The influence of bacterial structure on the host inflammatory response to *Neisseria meningitidis*

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This thesis is dedicated to

My parents,

Jo, Freya

And the one to follow

Abstract

The gram-negative bacterium *Neisseria meningitidis* is a significant cause of human disease worldwide. Systemic meningococcal disease is characterised by severe vascular endothelial injury and profound septic shock. Meningococci can be found associated with both endothelial cells and invading leukocytes in vasculitic lesions from patients with severe disease. Binding and migration of activated host inflammatory cells to endothelium is a central process in mediating vascular damage. The expression and function of cell adhesion molecules regulates the interaction between leukocytes and endothelial cells. This thesis is primarily concerned with how meningococci directly influence expression of these receptors on vascular endothelium.

To address this, the expression of the vascular cell adhesion molecules CD62E, ICAM-1 and VCAM-1 on cultured human endothelial cells in response to meningococci was explored. Exposure of endothelial cells to *Neisseria meningitidis* and a range of isogenic mutants revealed that both capsule expression and lipopolysacchide (LPS) structure significantly influenced capacity of meningococci to induce expression of cell adhesion molecules. The pattern observed in response to meningococci was different to that of purified LPS, which is considered to be the primary inflammatory mediator in *Neisseria meningitidis*. Organisms induced greater expression of CD62E but were equally effective at inducing ICAM-1 and VCAM-1 expression than LPS alone. Cell adhesion molecule expression, activation of nuclear transcription factors required for CD62E expression and the response to the presence of a potent inhibitor of LPS activity, was determined on endothelial cells stimulated with purified LPS, parent meningococci and an isogenic meningococcal mutant completely deficient in LPS. The results suggest

that potent induction of cell adhesion molecule expression is due to multiple signals from both LPS and non-LPS components within meningococci.

This thesis also explored the contribution of both LPS and non-LPS components of *Neisseria meningitidis* on dendritic cell maturation and cytokine production. Dendritic cells are critical in the generation of both innate and adaptive immunity to invading bacteria. The results showed that LPS is required to be present within intact bacteria to elicit high-level cytokine production. Taken together, the results presented in this thesis provide further insight into how the structure of *N. meningitidis* effects the host response to this organism, and hence the pathogenesis of meningococcal disease.

Declaration

The work presented in this thesis is the work of the candidate with the following exception:

Chapter 6

Dr. Phillipa Newton (Departments Genito-urinary Medicine and Immunology, Windeyer Institute, University College London) prepared the dendritic cell cultures and prepared samples for phenotypic analysis by flow cytometry.

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1. Introduction

1.1 Introduction

1.1.1. Background to this thesis.

Infections caused by *Neisseria meningitidis* remain an important cause of human disease (Jones 1995). The usual relationship between *Neisseria meningitidis* and the human host is a commensal one. However, for reasons that are still not completely understood, in a small number of cases this bacterium invades its host and proliferates in sufficient numbers to induce disease. It is the most common cause of bacterial meningitis in the UK, and in this situation, the prognosis is good. However, in other cases meningococci proliferate rapidly in the circulation, inducing severe septic shock with a rapidly progressive course. The mortality can approach 50% when there is severe shock and a high degree of vascular and tissue damage, with patients often succumbing quickly after presentation. Prompt recognition, early treatment and skilled intensive care have all contributed to a reduction in mortality from this disease in recent years (Kirsch et al. 1996). However, the overall mortality for all cases is around 10% in England and Wales and survivors may have extensive tissue injury that may require amputation and/or skin grafting.

There is much that is unclear about the process by which meningococci induce the vascular damage that is responsible for the characteristic clinical picture seen in severe disease. In common with other bacteria, meningococci have remarkable capacity to rapidly alter the expression of important components that enable them to adapt to different environments in the host. Changes in the expression of these components critically effects bacterial behaviour such as adhesion and invasion of host cells. How variations in bacterial structure effect interactions with host endothelial cells and the consequences this has for the host-inflammatory response in general is the principal concern of this thesis.

In addition, the same variations in bacterial structure may have very different effects with other cell types. This thesis also explores the consequences that these factors have on the interaction between meningococci and dendritic cells. Dendritic cells are antigen presenting cells that form a bridge between innate and adaptive immunity. Their effect on the adaptive immune response to invading microbial pathogens depends on their ability to generate critical signals. These are elicited on engagement of the same set of innate immune molecular recognition systems that are also responsible inducing severe inflammatory response in endothelial cells. How meningococci interact with the innate immune system lies at the heart of the host response to this organism.

1.1.2. Historical Perspectives

The Swiss physician Vieusseux has been accredited with the first recorded account of an outbreak of meningococcal disease in Geneva in 1805 (Cartwright 1995a). Reports of cerebrospinal fever sometimes associated with a haemorrhagic rash that occurred in clusters or even wider outbreaks and had a high mortality have been recorded since the time of Hippocrates. It seems likely that some of these outbreaks would have been caused by *Neisseria meningitidis*. The symptoms included severe headaches, vomiting and neck stiffness and the characteristic livid, non-blanching rash was noted, and was often fatal. When it occurred in outbreaks, the course was devastating, and there was very little to offer as a remedy. There were numerous reports of outbreaks in military institutions during the 18th, 19th and early 20th centuries, particularly amongst newly arrived recruits. Simple practical measures aimed at reducing transmission in military institutions, such as spacing out of sleeping accommodation, have been documented (Cartwright 1995a).

The development of sulphonamide antibiotics in the 1930's had a dramatic effect on mortality from meningococcal disease, and case fatality rates of 80% to

nearly 100% dropped to around 20% after the 2nd World War and after the introduction of penicillin (Cartwright 1995a). It is interesting to note that this figure did not improve until very recently. The overall mortality from all cases of meningococcal disease in England and Wales is currently between 8 and 10% (table 1). Despite this improvement, meningococcal disease is still responsible for 200 to 300 deaths per year in the UK, and many more worldwide. Recent, well-publicised outbreaks illustrate not only that it can cause significant mortality, but that it can instil a profound sense of worry and even panic amongst affected communities.

1.1.3. Epidemiology

N. meningitidis is a gram negative diplococcus which is an exclusive human pathogen. Meningococci are classified into a number of serogroups based on the structure of polysaccharide capsule. Serogroups A, B, C, Y and W135 are responsible for most human disease. There is wide variation in prevalence of serogroups worldwide. Group B is the most common in the UK, Scandinavia and New Zealand, with group C making up most of the rest of the isolates. Group C is most common in the USA and Canada. Group A predominates in sub-Saharan Africa, where it is responsible for widespread epidemics, mainly during the dry season. There has been a notable increase in serogroup C infections in England and Wales in recent years (Ramsay et al. 1997). Meningococcal disease shows seasonal variation, being most common in winter and spring in temperate climates. There is striking age related incidence of meningococcal disease, being highest under the age of 2 years, with another important peak in adolescents, which is mainly attributable to group C disease (Jones 1995). Current data of meningococcal disease in England and Wales are summarised in table 1 and figure 1.1

Statutory Notifications							
	1993	**1994**	**1995**	**1996**	**1997**	**1998**	**1999**
Meningitis	1053	938	1146	1164	1220	1152	1145
Septicaemia	398	430	707	1129	1440	1509	1822
Deaths							
All cases	1451	1368	1853	2293	2660	2661	2967
Meningitis	35 (3.32%)	19 (2.05%)	31 (2.7%)	44 (3.78%)	48 (3.93%)	35 (3.01%)	N/A
Septicaemia	122 (31%)	111 (25.8%)	154 (21.8%)	182 (16.1%)	182 (12.6%)	163 (10.8%)	N/A
All cases	157 (10.8%)	130 (9.5%)	185 (9.98%)	226 (9.86%)	230 (8.64%)	188 (7.44%)	N/A

Table 1. Statutory notifications and Deaths due to meningococcal disease in England and Wales 1993-1999
Cases are divided into either meningitis or septicaemia according to disease code entered. Deaths are divided using ICD9 codes 0360 (meningococcal meningitis) or 0362 (meningococcaemia). Source: Communicable Disease Surveillance Centre, Public Health Laboratory Service, Colindale, London, UK.

