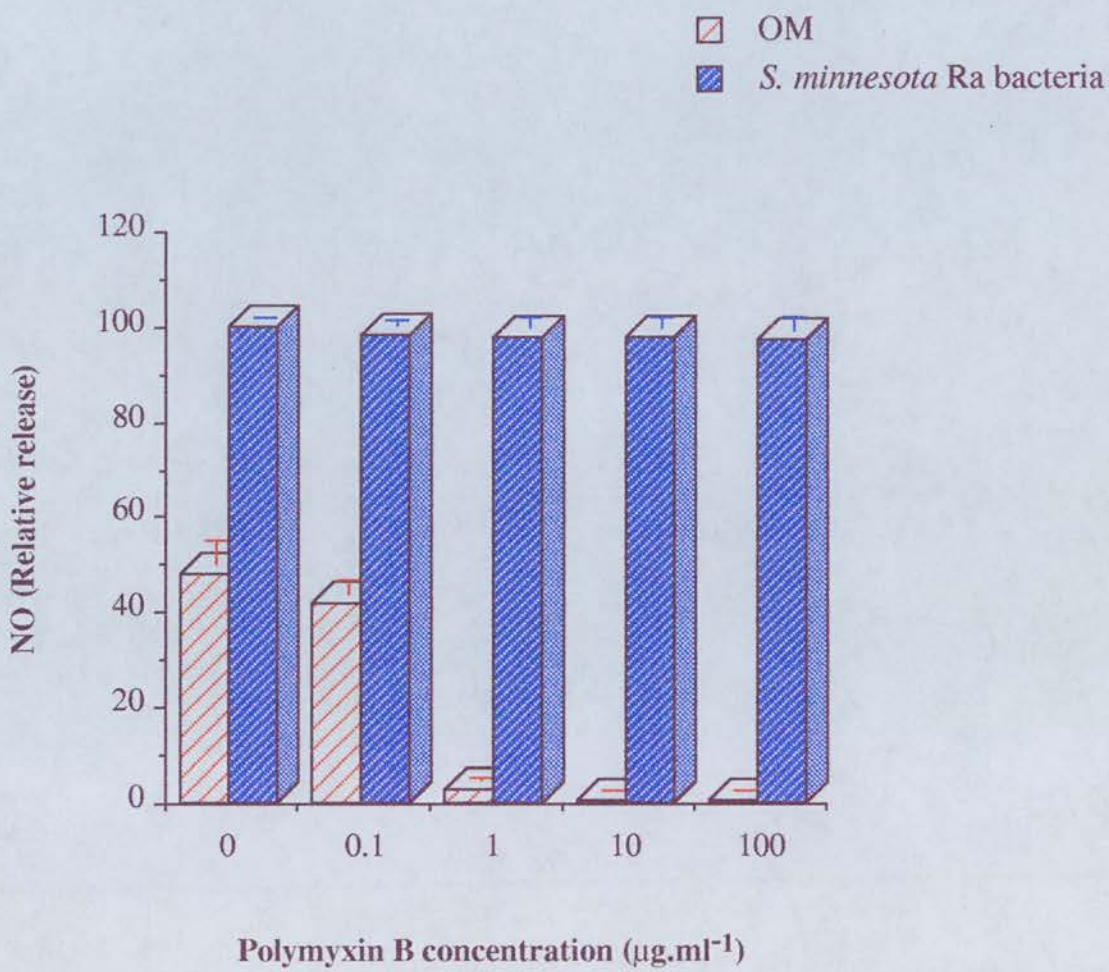

Figure 4.17: Inhibition of NO release by polymyxin B. THP-1 cells were stimulated by outer membrane fraction (OM) at a concentration of 10μg ml^{-1} or whole *S. minnesota* Ra at a dose of 10^8 ml^{-1} in the presence of different concentrations of polymyxin B sulphate. Each column represents the mean NO release of three experiments ± SEM

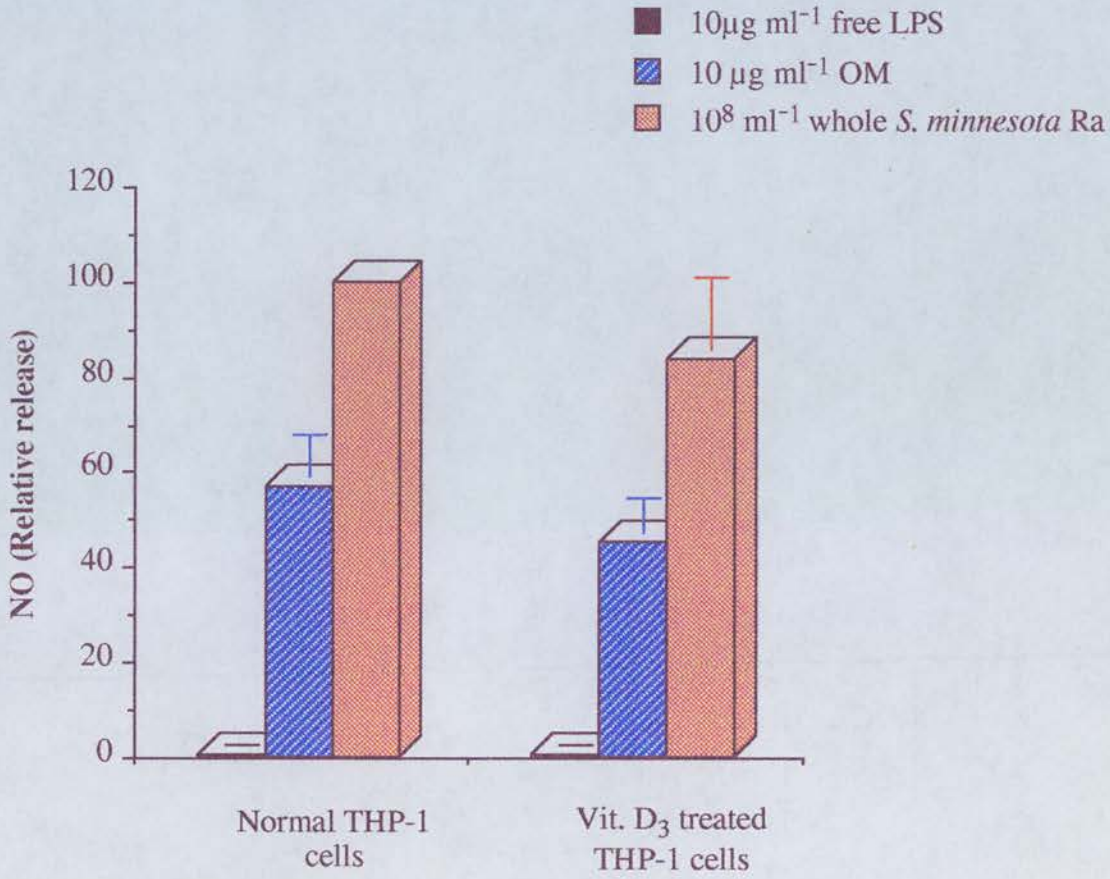

Figure 4.16: NO release from CD14-enhanced THP-1 and normal THP-1 cells in response to various forms of *S. minnesota* Ra LPS. Each column represents the mean NO release of three experiments ± SEM

(OM = outer membrane fraction).

in response to the OM preparation as compared to control (OM without polymyxin B treatment). Further increase in polymyxin concentration to 10 μg ml^{-1} and 100 μg ml^{-1} completely blocked NO production from THP-1 in response to OM preparation. The blockage of NO release from THP-1 was statistically significant ($p < 0.01$ for both concentrations).

4.4 Discussion

The aim of this study was to investigate the interaction between LPS and host cells. In the previous chapter an attempt was made to determine the direct interaction between various forms of LPS with host cells by studying the binding of bacteria to cells. This approach was abandoned due to the major technical difficulties in the use of FITC-labelled bacteria and the time consuming direct microscopic technique. The outcome of the interaction between LPS and host cells is the release of cytokines and other mediators by host cells. Measurement of the release of these molecules in response to various LPS preparations afforded a different approach to study the LPS-host cell interaction. Here the release of TNF and NO from host cells was investigated.

In addition to its potential role as a regulator of the normal immune response, TNF is also known to play a major role in systemic toxicity associated with sepsis (Tracey and Cerami, 1994). TNF might also be involved in the pathogenesis of AIDS (Folks *et al.*, 1989) as well as a number of autoimmune and inflammatory diseases.

The L929 mouse fibroblast bioassay for TNF quantitation was originally described by Flick and Gifford (1984) and has been used by many other investigators (Mohler *et al.*, 1993: Delahooke *et al.*, 1995). This assay is sensitive and relatively cheap. The L929 target cells also grow rapidly and are undemanding to culture.

THP-1 cells produced significant amounts of TNF in response to all forms of *S. minnesota* Ra LPS used. Whole *S. minnesota* Ra and OM fraction from *S. minnesota* Ra were more potent than free *S. minnesota* Ra LPS in stimulating the production of TNF from THP-1 cells. TNF production peaked after 4 hours of stimulation and was dose dependent for all forms of LPS used. Other investigators (Simon *et al.*, 1991) used THP-1 cells in their work. They reported that THP-1 cells produced TNF in response to free *E. coli* LPS and also to filtrates of *E. coli* killed by different antibiotics. They concluded that differences exist among bactericidal antibiotics in their ability to generate products capable of stimulating THP-1 cells to release TNF.

Gram-positive bacteria were used by other investigators (Timmerman *et al.,* 1993) to induce human peripheral blood monocytes to release TNF. These investigators reported that whole staphylococci and purified peptidoglycans isolated from three *Staph. epidermidis* and three *Staph. aureus* strains stimulated human monocytes to release TNF in a dose-dependent fashion. This is in agreement with this work in which *Staph. epidermidis* stimulated THP-1 cells to produce TNF in a dose-dependent fashion.

CD14 has been reported to be responsible for signalling mononuclear phagocytes to produce TNF and other cytokines when these cells interact with LPS (Shumann *et al.,* 1990; Dentener *et al.,* 1993), therefore a role of CD14 in signalling THP-1 cells to produce TNF was investigated. CD14 receptors appears not to be involved in the process of TNF production from THP-1 cells in response to all forms of LPS used. This is because of: a) THP-1 cells with increased expression of CD14 receptors released comparable amounts of TNF as compared to THP-1 cells without enhancement for CD14 and b) anti-CD14 mAb did not inhibit TNF release from THP-1 cells. Other investigators (Delahooke *et al.,* 1995) have used *Bacteroides* LPS to stimulate TNF production from THP-1 cells (with and without enhancement for CD14) in their work. They studied the capacity of LPS extracted by aqueous phenol method from four species of *Bacteroides* to induce TNF. They reported that there was no difference in TNF production by *B. fragilis* NCTC 9343 LPS from THP-1 cells with or without enhancement for CD14. The same workers reported that anti-CD14 mAb did not inhibit TNF production from THP-1 cells with or without enhancement for CD14 in response to *Bacteroids fragilis* LPS. They suggested that TNF production from THP-1 cells in response to *Bacteroids fragilis* LPS was independent of CD14. These results are in agreement with my work on TNF production from THP-1 cells in response to various forms of LPS from *S. minnesota* Ra.

The role of *S. minnesota* Ra LPS, in all forms used (free LPS, OM and whole bacteria) in inducing THP-1 cells to produce TNF was investigated using polymyxin B sulphate, an agent known to bind to lipid A region of LPS molecule. Polymyxin B sulphate at

concentrations of 0.1, 1, 10 μg ml^{-1} completely blocked TNF release from THP-1 cells in response to free *S. minnesota* Ra LPS and OM preparation from *S. minnesota* Ra. Polymyxin B sulphate at all concentrations used however, did not inhibit TNF production from THP-1 in response to whole *S. minnesota* Ra. One possible explanation for this is that free LPS and OM fraction bind to the surface of THP-1 cells and trigger TNF and therefore their affect was neutralised by polymyxin. Whole bacteria trigger by another method, perhaps by being internalised where polymyxin is released or inactivated.