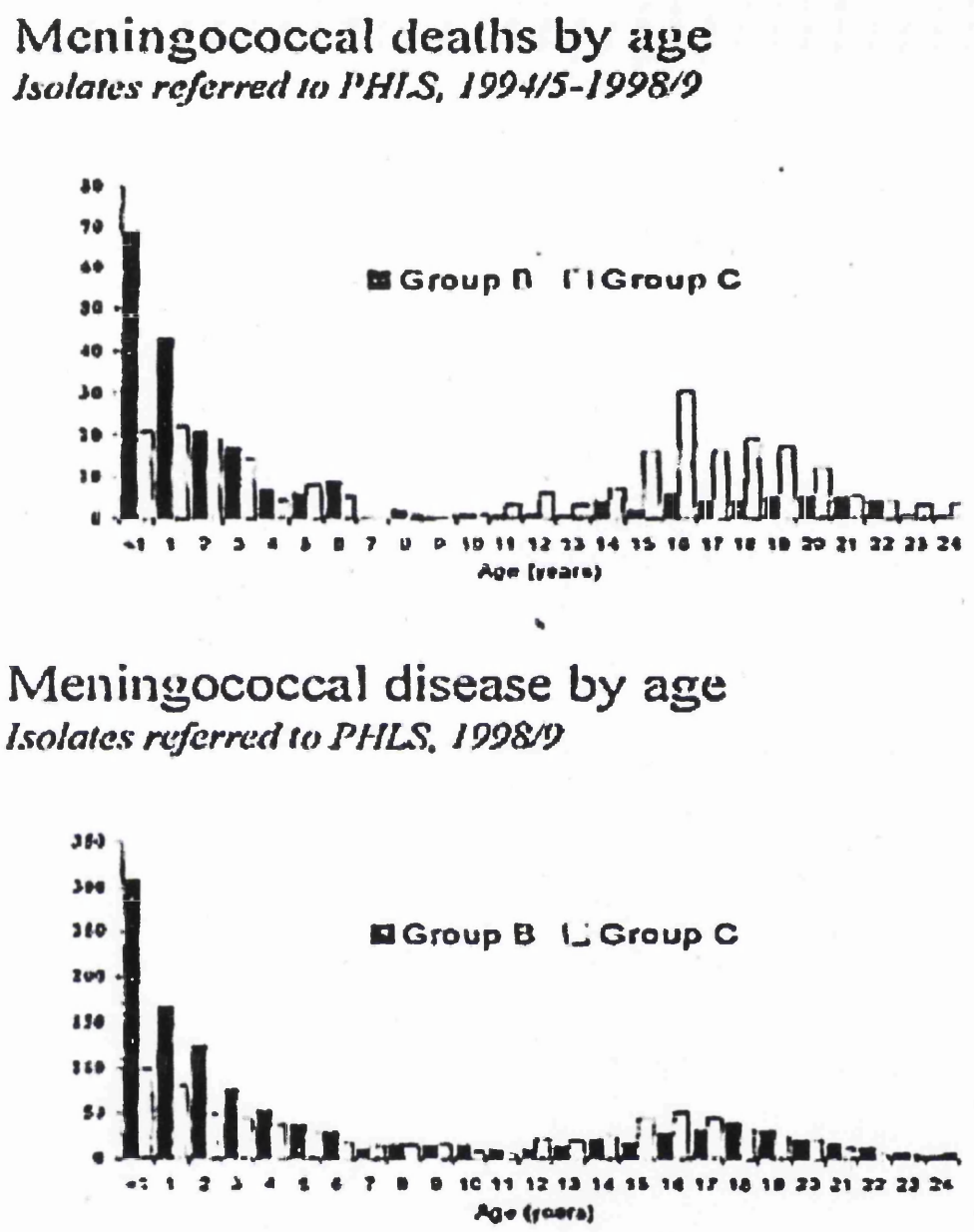

Figure 1.1 Meningococcal disease and meningococcal deaths by age and serogroup in England and Wales.

Source: Communicable Disease Surveillance Centre, Public Health Laboratory Service, Colindale, London, UK. Reproduced with permission.

1.1.4. Transmission, colonisation and acquisition of natural immunity

One of the striking features of the relationship between *N. meningitidis* and humans is that in most cases, it causes no disease at all. Up to 20 % of the population will carry *N. meningitidis* as a commensal in the upper respiratory tract

(McLeod Griffiths 1995). The establishment of the commensal relationship between meningococci and human host is a highly complex but important process. From an early age, children will be exposed to and colonised by *N. meningitidis*. These strains will be highly variable in their antigenic composition (McLeod Griffiths 1995). Colonisation is an active process involving attachment of meningococci to naso-pharynx. Establishment of mucosal immunity will depend on interactions with, and immune response to multiple surface antigens on meningococci. This will be discussed in following section. Antigenic challenge with related commensals like *Neisseria lactamica* (Gold et al. 1978) or other species of bacteria with related antigenic structure may have a role in this process (Grados & Ewing 1970). What appears to be the case is that the people most at risk are non-carriers, as the ability to establish a commensal relationship with a new strain is undetermined (McLeod Griffiths 1995).

The presence of immunity in a given population is complex and is constantly changing. However, most authorities agree that where there are high levels of immunity, occurrence of meningococcal disease is likely to be low. However, as immunity in a population is constantly changing, so does the susceptibility to a particular strain. This may in some way explain the occurrence of outbreaks, seen in newly arrived college students or military recruits or in new jail inmates (Tappero et al. 1996). In these situations, level of immunity in newcomers may be low, which together with crowding, close contact and changes in social behaviour, leads to a greater potential for transmission and in some cases, invasive disease (Neal et al. 2000). What determines whether an individual becomes a carrier, or if the organism invades the naso-pharyngeal epithelium, enters the circulation and causes disease is still not well understood. Still unresolved is whether in the carrier state the organism invades and then is

eliminated or whether they do not invade and then colonise the nasopharynx. However, it is likely that the establishment of local immunity is critical in keeping bacteria at the mucosa and thus maintaining the commensal state.

Meningococci can be transferred from one individual to another by close, 'kissing' contact, or by droplet spread over short distance (Nelson 1996). Host factors, such as preceding viral infections may be important in increasing attack rates. The most convincing evidence is the link between influenza A infections and increased susceptibility to meningococcal disease (Cartwright et al. 1991). There is a suggestion that this is due to immune suppressive action of a preceding influenza A infection, as occurs with both pneumococcal and staphylococcal infections. However, there are a number of confounding factors such as the possibility that influenza infection may itself increase transmission (Hubert et al. 1992). There is a lack of convincing evidence for a link with a number of other respiratory tract infections and increased risk of meningococcal disease, such as mycoplasma and RSV infection (Cartwright 1995b). Smoking is known to increase carriage rates significantly in a dose dependent manner, as does passive smoking (Stuart et al. 1989). Interestingly, smoking habits were not included in many older epidemiological studies investigating carriage rates and this may explain in part the variation observed in some studies.

Most work suggests that invasive disease occurs within approximately one week after exposure to a bacterium (Bierhaus et al. 1995;Marks, Frasch, & Shapera 1979). If the organism does not invade by then, the person will become a carrier and unlikely to get invasive disease. However, longer periods between exposure and disease onset have been reported (Tracey et al. 1986). What is clear is that meningococci employ highly complex molecular mechanisms that vary their phenotypic and antigenic characteristics as they adapt to different micro-

environmental situations both as a commensal and in those rare cases where they invade the mucosal barrier and cause disease.

1.1.4 Structure of *N. meningitidis*

The outer surface of meningococci most closely associated with the host, and it is not surprising that it contains a number of structures critical in the processes of adhesion, colonisation, invasion and disease pathogenesis (Poolman, van der Ley, & Tommassen 1995). The surface structure of *N. meningitidis* is illustrated in Fig 1.2. Almost all pathogenic meningococci possess polysaccharide capsule. Capsule of groups B, C, Y and W135 are formed by polymers of sialic acid (α-2,8 and α-2,9 linked, respectively for groups B and C), whereas group A is formed from N-acetyl mannosamine-1-phosphate (Jennings et al. 1977;Liu et al. 1971a;Liu et al. 1971b). Within the capsule is a cell wall, which is composed of an inner cytoplasmic membrane, a peptidoglycan layer, followed by an outer membrane, which is the characteristic of gram-negative bacteria. This outer membrane is composed of lipid bilayer, containing lipopolysaccharide (LPS/endotoxin) in the outer layer, lipoproteins in the inner layer, and spanning both these layers are numerous outer membrane proteins (OMP's). These have been used to classify meningococci (Poolman, van der Ley, & Tommassen 1995). The class 5 OMP's, are the opacity proteins Opa (A, B, C, D etc.) and Opc, which mediate invasion and adhesion to host cells (Virji et al. 1993a). Other proteins include the Transferrin Binding Proteins (Tbp-1 and 2), and the Porins, PorA (class 1 OMP), and PorB (Class 2/3 OMP). In addition, there are numerous other proteins, including enzymes such as IgA proteases, and enzymes involved in sialylation of lipopolysaccharide. Pili (Class 1 and 2) are long, filamentous processes that project from the bacterial surface, and mediate attachment to host cells (Heckels 1989).