In addition to other activities NO has been proposed as an antimicrobial and antitumoural effector system of mononuclear phagocytes of laboratory animals. Over the last five years more observations on the antimicrobial and cytotoxic effects of NO and the regulation of its production by macrophages have appeared in the literature, pointing to the increased interest in this effector system (Nathan and Hibbs, 1991; Moncada *et al.*, 1991; Nathan 1992). The increasing knowledge of the NOS of rodent macrophages contrasts with the scarcity of data on a counterpart of this enzyme system in human mononuclear phagocytes

NO production is measured by a colorimetric assay in which nitrite is measured in the supernatants of mononuclear phagocytes in culture. This method has been used by many other investigators to measure NO in the supernatants of mouse macrophage cell lines stimulated with E. coli LPS, BCG, lymphokines, IFN-γ and Zymosan (Stuehr and Marletta, 1987), peritoneal macrophages of different mouse strains stimulated with *E. coli* LPS (Zhang *et al.*, 1994) and human, mouse and rabbit macrophages stimulated with *E. coli* LPS and IFN-γ (Schneemann *et al.*, 1993).

This part of the study used the production of NO to investigate the interaction of mouse peritoneal macrophages with various forms of LPS and different bacterial species. Three mouse strains including the LPS hyporesponsive strain, C3H/HeJ, were used in the investigation.

Peritoneal macrophages of CBA mice produced measurable amounts of NO in response to all forms of LPS used. OM preparations were more potent than free LPS in stimulating peritoneal macrophages of CBA mice to produce NO. The most potent stimulus for NO production was whole *S. minnesota* Ra. High concentrations of free LPS, OM preparation (100 μg ml^{-1}) and whole *S. minnesota* Ra (10^9 ml^{-1}) apparently killed the cells because there was no measurable amounts of NO in the supernatants of macrophages stimulated with these concentrations. Low concentrations of free LPS and OM preparation (1 μg ml^{-1}) failed to stimulate macrophages to produce NO. *E. coli* and *N. meningitidis* stimulated peritoneal macrophages of CBA mice to produce NO at all bacterial doses used. NO production was the highest at a dose of 10^8 ml^{-1} for both microorganisms. *B. fragilis* and *Staph. epidermidis* stimulated peritoneal macrophages of CBA mice to produce small amounts of NO. There therefore appear to be variation in the ability of different bacteria and LPS preparations to stimulate NO production.

Peritoneal macrophages of BALB/c mice were stimulated with various forms of LPS and also with different bacteria to see the effect of a different genetic background on NO production. Peritoneal macrophages of BALB/c mice produced larger amounts of NO than those produced by peritoneal macrophages of CBA mice in response to free LPS, whole *S. minnesota* Ra and all other bacteria used. The best stimulus of NO for both strains of mice was whole *S. minnesota* Ra at a concentration of 10^8 ml^{-1}. In another study (Schneemann *et al.*, 1993) resident and thioglycollate elicited peritoneal macrophages of BALB/c and C3H/HeN mice were used to study NO production in response to free *E. coli* LPS alone or in combination with IFN-γ. The workers reported that thioglycollate elicited peritoneal macrophages of BALB/c and C3H/HeN produced NO (10.5 μmol/10^6 cells/day) in response to free LPS alone. Their finding is in agreement with this work in which resident BALB/c peritoneal macrophages produced NO amounts of 12.3 μmol/10^6 cells/day in response to free LPS. They also reported that IFN-γ enhanced the production of NO (51.3 μmol/10^6 cells/day) from thioglycollate elicited peritoneal macrophages of BALB/c and C3H/HeN in response to LPS. Other forms of LPS were not used in their study.

Having tested peritoneal macrophages of CBA and BALB/c mice with various stimuli, the ability of peritoneal macrophages of the hyporesponsive mouse (C3H/HeJ) to produce NO was tested using the same stimuli. The C3H/HeJ mouse strain is a mutant inbred strain which has been demonstrated to be refractory to the in vivo inflammatory and lethal effects of LPS (Sultzer 1968). This hyporesponsiveness to LPS is manifested in B and T lymphocytes, fibroblasts and macrophages of C3H/HeJ mouse strain (Morrison 1986).

The most potent form of *S. minnesota* Ra LPS in stimulating peritoneal macrophages of C3H//HeJ was whole *S. minnesota* Ra at a dose of 10^8 ml^{-1}. OM preparation stimulated peritoneal macrophages of C3H/HeJ to produce significant amounts of NO and was more potent than free LPS which stimulated peritoneal macrophages of C3H/HeJ to produce negligible (< 1 μmol/ 10^6 cells/ day) amounts of NO. *E. coli* was the most potent organism in stimulating peritoneal macrophages of C3H/HeJ to produce NO. *N. meningitidis* also stimulated substantial amounts of NO. Again as with other mouse strains *Staph. epidermidis* and *B. fragilis* were the least stimulating organisms for the production of NO. The level of NO produced by peritoneal macrophages of C3H/HeJ mice was similar to the NO level produced by peritoneal macrophages of CBA mice. Other investigators (Zhang *et al.*, 1994) used thioglycollate elicited peritoneal macrophages of C3H/HeJ mice in their work. They stimulated the thioglycollate-elicited C3H/HeJ peritoneal macrophages with either free *S. minnesota* Ra LPS (Ra-LPS) or free *E. coli* smooth LPS (S-LPS) and measured NO production. They found that neither Ra-LPS nor S-LPS could initiate the production of NO in those macrophages when used as stimuli on their own at 10 μg ml^{-1}. This finding is in agreement with my work on resident peritoneal macrophages of C3H/HeJ mice using similar concentration of free *S. minnesota* Ra LPS. Zhang and co-workers (1994) found that the ability of thioglycollate elicited peritoneal macrophages of C3H/HeJ to produce NO in response to free LPS was restored when LPS was used in combination with IFN-β or IFN-γ. Other forms of LPS have not been used in their study. These studies agree with in vivo response seen in

C3H/HeJ mice to LPS. However it appears that these mice respond to whole bacteria and outer membrane preparations by producing NO.

Human cells such as hepatocytes, endothelial cells and neutrophils have been shown to express an inducible NO synthase (NOS), but the presence of a similar pathway in human monocytes/macrophages has been questioned by many investigators and is a subject of great controversy.

THP-1 cells and human peripheral blood monocytes were used to investigate whether human mononuclear phagocytes can produce NO in response to various forms of LPS and other bacteria. THP-1 cells did not produce any significant amounts of NO in response to stimulation with different concentrations of free *S. minnesota* Ra LPS. In contrast these cells produced significant amounts of NO in response to the three concentrations of OM and whole *S. minnesota* Ra used. A dose of 10^8 ml^{-1} whole *S. minnesota* Ra was the most potent stimulus of NO from THP-1 cells. Whole *E. coli* also stimulated the production of significant amounts of NO from THP-1 and was more potent than other organisms except for *S. minnesota* Ra. THP-1 cells also responded to the stimulation of whole *N. meningitidis*, *B. fragilis* and *Staph. epidermidis* by producing small significant amounts of NO.

Human peripheral blood monocytes (PBMC) were tested to find if they could produce NO in response to various forms of LPS and different bacteria. In response to free LPS human peripheral blood monocytes did not produce any significant amounts of NO. In response to all OM preparation concentrations used human PBMC produced significant amounts of NO. The production of NO from human PBMC was dose-dependent. Human PBMC also produced significant amounts of NO in response to whole *S. minnesota* Ra at doses of 10^8 ml^{-1} and 10^9 ml^{-1}.

Both THP-1 and human PBMC behaved comparably in that both responded to OM preparation and whole *S. minnesota* Ra by producing NO and both of them did not produce NO in response to free LPS. OM preparation was more potent in stimulating the

production of NO from PBMC than whole *S. minnesota* Ra which was more potent than OM in stimulating the production of NO from THP-1 cells.

Other investigators (Schneemann *et al.,* 1993) reported that both human PBMC and human peritoneal macrophages were unable to produce NO in response to either free *E. coli* LPS alone or free LPS combined with IFN-γ or in response to a combination of free LPS with IFN-γ and tetrahydrobiopterin (BH_4). These investigators claimed that human mononuclear phagocytes do not synthesise BH_4 (an essential cofactor of NOS); therefore, they supplemented human mononuclear phagocytes with exogenous BH_4 to investigate if it would give rise to NO production. They concluded that human mononuclear phagocytes cannot produce NO under the conditions tested and that inducible NOS was not a constituent of the antimicrobial system of human mononuclear phagocytes. Part of our work agrees with Schneemann and co-workers (1993) in that human mononuclear phagocytes do not produce NO in response to free LPS; however this study shows that human mononuclear phagocytes have the ability to produce NO in response to bacterial OM preparations and whole bacteria of different species. Other investigators (Munoz-Fernández *et al.,* 1992) have reported that human PBMC can kill intracellular *Trypanosoma cruzi* by NO dependent mechanism when activated by IFN-γ and TNF-α. They also reported that NO production and trypanocidal activity induced in these monocytes by TNF-α or IFN-γ alone or in combination, were inhibited by *N*-monomethyl-L-arginine (*N*-MMLA), a competitive inhibitor of NO synthase activity.

In this work formaldehyde-killed bacteria were used to stimulate host cells. Heat killing is another method of killing bacteria but it has not been used because this method might cause the shedding of LPS from Gram-negative bacteria. The shed LPS would be washed away during the washing of bacteria and consequently would affect the results of experiments in which whole Gram-negative bacteria were used as another form of LPS. Heat killing of bacteria might also cause lysis that might affect the results of experiments in which whole Gram-positive and Gram-negative bacteria were used to stimulate host cells. The effect of both methods of killing of bacteria on NO production from THP-1

cells was investigated in this work. There was no significant difference in NO release from THP-1 cells in response to both formaldehyde-killed and heat-killed bacteria. This applied to both Gram-negative and Gram-positive bacteria; therefore, the work continued using formaldehyde-killed bacteria.

IFN-γ was reported to activate murine macrophages to produce NO in response to bacterial products (Amber *et al.*, 1988; Schneemann *et al.*, 1993). THP-1 cells and human PBMC were used to determine if they behave similarly to murine macrophages in response to activation with human recombinant IFN-γ. IFN-γ alone or in combination with free LPS did not induce the cells to produce NO. Also IFN-γ did not enhance the production of NO from either THP-1 or human PBMC in response to OM preparation or whole *S. minnesota* Ra. This work is in agreement with other investigators (Schneemann *et al.*, 1993; Murray and Teitelbaum, 1992; Padgett and Pruett, 1992) who reported that human mononuclear phagocytes treatment with IFN-γ alone or in combination with LPS did not induce these cells to produce NO. In contrast to our results, other investigators (Munoz-Fernández *et al.*, 1992) reported that IFN-γ–stimulated human PBMC-derived macrophages produce NO in response to an infection with *Trypanosoma cruzi*. The rIFN-γ used in my work was not tested in other assays; therefore, it is possible that it was inactive.

The possible role for CD14 receptors in signalling THP-1 cells to produce NO was also investigated. It seems that CD14 receptors are not involved in the process of producing NO from THP-1 cells because increased expression of CD14 on THP-1 did not enhance NO production from these cells. Also anti CD14 monoclonal antibodies failed to inhibit NO production from THP-1 cells in response to OM preparation and whole bacteria (data not shown).

The role of LPS in OM and whole bacteria preparations in inducing THP-1 cells to produce NO was investigated using polymyxin B sulphate. Polymyxin B sulphate at concentrations of 1 μg ml^{-1}, 10 μg ml^{-1} and 100 μg ml^{-1} completely blocked NO production from THP-1 cells in response to OM preparation, this finding points to the

involvement of the LPS molecule, in particular the lipid A region, in the induction process of THP-1 to produce NO. As for TNF polymyxin B sulphate did not inhibit NO production in response to whole *S. minnesota* Ra probably for the same reasons. Two monoclonal antibodies (SZ 27.19 and WN1) directed against *Salmonella* and *E. coli* LPS were used to inhibit the production of NO from THP-1 cells. Both antibodies at several dilutions failed to inhibit NO production from THP-1 cells in response to OM preparation and whole *S. minnesota* Ra. This might be because these antibodies were binding to an epitope on the LPS core that is different from the one which is recognised by host cell receptors. SZ was also used to inhibit NO production from PBMC in response to OM preparation and *S. minnesota* Ra. SZ did not inhibit NO production from PBMC at any dilutions used. Interestingly, SZ was found to stimulate PBMC to produce small amounts of NO in control samples (data not shown). The results with polymyxin B also suggest that the results with OM and whole bacteria are due to LPS and not other molecules.