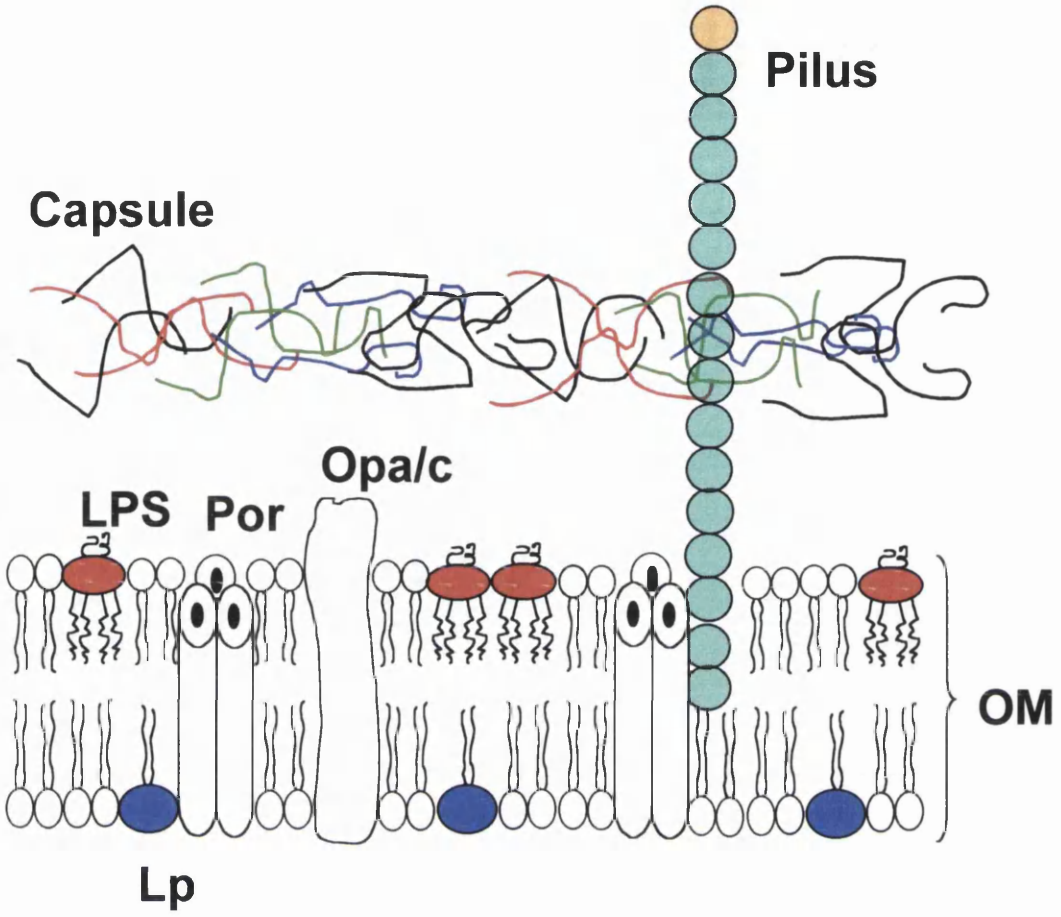

Figure 1.2 Surface structures of meningococci.

Only outer membrane and associated structures and capsule are shown. LPS, Lipopolysaccharide; Lp, lipoprotein; Opa/c, Opacity proteins; Por, porins; OM, outer membrane. Adapted from (Poolman, van der Ley, & Tommassen 1995).

As has been previously mentioned, basic serological classification of meningococci is based on type of polysaccharide capsule present (serogroup). Further serological typing includes serotype (Class 2 and 3 OMP's) and serosubtype (class 1 OMP's). In addition, immunotyping based on different structures of LPS is used (Mandrell & Zollinger 1977). A new generation of new typing techniques has had a dramatic impact on characterising meningococcal isolates mainly for epidemiological studies. Multilocus enzyme electrophoresis (MLEE) employs natural variability in electrophoretic mobility of some outer membrane proteins and cytoplasmic enzymes, which defines the electrophoretic type (ET). Groups of related ET's are then designated a subgroup (for serogroup

A), cluster or complex (for serogroups B and C) (Achtman 1995). This method has identified a number of complexes and clusters responsible for outbreaks of meningococcal disease, such as the group B ET-5 complex (Achtman 1995). There is wide variation of serological markers on meningococci from a given ET complex like ET-5 isolated different countries, making tracking patterns of spread difficult. Molecular techniques, such as restriction fragment length polymorphism (RFLP) and polymerase chain reaction (PCR) have been use to epidemiological analysis of certain strains (Fox et al. 1991;Woods et al. 1994).

The highly variable nature of meningococcal surface antigens from different strains of *Neisseria meningitidis* makes the study of their interaction with the human host a challenging one. Nonetheless, since a number of these have been identified as being major determinants of virulence and pathogenicity of meningococci, specific interactions between these and host cells are of great interest and will be discussed in detail in section 1.3. As will be discussed in a later chapter, a number of these surface bacterial components have a critical role in initiation of inflammatory and innate immune responses.

1.2. Clinical Manifestations

1.2.1. Introduction

Invasive meningococcal disease forms a spectrum of clinical presentations, ranging from primary meningitis, to combined sepsis and meningitis through to fulminant meningococcal septicaemia. Why it is that a certain patient presents with a particular manifestation is an intriguing aspect of this disease.

1.2.2. Meningitis

Meningitis occurs due to direct invasion of the meninges by meningococci, with the inflammatory process occurring within the sub-arachnoid space. The patient can present with the typical symptoms of meningism such as neck stiffness, photophobia, headache and vomiting or in the case of young infants often just irritability or lethargy. With prompt recognition and administration of antibiotics, meningococcal meningitis has a relatively good prognosis (Havens et al. 1989). Deaths, when they occur, are primarily due to effects of cerebral oedema, raised intracranial pressure and subsequent cerebral herniation. Such cases are sometimes referred to as meningoencephalitis (Nugent et al. 1979). A number of survivors will suffer form neurological sequelae, such as sensorineural deafness, seizures and learning difficulty (Baraff, Lee, & Schriger 1993).

1.2.3 Systemic meningococcal disease

The most severe form of the disease is systemic meningococcal disease (SMD), with severe hypotension, appearance of the characteristic purpuric lesions, multi-organ dysfunction syndrome with a rapidly progressive course and occasionally fatal outcome (Kirsch et al. 1996). The history can be short, with sudden onset muscle cramps, abdominal pain and occasionally diarrhoea, or malaise and chills. Sometimes these can be accurately described and time of onset given. There may have been a preceding prodromal illness such as upper respiratory tract infection. In the infant or younger child however, such a history will be unavailable and symptoms will be non-specific. Many of these initial symptoms can easily be mistaken for those due to influenza or other viral illness. The child can then become rapidly unwell and lethargic. Fever can be absent, or the child may be hypothermic.

The characteristic features of an infant with SMD are shown in Fig 1.3. This illustrates the fundamental features of this condition; capillary leak as shown by the presence of oedema, multi-organ failure, and vasculitic rash which are all evidence of severe vascular dysfunction. Cold extremities, skin mottling and increased capillary refill time are all evidence of poor peripheral perfusion and severity of shock. There may be appearance of the typical purpuric lesions, but these can be subtle and not noticed in the early stages. As the disease progresses, the size of the lesions may enlarge rapidly and can become confluent and necrotic. Hypotension can be severe and persistent, requiring intensive fluid and inotropic support.

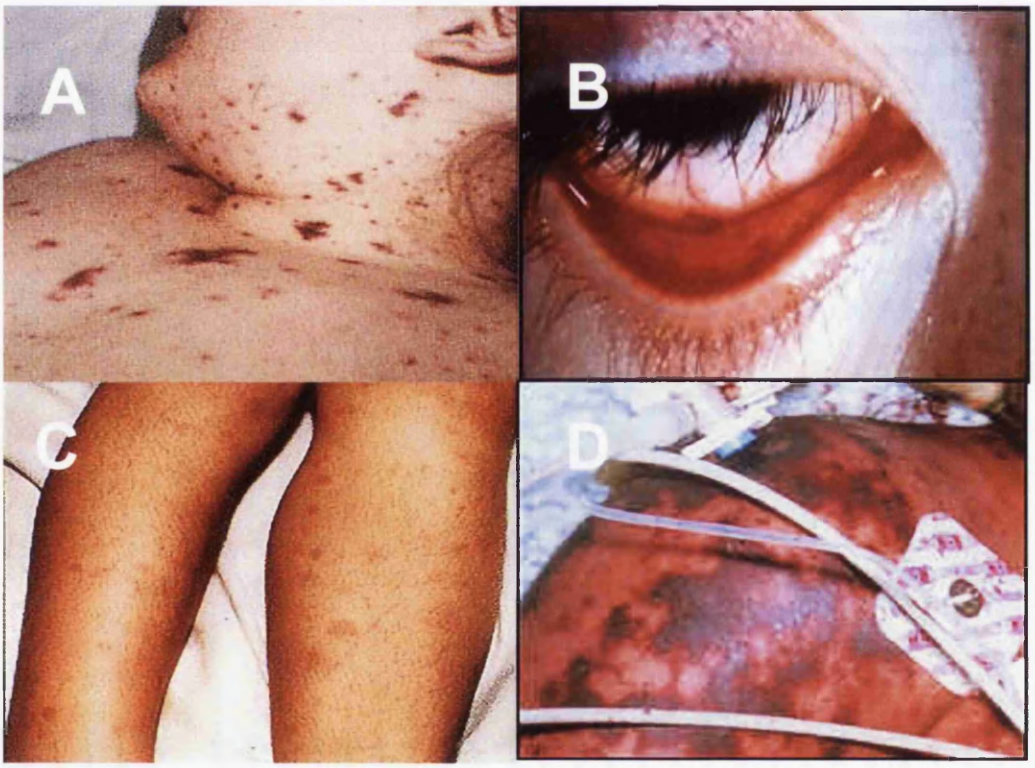

Figure 1.3. Systemic meningococcal disease

Images of meningococcal disease. Appearance of vasculitic rash at different stages and severity of disease. A) Widespread petechiae on trunk in established disease; B). conjunctival petechiae, often missed; C). Early appearance of lesions, occasionally mistaken as macular viral rash; D), severe purpura in advanced disease. University of Nottingham, published with permission.

Maximal intensive care is required, with mechanical ventilation, occasionally renal dialysis or haemofiltration in the case of renal failure, and

prolonged ventilation with acute respiratory distress syndrome. Extra-corporeal membranous oxygenation as rescue therapy has been known to help in some of the most severe cases (Goldman et al. 1997).

Other patients present with signs of both sepsis and meningitis. Interestingly, prognosis is better, the degree of shock and vascular damage is less in these cases than cases of sepsis where meningitis is not a feature. Indeed, absence of meningitis is included in the prognostic scoring systems such as Glasgow Meningococcal Prognosis Score and indicates a worse prognosis in systemic meningococcal disease. The relevance of this observation will be discussed in more detail in a later section.

The overall mortality is around 10% for all cases of meningococcal disease (table 1.1). The prognosis for those with meningococcal septicaemia has improved steadily over the last 10 years. However, there are still a small number of patients presenting with a fulminant course in which the mortality can be as high as 50% (Havens et al. 1989). Most patients who do so succumb quickly, often within the first 24 hours (van Deuren 1998). Intensive care may be prolonged and difficult, with complications of multi-organ failure, as detailed above but these complications are rarely fatal in those who survive the first 24 hours. However, in survivors, extensive tissue damage requiring amputation of digits and limbs, skin necrosis requiring skin grafting, or end-organ damage leading to chronic renal failure are seen. With increasing survival from systemic meningococcal disease, it is likely that the burden of such sequelae will increase.

1.2.3 Other manifestations of meningococcal infection

There are a number of uncommon presentations of primary disease. Chronic benign meningococcaemia occurs in a small number of patients with

recurrent fever, arthritis and rash (Jennens, O'Reilley, & Yung 1990;op de Coul & Kardos 1996). Primary pneumonia, conjunctivitis, endopthalmistis, arthritis have all been described. In a number of patients recovering from systemic meningococcal disease, prolonged or recurrent fever, with reappearance of the rash (often mild) and arthritis occurs. This is as a result of immune complex deposition and although alarming and worrying is not due to persistent bacteraemia (Edwards & Baker 1981).

1.3. Pathophysiology

1.3.1 Introduction

Invasive disease due to *N. meningitidis* is a rare event. In order for the meningococcus to cause disease, several things must occur. Firstly, an individual must be exposed to a virulent strain. The bacteria must then attach to and then invade the mucosal epithelium of the nasopharyngeal mucosa, and lastly, bacteria must survive and multiply in the bloodstream in sufficient numbers to result in clinical disease (van Deuren 1998). These steps are influenced by numerous factors, including bacterial properties, host factors (importantly age, immune status, concomitant illness), and social, environmental and climatological factors. Whilst there have been advances in our understanding of this process, there is still much that is unknown or incomplete. The present state of knowledge is described in the next section.

1.3.2 Bacterial factors

1.3.2.1 Introduction

Neisseriae meningitidis has a remarkable capacity to vary expression of surface structures, several of which are linked with virulence. A number of mechanisms by which meningococci rapidly and reversibly switch expression of certain surface structures has been identified (de Vries et al. 1996). Such phase variable expression of surface antigen may allow for different types of interactions in different microenvironments within the host. Some of these may have evolved as sophisticated mechanisms to evade host immune attack. However, a number of these factors may also be temporarily switched off in order to promote adherence and invasion of host cells, as will be discussed in the following sections.

1.3.2.2 Colonisation of nasopharyngeal mucosa

Colonisation of naso-pharyngeal epithelial cells by meningococci is highly complex. They can occupy different sites of the mucosa, which probably requires alterations in surface phenotype (Stephens & McGee 1981). Encapsulated, piliated organisms adhere to non-ciliated nasopharyngeal epithelial cells. Specific ligand – ligand interactions between pili, and other outer membrane proteins and host cellular receptors promote adherence, as described below. The LPS structure of the majority of carrier strains is different to that of disease isolates (Jones et al. 1992). Some work suggests that colonisation is associated with local invasion and generation of local immune response (Brandtzaeg 1995). The mechanisms that determine which organisms gain access to the circulation are not well understood. Interestingly, it is known that meningococci can exchange genetic information both *in vitro* and *in vivo* (Frosch & Meyer 1992;Swartley et al. 1997). Recently, it has been shown that rapid switching of serogroup from C to B can occur *in vivo*

(Vogel, Claus, & Frosch 2000), suggesting that major virulence factors can be horizontally transferred and result in invasive disease *in vivo*.

1.3.2.3 Adhesion and invasion of host epithelial cells

Encapsulated organisms initially attach to naso-pharyngeal epithelial cells by long, filamentous structures known as pili (McGee & Stephens 1984). Pili expression is both phase and antigenically variable, which may be important in determining the ability of some strains to adhere to and invade mucosal epithelium (Heckels 1989). CD46 has been identified as a host cellular receptor for pilin (Jerse & Rest 1997), and there are likely to be many other host ligands involved. The outer membrane proteins, Opc and Opa are considered to mediate firm attachment and invasion to both epithelial and endothelial cells. This is particularly important for unencapsulated organisms (Virji et al. 1992a). Previous studies have shown that unencapsulated mutants are more adherent to epithelial cells than capsulated strains, although this varies according to the nature of the epithelial cells used (Stephens, Spellman, & Swartley 1993). Certain Opa proteins may be more important than others in mediating attachment to epithelial cells *in vitro*. In addition, these interactions are inhibited in the presence of capsule and sialylated LPS (Virji et al. 1993a).

Encapsulated meningococci can be endocytosed in membrane bound vacuoles whereas unencapsulated bacteria may traverse the cytoplasm of epithelial cells without vacuoles (Stephens, Spellman, & Swartley 1993). This process might be occurring frequently with meningococci colonising the upper respiratory tract. As mentioned previously, there is evidence to suggest that during the passage through the epithelial layer, meningococci may modulate a number of surface antigens by phase variation. In one study, spontaneous switching from an encapsulated strain to a capsule deficient phenotype promoted both adherence

and invasion of human epithelial cells *in vitro* (Hammerschmidt et al. 1996a). This was due to the reversible inactivation of the *siaA* gene, which is involved in sialic acid biosynthesis, by a naturally occurring insertion sequence IS*1301*, resulting in deficient capsule formation and endogenous sialylation of LPS. Another study showed that a group B meningococcus that had invaded a primary human epithelial cell monolayer was unencapsulated and had an unsialylated LPS (de Vries et al. 1996). It was also found to express a 28-kDa-opacity protein, and bacteria lacking this protein were poorly invasive (de Vries et al. 1996). This was dependent on the type of epithelial cells used, either primary cell cultures or epithelial cell lines. For example, co-expression of Opc and the 28-kDa opacity protein together inhibited invasion in primary cultured cells whereas internalisation of bacteria into various epithelial cell lines was correlated with expression of Opc (de Vries et al. 1996).

Once meningococci have invaded epithelial cells and gained access to sub-mucosa, they will come into contact with both host phagocytic cells such as macrophages and dendritic cells and blood vessels. The subsequent course of events is critical, and will depend on the nature of the host immune response and behaviour of the bacteria themselves. There are thus two opposing processes occurring when pathogenic *N. meningitidis* invade the human host; those mechanisms that result in attachment and invasion and those that help the organism evade host immune attack. The complex interplay between the phenotypic characteristics of meningococci and host cell interactions is important for understanding the pathogenesis of meningococcal disease

1.3.2.4 Bacterial factors influencing resistance to host immune attack.

1.3.2.4.1 Capsulation

Disease causing isolates are almost always encapsulated, have sialylated LPS and express pili (DeVoe & Gilchrist 1975;Jones et al. 1992). Capsulation is a major factor in the pathogenicity of *N. meningitidis*. The protection afforded by the polysaccharide capsule of meningococci seems to be largely due to resistance to complement mediated lysis and phagocytosis by neutrophils (Hammerschmidt et al. 1994;Jarvis & Vedros 1987a). Specific anti-capsular bactericidal antibodies can be elicited against Groups A, C, Y and W (Goldschneider, Gotschlich, & Artenstein 1969a;Goldschneider, Gotschlich, & Artenstein 1969b;Gotschlich, Goldschneider, & Artenstein 1969)[illegible]major determinant of serogroup specific immunity (Goldschneider et al. 1973). However, capsule from group B is weakly immunogenic, due to its molecular similarity to human embryonic neural cell adhesion molecules (Finne, Leinonen, & Makela 1983).