Gram-positive bacteria were previously reported to induce the production of NO from rat bone marrow derived mononuclear phagocytes (Keller *et al.*, 1991); therefore, the ability of Gram-positive bacteria to stimulate host cells to produce NO was investigated. In this work Gram-positive bacteria represented by *Staph. epidermidis* stimulated peritoneal macrophages of different mouse strains and human macrophage cell line (THP-1) to produce small amounts of NO. Keller and co-workers (1991) used several Gram-positive and Gram-negative bacteria in their investigations of the interaction between bacteria and macrophages by measuring NO production and macrophage-mediated tumouricidal activity. They reported that several Gram-positive bacteria including a strain of *Staph. epidermidis* stimulated bone marrow-derived mononuclear phagocytes of rats to produce NO which is in agreement with the results of this work on mouse macrophages.

Free LPS did not stimulate human peripheral blood monocytes or THP-1 cells to produce NO, but whole bacteria and OM preparation stimulated these cells. Also NO production from murine resident macrophages was greater in response to whole bacteria and OM preparation than free LPS. This might indicate that other bacterial cell surface

component/s are stimulating host cells to produce NO by themselves and therefore enhancing the effect of LPS or they might synergise with LPS to stimulate host cells to produce NO. Because of the lack of an accurate method to estimate the amount of LPS attached to whole bacteria (the KDO assay is not accurate with whole bacteria), whole bacterial preparations might have greater amount of LPS than the amount used as free LPS or the amount of LPS attached to OM preparation.

In this chapter the production of TNF from the human monocytic cell line (THP-1) and NO from murine macrophages in response to various forms of LPS and different bacteria was investigated. The ability of human mononuclear phagocytes to produce NO in response to particular forms of LPS and to different bacteria was shown indicating that human mononuclear phagocytes possess NOS activity which might form part of their antimicrobial armoury.

Chapter 5

General discussion

Lipopolysaccharides (endotoxins) are major components of the outer membrane of all Gram-negative bacteria. They are essential for the physical organisation and function of these membranes and for bacterial growth and multiplication (Rietschel *et al.,* 1990). They form a hydrophobic barrier that restricts the entry of harmful substances. The O-side chains (O-antigen) of LPS form a carbohydrate layer in organisms lacking capsules. This layer increases the hydrophilicity of the cell surface enabling the cells to avoid phagocytosis. Avoiding phagocytosis is necessary for survival of pathogens of animals (Cunningham *et al.,* 1975). Endotoxins have the ability to influence both cellular and humoral limbs of the immune response (Rapson, 1988). The endotoxicity is determined mainly by the structure of the lipid A with the determining factor being the fatty acid composition and substitution pattern and the phosphate substitution.

Endotoxins have been implicated in the pathogenesis of several clinical disorders. Of these the most familiar is Gram-negative septic shock in which the role of endotoxins is most clearly established (Morrison and Ryan, 1987). Gram-negative septic shock is a life-threatening disease associated with high mortality especially in immuno-compromised patients. The mechanism by which Gram-negative bacteria induce septic shock is complex with little experimental evidence pointing to a single toxic factor. When purified from organisms and injected into experimental animals, endotoxin induces many of the same pathophysiological changes seen during Gram-negative infections (Manthey and Vogel, 1992). Based on an increasing body of data, it is now felt that many of the symptoms of septic shock are mediated by the action of host-derived mediators released in response to endotoxin. These mediators play important roles in the normal host response to insult but their overproduction can have important consequences for the host. Several of these mediators have been recognised for some years. Macrophages are the principle source of endotoxin-

induced mediators which include tumour necrosis factor (TNF) (Manthey and Vogel, 1992).

For these mediators to be released LPS must interact with host cells. At least three classes of molecules on leucocytes have been recognised to be LPS receptors. CD18 molecules and the scavenger receptor are involved in the recognition and disposal of LPS without initiating mediator release. CD14 recognises LPS complexed to a serum protein, lipopolysaccharide binding protein (LBP) and this leads to both phagocytic uptake of bacteria and TNF release in response to very low concentrations of LPS-LBP complexes. High concentrations of LPS might initiate TNF release in the absence of serum or CD14 therefore another LPS receptor of low affinity might exist (Wright 1991). The pathways used by different forms of LPS (pure, OM and bacterial-associated) to signal release of mediators are not known. Also differences between LPS from different bacteria exhibit different levels of endotoxicity. It is important to know if variation in responses is linked to differences in interaction of LPS with cells.

In this study the binding of *S. minnesota* Ra to mouse peritoneal macrophages was studied to investigate the role of LPS in mediating the binding of Gram-negative bacteria to macrophages. The outcome of the interaction between LPS and host macrophages was also studied by measuring TNF and nitric oxide (NO) release following the stimulation of macrophages using various forms of LPS. An attempt was made to investigate the role of CD14 in signalling of macrophages to release TNF and NO.

The binding of Gram-negative bacteria to mouse peritoneal macrophages, human macrophage cell line (THP-1), human peripheral blood monocytes and neutrophils was measured using flow cytometry. This method apparently overcame some of the difficulties concerning the study of bacterial binding to host cells. One important

advantage of flow cytometry is that it allowed studies involving mixed cell types that can be distinguished on the basis of light scatter. Flow cytometry is objective; it is free of the possible experimental bias of microscopic binding studies. Flow cytometry also allowed the analysis of a large number of samples as well as a large number of cells in each sample in a short period of time. By using flow cytometry the optimum binding conditions of *S. minnesota* Ra to peritoneal macrophages of C3H mouse strain were determined. THP-1 cells exhibited similar binding pattern to those of mouse peritoneal macrophages when tested under the same conditions; however, the level of the binding of *S. minnesota* Ra to THP-1 cells was lower than the level of the binding of *S. minnesota* Ra to mouse peritoneal macrophages. This might be because of the maturation state of THP-1 cells which are immature macrophages probably expressing fewer receptors than the mature mouse peritoneal macrophages or it might be due to a variation in the binding ability between different species. Human peripheral blood monocytes and neutrophils from different individuals showed variable binding characteristics. This variation in the ability of cells from different individuals to bind bacteria could be because of genetic and/or environmental factors.

In this work the role of LPS in mediating the binding of Gram-negative bacteria to host macrophages was investigated using various forms of LPS. Free *S. minnesota* Ra LPS and outer membrane fraction from *S. minnesota* Ra inhibited significantly the binding of FITC-labelled *S. minnesota* Ra to mouse peritoneal macrophages. This clearly indicated the involvement of LPS in the attachment of FITC-labelled Gram-negative bacteria to mouse peritoneal macrophages. The inhibition of binding of FITC-labelled bacteria by using whole unlabelled bacteria (as another form of LPS) produced unexpected results in that whole unlabelled *S. minnesota* Ra failed to inhibit the binding of FITC-labelled *S. minnesota* Ra. This finding raised a few questions. First, the binding of unlabelled bacteria to mouse peritoneal macrophages

was questioned; therefore, the binding of unlabelled bacteria was investigated using mAb directed against *Salmonella* LPS and FITC-conjugated antimouse immunoglobulin. Although the level of the binding detected using this method was lower than that seen by using directly labelled bacteria with FITC (see section 3.3.3.4), this method confirmed that unlabelled bacteria were binding to mouse peritoneal macrophages in the absence of serum. This method was not suitable for inhibition work in which various forms of LPS were used to inhibit the binding of *S. minnesota* Ra to host cells.

Other questions concerning the specificity of the binding of FITC-labelled bacteria to macrophages were also raised. To investigate this a visual assay was used. With this method a direct comparison between the binding of FITC-labelled and unlabelled bacteria was possible. Using the visual assay, FITC-labelled *S. minnesota* Ra were found to bind to macrophages at much higher levels than unlabelled *S. minnesota* Ra. This enhancement of the binding seen with FITC-labelled bacteria was further investigated to find out whether this enhancement was specific to the FITC itself or it was because of the high pH of FITC coupling buffer. The effect of FITC labelling of other bacterial species on their binding to mouse peritoneal macrophages was also investigated. The enhancement of binding seen with FITC-labelled bacteria was found to be specific to the FITC itself and not to the FITC coupling buffer and also was not specific to the strain of *S. minnesota* Ra used in this study but was also applied to other bacterial species. The mechanism by which FITC enhanced bacterial binding is not known. It might be that FITC is non-specifically recognised by macrophages or have some kind of charge effect. FITC might also be altering the structure of bacterial cell surface component/s or it might be changing the hydrophobicity of bacterial cells. Because the results obtained using flow cytometry were brought into question, the visual assay was used to re-investigate the role of LPS in mediating the binding of Gram-negative bacteria to mouse peritoneal

macrophages. With the visual assay free *S. minnesota* Ra LPS inhibited the binding of unlabelled and FITC-labelled *S. minnesota* Ra to macrophages. These results confirmed the inhibition results obtained by using flow cytometry and also confirmed the conclusion that LPS is involved in mediating the binding of Gram-negative bacteria to macrophages. Consistent with this observation is the work of Wright and Jong (1986) who concluded that human peripheral blood-derived macrophages recognised *E. coli* in the absence of serum opsonins by recognising LPS. They reported that free *S. minnesota* R595 (Re) LPS and a biosynthetic precursor of LPS termed lipid IVa inhibited the binding of *E. coli* to human macrophages.

It has been found in this work that FITC labelling of different bacteria increased their binding capacity to host cells. Many other investigators (Wright and Jong, 1986; Zorgani *et al.,* 1994; Raza *et al.,* 1993; Saadi *et al.,* 1993; Raybourne and Bunning, 1994) have used FITC-labelled bacteria in their studies on bacterial binding to host cells. The results of their studies using FITC-labelled bacteria in vitro might be affected by the use of FITC and might not apply in vivo since FITC-labelled bacteria do not exist physiologically. Therefore it might be important to re-evaluate their results.

Free LPS and outer membrane fraction inhibited the binding of *S. minnesota* Ra to a similar degree. Both preparations did not block the binding of bacteria to host cells completely which can be explained by the presence of other bacterial cell surface component/s that are also involved in part of the binding such as carbohydrate residues which recognised by mannose-fucose receptors. Free *S. minnesota* Ra LPS also inhibited the binding of other FITC-labelled bacteria (*E. coli, Pseudomonas aeruginosa* and *Bacteroides fragilis*) to a variable degree (data not shown). This indicated that the inhibition of binding by free LPS from *S. minnesota* Ra was relatively non-specific as far as the LPS is concerned. It therefore appears probable

that conserved parts of the LPS, i.e. core and lipid A, are involved in the binding. Because of the difficulties in the use of FITC-labelled bacteria and because the use of direct microscopy is time consuming and also subjective to some extent, this line of investigation was abandoned.

The outcome of the interaction between LPS and host macrophages is the release of cytokines and other mediators by host macrophages. In this study the release of TNF from THP-1 cells was studied briefly. Nitric oxide release from different mouse strains and from human peripheral blood monocytes and THP-1 cells was also investigated. This afforded another read out system to study the interaction of various forms of LPS with host cells.

THP-1 cells released significant amounts of TNF in response to all forms of *S. minnesota* Ra LPS used. TNF release from THP-1 cells was dose dependent and peaked after 4 hours of stimulation of the cells. It seems that other bacterial cell surface component/s synergise with LPS in inducing TNF in that outer membrane fraction and whole *S. minnesota* Ra were more potent than free *S. minnesota* Ra LPS in this respect. It was hoped to use murine macrophages to measure TNF release upon stimulation with various forms of LPS, but it was not possible at the time due to a high background of TNF production by control samples. This might have been due to an infection in mouse colony or contamination with LPS during the harvesting and handling of the macrophages.