1.3.2.4.2. Lipopolysaccharide Structure

Lipopolysaccharide of *N. meningitidis*, like that of any gram-negative bacterium, is an integral, structural component of the outer cell membrane. It is an ampiphilic molecule, with a hydrophobic lipid A portion inserted in to the outer layer of the cell membrane. This is attached to a carbohydrate core via two 2-keto-3-deoxy-octulosonic acid (KDO) residues, to which are attached oligosaccharide chains of varying length and composition. There is considerable structural diversity in meningococcal LPS (Andersen 1997). The predominant structure found in invasive strains of meningococci is of the L3, 7,9-immunotype (Jones et al. 1992), which is shown schematically in Figure 1.4. Lacto-N-neotetraose originates from a heptose residue which forms part of the inner core. The enzyme α–2,3-

sialyltransferase catalyses the sialylation of lacto-neo-tetraose from *N*-acetyl-neuraminic acid (CMP-NANA), which can be either exogenously acquired or endogenously synthesised. (Gilbert et al. 1996a;Mandrell et al. 1991).

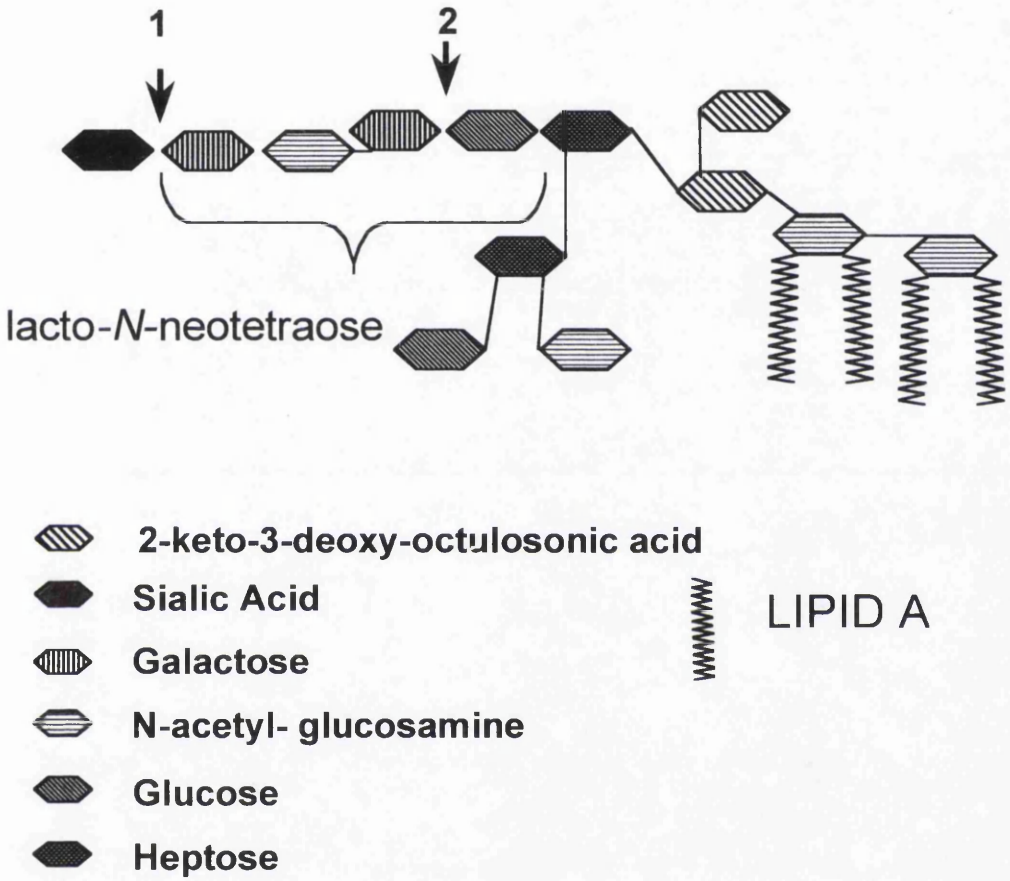

Figure 1.4. Schematic representation of Meningococcal L3, 7, 9 LPS.

Arrows indicate acceptor site for terminal sialylation of lacto-neo-tetraose (1), and truncation point of galE LPS (2). The lipid A structure is considered to be responsible for inflammatory response in host. Adapted from Jack and Schneider 1991.

Sialylation of LPS may confer resistance resistance to host immune attack to meningococci, as seems to be the case with the. *N. gonorrhoeae* (de-la Paz H., Cooke, & Heckels 1995;Ram et al. 1998). Some studies suggest that sialylation of meningococcal LPS masks lacto-N-neotetraose thus inhibiting serum bactericidal activity (Estabrook, Griffiss, & Jarvis 1997). However, *lst* isogenic mutants of both group B and C meningococci that lack α–2,3-sialyltransferase, hence are unable to

sialylate LPS, are fully serum resistant and can induce bacteraemia in infant rats (Vogel et al. 1997;Vogel et al. 1999a). This suggests that LPS sialylation may not play a major role in resistance to serum bactericidal activity. *galE*- mutants, which lacks the enzyme UDP-glucose 4 epimerase, produce a truncated LPS lacking the lacto-N-neotetraose, and thus cannot be sialylated (see Fig 1.4). Some *galE*- mutants are serum sensitive (Hammerschmidt et al. 1994;Vogel et al. 1999a;Vogel, Hammerschmidt, & Frosch 1996), whilst others have been found to be serum resistant (Kahler et al. 1998). The reason for these discrepancies is not entirely clear. The serum resistance seen in one *galE*- mutant from strain NMB may be due to the addition of glucose extensions to heptose core (Kahler et al. 1998). It is clear however, that even small alteration to oligosaccharide structure of LPS can effect sensitivity to host immune attack. In addition, serum resistance will depend on factors other than LPS in disease causing isolates which do not sialylate LPS either endogenously or exogenously (Vogel et al. 1999a).

1.3.2.4.3 Other Bacterial Factors

The ability of meningococci to survive and multiply in the bloodstream is central to the subsequent course and severity of meningococcal disease. There are a number of other bacterial factors that may promote survival of bacteria, in addition to capsulation status and LPS structure discussed above. Neisserial porins may inhibit neutrophil phagocytosis and down-regulate opsonin receptors FcRγ and complement receptors (Bjerknes et al. 1995). Meningococcal IgA proteases liberate IgA f $(ab)_2$ fragments that inhibit binding of host IgM and IgG to bacteria (Mulks & Plaut 1978). These are only a few of numerous mechanisms employed by meningococci to evade the host immune response.

1.3.3 Host factors related to susceptibility to meningococcal disease.

1.3.3.1. Age

The best defence against invasive meningococcal disease is specific antibody, causing complement dependent bacteriolysis and opsonic cellular cytotoxicity (Goldschneider, Gotschlich, & Artenstein 1969a;Goldschneider, Gotschlich, & Artenstein 1969b). Children aged between 6 months to 2 years have the lowest presence of specific antibody and also have the highest incidence of disease. However, incidence is still relatively high in neonatal period, indicating that passive immunity from maternally derived antibody is incompletely protective. In England and Wales, in common with many other developed countries, this is mainly due to serogroup B. Age related incidence falls after the age of 2 but then rises again in late teenage years, mainly due to serogroup C disease. This increase in attack rate is not clear, but may relate to changing social habits in late school years and at college and relatively low immunity against serogroup C(Jones 1995). All age groups may rely upon the innate immune system for protection against this organism, but this maybe more important in those with low antigen specific immunity.

1.3.3.2. Specific Immune deficiencies

It has been well recognised that susceptibility to meningococcal disease can be familial. The classic example of this are inherited deficiencies in the complement pathway. Males with X-linked properdin deficiency, who have defective alternative pathway activation, succumb to devastating meningococcal disease, especially in the absence of humoral immunity (Braconier, Sjoholm, & Soderstrom 1983). Patients with deficiencies in the terminal, membrane attack complex components of the complement system are prone to recurrent

meningococcal infections, often of a less severe nature, and due to uncommon serogroups (Figueroa & Densen 1991a;Fijen et al. 1989). Although rare, these conditions illustrate that complement plays a major role on immune defence against meningococci. Recently, genetic polymorphism in mannose binding lectin (MBL) gene associated with MBL deficiency, has been shown to predispose individuals to meningococcal disease (Turner 1996). Defects in Immunoglobulin receptor, FcγR2a may be associated with risk of meningococcal disease (Bredius et al. 1994). Splenectomy does not appear to be a major risk factor (Loggie & Hinchey 1986). Interestingly, defects of the cellular immune system, in particular neutropaenia, have not been shown to be risk factors for meningococcal disease.

The majority of meningococcal disease occurs in individuals who do not have defined defects in immune system described above. However, there is increasing interest in host factors hitherto unrecognised that may predispose an individual to this disease, or influence its severity. It is clear that the host response to many infections is in part determined by genetic background of that individual. Some of this may involve genetic polymorphism in genes that have fundamental effects on inflammatory response and regulation of immunity, such as cytokines TNF-α and IL-10. Specific example of these will be discussed in following sections.

1.3.4. The inflammatory response in meningococcal disease

1.3.4.1. Introduction

Severe meningococcal disease is associated with a systemic inflammatory response syndrome (SIRS). For many years, it was assumed that the severity of SIRS, the whether it be due to bacterial sepsis or other causes, was a result of overwhelming activation of host, pro-inflammatory processes (Bone 1991). This activation leads to a cascade of cellular and humoral mechanisms thought to result

from the action of powerful, pro-inflammatory cytokines such as TNF-α, IL-1, and other inflammatory mediators. This was based on a number of experimental observations. Patients with SIRS often had high levels of these circulating mediators that correlated with poor outcome. SIRS can be induced experimentally in animals by infusing either TNF-α, or IL-1, and the effect abrogated by inhibiting these by blocking their action (Cannon et al. 1990;Tracey et al. 1986)

The best-characterised bacterial initiator of the systemic inflammatory response is gram negative LPS. Picomolar quantities of LPS can induce SIRS experimentally and has been used in models of gram negative sepsis (Cannon et al. 1990). It is not surprising that bacterial LPS is considered be the primary initiator of the host inflammatory response seen in gram negative sepsis in general and meningococcal disease specifically. This view has greatly influenced thinking on how to approach the design of therapeutic interventions against this condition.

1.3.4.2. The role of bacterial lipopolysaccharide/endotoxin in pathogenesis of meningococcal disease

The terms endotoxin and lipopolysaccharide are often used interchangeably. Endotoxin was first used to describe the biological activity of lysed gram-negative bacteria (Westphal, Westphal, & Sommer 1977), and referred to all the outer membrane components, including lipopolysaccharide, lipoproteins, phosholipids and outer membrane proteins. More recently, endotoxin refers only to the activity of lipopolysaccharide (LPS), particularly the principal biologically active moiety lipid A (Ulevitch & Tobias 1995). The term endotoxin should probably be used to denote the biological activity of outer membrane components of gram-negative bacteria, whereas LPS refers to the defined molecular structure.

The structure of meningococcal LPS has been described previously. However, there area number of points that should be made about the structure of

LPS in general. Firstly, this structure varies widely between species and strains of bacteria. One of the most extensively studied LPS structures is derived from *Escherichia coli.* The carbohydrate portion is composed of an inner core (KDO) which is covalently linked to lipid A and a relatively conserved outer core. Attached to the outer core is a highly variable O antigen composed of multiple sugar residues. It is to this portion that the majority of specific immune response is generated. There is good evidence that lipid A is the principal component responsible for the inflammatory response initiated by LPS (reviewed in (Ulevitch & Tobias 1995)). For example, synthetic lipid A has full endotoxin activity (Takada & Kotani 1989), whereas chemical alteration of lipid A, by deacylation completely abolishes this activity (Munford & Hall 1986).

The mechanisms by which host immune system recognises and responds to bacterial LPS has been subject to intense investigation over many years. Bacterial LPS is not itself directly toxic to host cells. Rather, its effects seem to be mediated by the production of powerful host inflammatory mediators, which are induced when LPS is recognised by host and cause activation of specific signal transduction pathways. This highly evolutionarily conserved mechanism has evolved primarily as a defence against invading microbial pathogens, and is critical to host survival. However, the same process, when overwhelmingly activated, leads to the devastating consequences such as occurs in gram-negative shock. However, the mechanism by which this occurs, was until quite recently, not characterised.

It was discovered initially that a 60-kDa-serum protein named LPS binding protein (LBP) binds lipid A of LPS (Tobias, Soldau, & Ulevitch 1986). The glycosylphosphatidylinositol (GPI) anchored membrane protein mCD14, a marker for myeloid lineage cells such as monocytes and macrophages, was identified as the cellular receptor for LPS/LBP complex (Wright et al. 1990). It was then

discovered that serum CD14 (sCD14) could bind to LPS/LBP complexes and activate endothelial and epithelial cells, which do not bear mCD14 (Pugin et al. 1993). Other evidence emerged that it was sCD14/LPS complex that is absolutely required for activation of these cells (Arditi et al. 1993;Pugin et al. 1993), whereas LBP acts as a catalyst for the formation of CD14/LPS complex (Hailman et al. 1994). LBP therefore enhances the activation at low doses of LPS. Immunodepletion of LBP from plasma markedly reduces LPS induced activation (Schumann et al. 1990). LBP may also be thought of as an opsonin for LPS bearing particles like gram negative bacteria (Ulevitch & Tobias 1995). There are a number of other molecules in serum that also bind LPS, such as high-density lipoproteins (HDL's) and c reactive protein (CRP) and for this reason it has been postulated that catalysing the sCD14/LPS complex by LBP is important (Ulevitch & Tobias 1995).

The action of mCD14 in LPS induced signal transduction is dependent on two functional properties. Firstly, binding of LPS and secondly, interacting with the putative, multimeric cellular receptor, which until recently remained elusive (Ulevitch & Tobias 1995). The evidence for this is strong. Neutralising monoclonal antibody to mCD14 completely abolishes activation by LPS, at least at low doses, but does not inhibit binding of mCD14 to LPS (Peters et al. 1999). In contrast, deacylated LPS binds to CD14 and is taken up by cells but fails to activate them (Kitchens, Ulevitch, & Munford 1992). A number of other experimental findings suggest that although CD14/LBP is an essential component of LPS recognition, this is by no means the whole story. LBP can transfer phosholipids and mCD14 can bind phosholipids (Yu, Hailman, & Wright 1997), and thus some workers have argued that neither have the binding specificity to act as "pattern recognition receptors" in the true sense of the term (Thieblemont, Thieringer, & Wright

1998). In addition, in one study using a lipid A analogue that was shown to be antagonist to human cells but agonist in murine cells, and this effect was dependent on the species of cell, and not species of mCD14 (Delude et al. 1995). It also seems that internalisation, intracellular transport and processing of LPS involves some processes that are independently regulated from activation, but are functionally interrelated (Lentschat et al. 1999;Thieblemont, Thieringer, & Wright 1998). This probably reflects processes involved in deactivating and clearing LPS once a cell takes it up.

Although a major discovery, one fundamental question remained unanswered. Since membrane bound CD14 is GPI linked and hence has no cytoplasmic component capable of signalling within the cell, this meant that cellular receptor for LPS that could initiate signal transduction remained unknown (Ulevitch & Tobias 1995). The putative receptor for CD14/LPS complex, human Toll-like receptor 4 (TLR4) has now been identified and appears to impart ligand specificity and ability to initiate downstream signalling events (Beutler 2000;Lien et al. 2000). TLR4 is part of a highly evolutionary conserved family of TLR's that appear to be critical to recognition of microbial pathogens from diverse sources (reviewed in (Anderson 2000)). The subsequent signalling events initiate numerous inflammatory and immune responses (Kopp & Medzhitov 1999a;Medzhitov & Janeway-CA 1998;Medzhitov, Preston, & Janeway-CA 1997). This includes activation of nuclear transcription factors, particularly nuclear factor kappa B (NF-κB) which control expression of a large number of genes critical to the inflammatory response, including cell adhesion molecules (Hatada, Krappmann, & Scheidereit 2000). This will be discussed in greater detail in chapter 5.

1.3.4.3. The roles of bacterial numbers and levels of endotoxin

There is little doubt that levels of endotoxin are a critical determinant of the severity and outcome of meningococcal disease. Brandtzaeg and workers showed that high plasma endotoxin levels (>700ng/L) were directly correlated with severe septic shock, multiple organ failure and death (Brandtzaeg et al. 1989a). This important study also showed that endotoxin levels always decreased after administration of antibiotic therapy, indicating that fears that antibiotic therapy may cause release of endotoxin and worsening disease may be unfounded. It had always been assumed that meningococci contained a "filtrable" component of its cell wall that was responsible for its toxicity. It was not until the limulus amoebocyte lysate (LAL) assay was standardised (Iwanaga et al. 1978) that more accurate information regarding levels of endotoxin in meningococcal disease was gained (Brandtzaeg 1995). In one study, endotoxin levels were measured in one patient (an 18-month-old boy) throughout the course of the disease. At presentation, endotoxin levels were 10500ng/L (10.5 ng/ml), which declined progressively after initiation of antibiotic treatment until the patient died (van Deuren et al. 1992). In another study, endotoxin levels (as measured by LAL assay) were significantly higher in patients who had severe shock and the highest mortality (van Deuren et al. 1995).

Why are levels of endotoxin so high in severe disease? The critical determinant of circulating endotoxin levels, and given the evidence above, outcome of disease, is very likely related to the number of viable organisms in the patient. This is best illustrated by the fact that endotoxin levels, even if very high, fall after hospital admission and administration of antibiotics, as previously mentioned. However, quantification of bacterial numbers in patients is difficult.