It has been established that bacterial LPS binds to the plasma protein LPS-binding protein (LBP) forming high affinity complexes (LPS-LBP) which are potent agonists for monocytic cells. CD14 has been reported to mediate monocyte recognition of LPS-LBP complexes leading to cytokine production (Wright *et al* 1990b); therefore, a role for CD14 receptors in the production of TNF in response to different forms of *S. minnesota* Ra was investigated. THP-1 cells with enhanced expression of CD14

were stimulated with various forms of LPS and produced amounts of TNF comparable to those produced by unenhanced THP-1 cells in response to the same forms and concentrations of LPS. One possible explanation for this finding is that CD14 receptors expressed on normal THP-1 cells were sufficient to mediate maximum stimulation of the cells and therefore, an increased expression of CD14 receptors does not have an effect. An other explanation is that other pathways for the stimulation of the cells to produce TNF might be of importance. In line with this hypothesis is the finding that anti-CD14 mAb did not block or inhibit the production of TNF. This is consistent with other investigators' work (Lynn *et al.*, 1993) in which they reported that CD14 and serum were not absolutely necessary for the activation of mononuclear phagocytes by bacterial LPS. They also suggested that a CD14-independent pathway might be of importance in local sites of infection where the concentration of LPS might be high.

The role of various forms of LPS in stimulating THP-1 cells to release TNF was investigated using polymyxin B sulphate, an agent known to bind to the lipid A region of the LPS molecule. Polymyxin B completely blocked TNF production from THP-1 cells in response to free *S. minnesota* Ra LPS and to the outer membrane fraction of *S. minnesota* Ra. The blockage of TNF production from THP-1 cells caused by polymyxin B indicated that lipid A is the active part of LPS molecule in stimulating the cells. It also seems that other bacterial cell surface components are not involved in the process of stimulation of THP-1 cells in that polymyxin B blocked TNF release in response to outer membrane fraction of *S. minnesota* Ra; however, polymyxin B did not block or inhibit TNF production from THP-1 cells in response to whole *S. minnesota* Ra. One possible explanation is that the whole bacteria might have been phagocytosed and polymyxin B might be released or inactivated after the binding or internalisation of whole Gram-negative bacteria by

host cells. In this situation the bacteria might then stimulate host cells via an intra- or extracellular mechanisms.

The production of NO and other reactive nitrogen intermediates by cytokine-activated rodent cells is an important component of antimicrobial and/or antineoplasic activities of these cells. The pathway of production involves the oxidation of arginine to citrulline with the concomitant release of NO by the inducible form of NO synthase (iNOS) (Denis, 1994). Numerous cell types express iNOS after stimulation with bacterial products such as LPS and/or cytokines. Although human cells such as hepatocytes and endothelial cells have been shown to express iNOS, the presence of such a pathway in the human monocyte/macrophage lineage has been questioned and is the subject of great controversy (Denis, 1994).

In this work NO release from different mouse strains including LPS hyporesponsive strain (C3H/HeJ), human macrophage cell line (THP-1) and human peripheral blood monocytes was investigated using various forms of *S. minnesota* Ra LPS and other bacteria. Peritoneal macrophages of CBA and BALB/c mice responded to various forms of *S. minnesota* Ra LPS by producing measurable amounts of NO. In both of these mouse strains whole *S. minnesota* Ra organisms were the most potent form of "LPS" in stimulating NO production. In CBA mice the OM fraction from *S. minnesota* Ra was more potent than purified *S. minnesota* Ra LPS. Different bacteria were also used to stimulate NO production from the peritoneal macrophages of CBA and BALB/c mice. *E. coli* was more potent than *N. meningitidis* in stimulating peritoneal macrophages of CBA mice whereas *N. meningitidis* was more potent than *E. coli* in stimulating peritoneal macrophages of BALB/c mice; however peritoneal macrophages of BALB/c mice produced larger amounts of NO than those of CBA mice in response to all forms of LPS and different bacteria used. *B. fragilis* and *Staph. epidermidis* were the least potent stimuli in both mouse strains.

Other investigators (Delahooke *et al.*, 1995) have reported that *B. fragilis* and *E. coli* O18K$^-$ LPS stimulated TNF production from peritoneal macrophages of C3H/HeN mice (responder) and also reported that peritoneal macrophages of C3H/HeJ mice (hyporesponsive) produced TNF in response to *B. fragilis* LPS but not to *E. coli* LPS. Other workers (Schneemann *et al.*, 1993) have used E. coli O26: B6 LPS to stimulate NO production from resident and thioglycollate elicited peritoneal macrophages of BALB/c and C3H/HeN mice. They reported that peritoneal macrophages of both mouse strains produced NO in response to *E. coli* LPS and also reported that IFN-γ enhanced the production of LPS-induced NO from these macrophages. These investigators work support the results of my work on peritoneal macrophages of different mouse strains when stimulated with LPS.

The C3H/HeJ mouse strain is a mutant inbred strain which has been demonstrated to have an inherited defect in the ability to respond to LPS. Recently, specific LPS binding proteins have been isolated on lymphocytes and other cells of C3H/HeJ. However a receptor which transduces an activation signal has not been isolated yet. It appears that the defect in C3H/HeJ cells resides in the signal pathway leading to gene activation and proliferation (Sultzer *et al.*, 1993). However there is growing experimental evidence that C3H/HeJ mice can recognise R-chemotype LPS.

In this work peritoneal macrophages of C3H/HeJ responded to the stimulation of whole *S. minnesota* Ra and OM fraction from *S. minnesota* Ra by producing NO, but they did not respond to the stimulation by free LPS. Peritoneal macrophages of C3H/HeJ also responded to different bacteria by producing NO. As for the other mouse strains used whole *S. minnesota* Ra bacteria were more potent than the OM fraction. *E. coli* was the most potent organism in stimulating C3H/HeJ whereas *S. minnesota* was more effective than *N. meningitidis*. Again *B. fragilis* and *Staph. epidermidis* were the least potent stimuli for NO production from C3H/HeJ mice. It seems that free LPS lacks a necessary component/s required to initiate LPS-induced

NO from C3H/HeJ and that this component/s is present in OM fraction and whole bacteria. Others (Zhang *et al.*, 1994) have reported that peritoneal macrophages of C3H/HeJ mice did not respond to stimulation with either *E. coli* S-LPS or *S. minnesota* R60 LPS. They also reported that addition of exogenous IFN-β or IFN-γ corrected the hypo- responsiveness of C3H/HeJ macrophages enabling them to produce TNF and NO.

In this work NO production from human cells (THP-1 and peripheral blood monocytes) was investigated using various forms of LPS and different bacteria. Both THP-1 cells and human monocytes did not respond to free *S. minnesota* Ra LPS. THP-1 cells responded to the stimulation of whole *S. minnesota* Ra bacteria and the OM fraction from *S. minnesota* Ra by producing NO. THP-1 also responded to other bacteria by producing measurable amounts of NO. *S. minnesota* Ra was the most potent organism in stimulating THP-1 cells. *E. coli* was more potent than the other organisms used. *N. meningitidis*, *B. fragilis* and *Staph. epidermidis* were the least potent organisms in stimulating THP-1 cells. Similar to THP-1 cells human peripheral blood monocytes produced NO in response to whole *S. minnesota* Ra and OM fraction from *S. minnesota* Ra. Although THP-1 cells and human monocytes behaved comparably in that both released NO in response to whole *S. minnesota* Ra and OM fraction, THP-1 cells produced greater amounts of NO than human monocytes. This might be due to differences in the state of maturation between THP-1 cells and human monocytes. The inability of free LPS to stimulate human cells might be because of the binding of free LPS to the cells was not efficient enough to stimulate these cells to release NO.

It appears that a variation exists in the ability of different bacteria to stimulate TNF and NO production from host cells. This might be due to variations in the structure of LPS in different Gram-negative bacteria, and Gram-positive bacteria must use entirely different stimulus. It might be due to a variation in the ability of host cells to

bind to different bacteria due to genetic and/or environmental factors. A study of this was part of the aims of this thesis. The binding of different bacteria to peritoneal macrophages of C3H mice (Figure 3.12) showed different levels of binding (this part of the work could not be extended due to the difficulties of using flow cytometry and visual assay).

It has been reported that *B. fragilis* contains different fatty acids from enterobacterial LPS, is monophosphorylated and generally accepted as being less endotoxic (Lindberg *et al.,* 1990). In this work *B. fragilis* stimulated mouse macrophages and THP-1 cells to produce NO. Other workers (Delahooke *et al.,* 1995) also reported that *B. fragilis* LPS extracted by the aqueous phenol method was as effective as *E. coli* O18K$^-$ LPS in stimulating TNF production from THP-1 cells. These observations might be of clinical significance when we consider that *Bacteroides* species are 1000-fold greater in number than *E. coli* in the gut. Although it is not known if these in vitro observations will occur in vivo, it could be of importance in terms of immunotherapy if bacteria or bacterial products translocate from the gut into the circulation; therefore, if *Bacteroides* LPS is a major inducer of cytokine and mediator responses, it should be targeted with antibody therapy instead of *E. coli* LPS.

The most potent form of LPS in stimulating TNF and NO was whole bacteria and the OM fraction was more potent than free LPS. This might be due to different signalling mechanism or pathways (bacteria might stimulate the cells after being internalised) or the ability of host cells to bind whole bacteria might be greater than that of the OM fraction and free LPS. The binding of OM fraction to host cells might also be more efficient than free LPS. Also there might be more LPS in whole bacteria than free LPS and OM fraction used in the same experiments. This is because of the lack of an accurate procedure to determine the amount of LPS in whole bacteria.

The production of NO from murine peritoneal macrophages in response to free *S. minnesota* Ra LPS is in contrast to the apparent hyporesponsiveness of human cells (THP-1 cells and human peripheral blood monocytes). Differences in the maturation and specialisation of the cells (THP-1 cells are immature macrophage cell line) could be one reason for the observed differences between species. Although human monocytes are mature cells, they did not respond; however, mice are much more resistant to the toxic effects of LPS. Mice will survive challenge with the same amount of LPS that would kill an adult human being. More work is needed on the signalling pathways in the two species to give possible insight into the mechanisms that mediate more production of cytokines by murine cells but make human beings more susceptible to the endotoxic shock.

Interferon-gamma was reported to enhance NO production from murine macrophages (Schneemann *et al.*, 1993; Zhang *et al.*, 1994). In this work human recombinant INF-γ did not enhance NO production from THP-1 cells. This might possibly be because the INF-γ used in this work was inactive.

The role of CD14 receptors in signalling THP-1 cells to produce NO was investigated. As with TNF production enhanced expression of CD14 on THP-1 cells had no effect on NO production as compared to THP-1 cells without enhancement for CD14.

The role of various forms of LPS in stimulating the production of NO was investigated using polymyxin B sulphate. As with TNF, polymyxin B completely blocked NO production from THP-1 cells in response to free *S. minnesota* Ra LPS and OM fraction from *S. minnesota* Ra but it did not block NO production from THP-1 cells in response to whole *S. minnesota* Ra.

In conclusion, LPS has been shown to interact directly with host cells and, therefore, probably plays a role in mediating the attachment of Gram-negative bacteria to host

macrophages. Host macrophages responded to the interaction with LPS by releasing TNF and NO. The lipid A region of the LPS molecule has been shown to be the active part in stimulating these responses. There is variation in the response of macrophages to stimulation by various forms of LPS and by different bacteria. The work suggests that pathways independent of CD14 are of importance in stimulating TNF and NO production. It might prove of importance to investigate the role of other LPS receptors in stimulating host responses. More knowledge of the mechanisms by which LPS stimulates host responses is necessary for the development of new strategies to neutralise the biological effects of endotoxins in Gram-negative bacterial infections and septic shock.

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