Blood culture techniques only estimate viable organisms in circulating blood. Nonetheless, numbers of bacteria in blood, using quantitative viability counts, has been correlated with outcome (Sullivan & LaScolea-LJ 1987). The number of Colony forming units (CFU's) detected per millilitre of blood may be higher in children than adults (Clementz, Zhou, & Raetz 1997). In one study, an adolescent with fulminant MD had a level of 10^5 CFU's/ml, whereas the co-primary case, who had meningitis with no shock, had a negative blood culture (Zwahlen & Waldvogel 1984). There is recent evidence that numbers estimated by blood cultures might significantly underestimate the level of bacteria in patients with meningococcal disease. A quantitative PCR technique has indicated that in the severest cases where there is fulminating disease the number of bacteria may be as high as 10^8CFU/ml (R.Borrow, personal communication). In addition, numbers of viable meningococci estimated from blood culture is likely to be a significant underestimate of the real bacterial burden, since it does not measure organisms adherent or invading vascular endothelium or within cutaneous lesions, or phagocytosed by neutrophils.

What is the relationship between levels of endotoxin activity (as measured by LAL assay) and LPS content of organisms? Endotoxin activity in plasma is related to the amount of LPS either on or released by organisms. Meningococci characteristically shed outer membrane vesicles called blebs, which contain LPS in addition to other outer membrane proteins (Poolman, van der Ley, & Tommassen 1995). It has been calculated that 1 nanogram of purified LPS is the amount that is found in approximately 10^5 organisms, but this relates only to *E. coli* (Berry 1985). Quantitation of the LPS content of *Neisseria meningitidis* based on spectrophotometric analysis of the LPS specific sugar 2-keto-3-deoxyoctonic acid (KDO) has demonstrated that there are approximately 1.5×10^5 molecules of LPS

per bacterium. This makes 100ng/ml of purified LPS equivalent to about 10^8 bacteria/ml. (M.van Deuren, personal communication). It is interesting to note therefore, that in the most severe cases, plasma endotoxin concentration was found to be 10ng/ml. This would relate to LPS content of just over 10^7 CFU/ml. However, for the reasons given above, this may underestimate of the total bacterial load. Thus, our current understanding of the real bacterial burden in meningococcal disease may have to be revised.

How do these considerations relate to clinical presentation, course and outcome form the disease? Patients who present with meningococcal disease fall into three broad categories: those with meningitis with no shock (A), those with meningitis and shock (B), and those with shock and no meningitis (C) (Halstensen et al. 1987). Work by M. van Deuren and colleagues has shown that the duration of disease symptoms prior to hospital admission was inversely related to disease severity (van Deuren 1998). Hence, time between onset of symptoms and presentation to hospital was longest in those patients with meningitis and no shock (group A), less with meningitis and shock (group B) and least of all with shock alone (group C). This is a very important observation. The explanation for this may depend on the balance between rate of bacterial proliferation and the ability of the host to clear the organism. In cases where there is predominantly meningitis, although there is bacteraemia, the number of organisms is relatively low, with low endotoxin levels and not symptoms or signs of systemic involvement. Presentation to hospital may be delayed until organisms invade the meninges and cause meningeal irritation and the associated symptoms. Blood cultures drawn at this time may be negative, whereas organisms maybe cultured from the cerebro-spinal fluid. In contrast, in fulminant septicaemia, bacterial proliferation is rapid, endotoxin levels higher and the ensuing symptoms of shock

bring the patient to medical attention. Sometimes, death may occur within hours after admission. In cases where there is both septicaemia and meningitis, duration between onset of disease and presentation is longer. This may reflect the fact that bacterial proliferation is less rapid and endotoxin levels will be lower. This may explain, in part, why such cases have a better outlook than the previous group. Hence, numbers of meningococci in the host at presentation of disease may be a critical determinant of severity outcome of this condition.

The reasons why difference in these presentations occurs are likely to be complex and due to a number of host or bacterial factors. Some evidence suggests that high level of opsonins are associated with milder disease due to greater killing of bacteria and reduced growth, whereas low levels are associated with fulminant disease (Halstensen et al. 1989). Some meningococcal strains are more virulent than others, but the mechanisms for this is not well understood. It seems likely therefore, that in the most severe and rapidly fatal cases, concentration of endotoxin and numbers of organisms in the body as a whole will be very high. Therefore, factors that favour bacterial proliferation over killing of the organism when it first invades is likely to have profound influence on the subsequent course of the disease.

1.3.4.4. Pathophysiology of shock

Fulminant meningococcal disease is associated with severe shock, characterised by severe capillary leak, profound hypotension, disseminated intravascular coagulopathy, and vascular damage and ultimately multi-organ failure. All these processes are causally interrelated. Shock is characterised by profound haemodynamic and cardiovascular disturbances. Capillary leak is severe, with loss of normal osmotic regulation resulting in decreasing intravascular volume (Mercier

et al. 1988). Alteration of endothelial osmotic barrier is related to alterations in endothelial function and occurs partly as a result of disruption of glycsoaminoglycans matrix (GAG's) on surface of endothelial cells (Klein et al. 1993). Many other factors, such as dysregulation of venous tone and venous pooling are also important (Astiz 1998). Myocardial depression can be due to circulating TNF-α, Il-1 and nitric oxide (Kumar et al. 1996), but also as a result of vasculitis and endocardial thrombosis. Cardiac function, as estimated by ejection fraction and end-diastolic volume can be severely impaired. Delayed onset congestive cardiac failure and conductive defects are not unknown (van Deuren 1998).

The systemic inflammatory response syndrome (SIRS) is a term used to described the cascade of cellular and humoral processes occurring in severe sepsis, in addition to a number of other causes, such as burns or severe trauma. It is assumed that bacterial endotoxin (or LPS) is the primary, initiating activator of this cascade. Of these, production of potent, pro-inflammatory cytokines leads to activation of the coagulation, complement, as well as the bradykinin-kallikrien pathways, all of which contribute to profound disturbance in haemodynamic function that is a hallmark of this condition.

1.3.4.5. Cytokines in meningococcal disease

The association between plasma levels of pro-inflammatory cytokine TNF-α and severity of meningococcal disease was first published by Waage and colleagues in 1987 (Waage, Halstensen, & Espevik 1987). Subsequently, the same workers found that high levels of IL-6 was associated with fatal outcome, and IL-1 in particular was seen in patients who had high levels of TNF-α, IL-6 and endotoxin and had a rapidly fatal outcome (Evans et al. 1969). It was thought that

co-ordinate, action of TNF-α and IL-1 was responsible for the severity of the systemic inflammatory response (Girardin et al. 1988) in humans as occurs in mouse models of endotoxic shock (Rothstein & Schreiber 1988;Waage & Espevik 1988). This is biologically plausible, since TNF-α infusion not only induces a shock-like state but also blocking TNF-α abrogates LPS induced shock experimentally (Bone 1991).

It soon became apparent that levels of a large number of cytokines, including those that exert a powerful anti-inflammatory effect, were also related to outcome in MD. Hence, IL-10 and IL-1 receptor antagonist, both anti-inflammatory molecules, were found to be far higher in patients with severe disease than those with less severe disease (van Deuren et al. 1995). Since then, patterns of both membrane bound IL-1RA and soluble IL-1s receptor type 2 in patients with meningococcal disease have revealed the complex nature of cytokine response in MD (van Deuren et al. 1997). Similarly, soluble TNF receptors, which have both anti and pro-inflammatory effects, are significantly higher in severely affected patients (van Deuren et al. 1995). What is clear, is that there is overwhelming activation of both pro- and anti-inflammatory cytokine mediators are seen in the most severely affected cases. The significance of this will be discussed in a later section

Two other very important observations are that cytokine levels are compartmentalised in cases of meningococcal disease. Hence, in cases of meningitis, very high levels of cytokines are found in the CSF but relatively low in levels in the blood, whereas the converse is true for cases of shock without meningitis (van Deuren et al. 1997). Similarly, LPS levels are also compartmentalised (Brandtzaeg, Ovsteboo, & Kierulf 1992) suggesting that meningococci proliferate within either the systemic circulation or in the sub-

arachnoid space and that, this is associated with release or production of these inflammatory mediators.

1.3.4.6. Disseminated intravascular coagulopathy

Disseminated intravascular coagulopathy (DIC) is characteristic of severe fulminant meningococcal disease. (Heyderman 1993). The purpuric lesions contain microthrombi in small vessels typical of consumptive coagulopathy (Hill 1947;Sotto et al. 1976). Since both coagulation and inflammatory pathways are tightly interrelated, knowledge of this process is likely to be crucial to the understanding of the pathogenesis of severe MD. Animal studies have demonstrated that pro-inflammatory cytokines such as IL-1 and TNF-α, and LPS induce a pro-thrombotic state (Levi et al. 1993;van der Poll et al. 1990). *In vitro* experiments have demonstrated the vascular endothelium is a critical component of this process (Nawroth & Stern 1986). Activated monocytes and endothelium in septic shock produce tissue factor, an essential part of the activation of both intrinsic and extrinsic coagulation pathways (Heyderman 1993). Tissue factor is up regulated on monocytes in patents with meningococcal septicaemia, and is markedly raised in fatal disease (Osterud & Flaegstad 1983). Levels of natural inhibitors of coagulation, such as protein C, anti-thrombin III are reduced in severe disease and are related to severity of shock and multi-organ failure (Brandtzaeg et al. 1989b). This is coupled to an inhibition of fibrinolytic activity, caused by increased levels of plasminogen activator inhibitor-1 (Brandtzaeg et al. 1990). Two new recent reports have demonstrated that patients who have polymorphism in the promoter controlling expression of PAI-1 gene, who have higher plasma levels of PAI-1, have a significantly increased risk of death from meningococcal shock (Hermans et al. 1999;Westendorp, Hottenga, & Slagboom 1999). Thrombin, a key component of thrombi, is itself pro-inflammatory, causing

up-regulation of vascular cell adhesion molecules and activation of monocytes (Johnson et al. 1998). Death from SMD is significantly correlated with low fibrinogen levels, indicating severity of DIC (Girardin et al. 1988). DIC is associated with a very poor outcome in meningococcal shock (Vik, Lote, & Nordoy 1978). Microvascular thrombosis also contributes to tissue hypoperfusion, hypoxia and tissue damage.

Activation of the intrinsic or contact coagulation system is also evident in septic shock, in which activation of Factor XII, the starting point of the intrinsic cascade, is seen (Kalter et al. 1985). Factor XIIa also activates complement system and formation of C3a and C5a and induces formation of bradykinins, all of which are potent vasodilators and contribute to hypotension. Factor XIIa also induces production of neutrophil elastase, thought to be important in the pathogenesis of acute respiratory distress syndrome (Donnelly et al. 1995).

1.3.4.7. Complement Activation.

The complement system has a critical role in the pathogenesis of meningococcal disease.. As mentioned previously in this section, hereditary defects in components of the terminal attack complex are associated with recurrent meningococcal infections, usually with good prognosis, whereas deficiencies in properdin can lead to fulminant sepsis and death (Figueroa & Densen 1991b). The complement system is a critical part of the host defence against *N. meningitidis* through bactericidal activity of terminal membrane attack complex (MAC), C3b mediated opsonophagocytosis, and intracellular killing (Frank, Joiner, & Hammer 1987;Ross et al. 1987). However, complement activation can also have detrimental effects. Levels of the complement activation products C3a and terminal attack complex SC5b-9 has been correlated with both endotoxin levels and fatality (Brandtzaeg, Mollnes, & Kierulf 1989). It has been shown that although both

classical and alternative complement pathways are activated in fulminant meningococcal sepsis (Brandtzaeg et al. 1996a). However, importantly, it was activation of the alternative pathway, as determined by levels of factor Bb that was significantly correlated with C3 activation products, membrane attack complex, LPS levels and shock (Brandtzaeg et al. 1996a).

This has a number of important implications. Since LPS levels reflect meningococcal proliferation in the blood (Brandtzaeg et al. 1989a;Brandtzaeg 1995), this suggests that either LPS, or meningococci themselves trigger complement activation, particularly the alternative pathway. It is known that LPS can activate both classical and alternative pathways (Morrison & Kline 1977). Lipid A may activate the classical pathway through binding to C1-q and polysaccharide components of LPS bind to factor B (Cooper & Morrison 1978;Grossman & Leive 1984). In addition, it has been shown that short chain sugar moiety of meningococcal LPS may activate the alternative pathway (Jarvis 1994). The conclusion drawn is that the predominant activation of complement in severely affected patients is via the alternative pathway on LPS oligosaccharide side chains (Brandtzaeg et al. 1996a).

It has also been speculated that this excessive alternative pathway activation might be directed against outer membrane vesicles released by bacteria in blood (Brandtzaeg et al. 1996a;van Deuren 1998). This intriguing idea would explain the paradoxical failure of this alternative complement activation to effectively kill meningococci, since this may act as a 'diversionary' tactic by bacteria to resist this attack. Whether this is true *in vivo* is not yet established. However, it is one of a number of examples of direct effect of bacteria on the host systemic inflammatory response that is likely to occur very early, especially in the face of

rapid unconfined, bacterial proliferation, and also prior to release and synthesis of pro-inflammatory cytokines.

1.3.4.7. The role of Platelets in pathogenesis of meningococcal disease

Experience with patients with meningococcal disease quickly demonstrates the pivotal contribution of platelets in this disease. Thrombocytopaenia is associated with a very poor outcome from MD (Kornelisse et al. 1997). A low platelet count in a patient with suspected MD in its early stages should alert the medical team of the severity of their condition, even in the absence of severe rash or hypotension. However, initial platelet counts can be normal on admission and fall rapidly as the disease progresses, underlining that extreme caution should be taken in ruling out a fulminant course on the basis of single platelet count (van Deuren et al. 1998).

Thrombocytopaenia is an indicator of DIC. Platelets have a critical role in the regulation of haemostasis. Activated platelets express adhesion molecules involved in formation of thrombus and also mediate conversion of prothrombin to thrombin (Flaherty et al. 1997). They are integral components of microthrombi. Activated platelets also release pro inflammatory mediators including the pro-inflammatory cytokine IL-1 (Hawrylowicz et al. 1989).

1.3.4.8. The role of Neutrophils in pathogenesis of meningococcal disease

Neutrophils are a critical component of the acute inflammatory response seen in many pathological states, including local and systemic infections, trauma, post surgery and burns (Wagner & Roth 1999). Whilst they may be important in clearance of meningococci, there is compelling evidence that they could mediate many processes involved in vascular injury (Klein et al. 1992;Smedly et al. 1986).

Activated neutrophils release a large number of inflammatory mediators such as the protease elastase and glycanase, also matrix metaloproteinases and reactive oxygen metabolites, which is augmented by adhesion of neutrophils to the endothelium (Henson & Johnston-RB 1987;Richter, Olsson, & Andersson 1990;Shappell et al. 1990). Meningococci are a potent stimulus for neutrophil activation and subsequent endothelial injury *in vitro* (Ison et al. 1995;Klein et al. 1996;McNeil & Virji 1997). Low peripheral white cell count, particularly of neutrophils, is associated with a very poor outcome in severe MD (Kornelisse et al. 1997). It is interesting to speculate why this should be the case. The reduction in peripheral neutrophil count could be due to widespread degranulation, apoptosis or death of neutrophils. However, there is strong evidence from histological examination of petechial lesions from patients with systemic disease that widespread adhesion of neutrophils to vascular endothelium may account for their loss from the peripheral circulation. The consequences of interactions between neutrophil activation and adhesion to activated endothelium, and how these processes are influenced by meningococci themselves will be discussed fully a later section.

1.3.4.9. The influence of pro inflammatory versus anti-inflammatory cytokine balance and outcome from meningococcal disease

Until quite recently, it was thought that the predominant cause of SIRS, especially due to bacterial infection was due to overwhelming activation of potent, pro-inflammatory mediators, which in the case of gram negative sepsis, are primarily initiated by the action of LPS (Luderitz et al. 1984;Morrison & Ulevitch 1978;Suffredini, Harpel, & Parrillo 1989). Since LPS itself is not directly toxic to mammalian cells it was thought that the damage mediated is by multiple cellular

and humoral mediators that it activates (Ulevitch & Tobias 1995). This view predominantly affected strategies designed to counteract the effects of pro-inflammatory cytokines such as TNF-α.

As mentioned in a previous section, in severest cases of meningococcal septicaemia both pro and anti-inflammatory cytokines are found at very high levels in plasma (van Deuren et al. 1995). IL-10, a cytokine with potent inhibitory actions on host cells, is found at high levels on admission in the patients with severest disease (Derkx et al. 1995). Furthermore, *ex vivo* production of pro-inflammatory cytokines in response to purified LPS in whole blood taken from patients with MD was shown to be suppressed, being most marked just after admission (van Deuren et al. 1994). This suggests a powerful, counter-regulatory mechanism by which the host inflammatory system was damped down as a consequence of infection. It was then shown that the major, deactivator of monocytes in meningococcal shock was IL-10 (Brandtzaeg et al. 1996b). In this study, plasma from patients with shock was strongly inhibitory of cytokine production and pro-coagulant activity in monocytes exposed to native meningococcal LPS *in vitro*. Removal of IL-10 by immunoprecipitation abrogated this inhibitory effect of shock plasma on monocyte activation by meningococcal LPS. The phenomenon of LPS desensitisation had been known for many years, but specific agent(s) and mechanisms responsible were hitherto unknown. It was assumed that the presence of anti-inflammatory cytokines such as IL-10 was a homeostatic mechanism to prevent overwhelming inflammatory activation, since Il-10 had been found to protect against experimental, endotoxin mediated lethality (Gerard et al. 1993). It has been speculated that such anti-inflammatory mediators could have a role in dampening the overwhelming effect of pro-inflammatory mediators. It now seems clear however, that IL-10 is only one of a number of mechanisms

responsible for hyporeposnsiveness to inflammatory stimuli that occurs in patients with SIRS due to diverse pathological insults.

With so much emphasis on the effect of high levels of pro and anti- some researchers asked whether genetic host factors could influence the capacity of an individual to respond to infectious agents. TNF-α production is controlled by promoter region containing several polymorphisms. These have been shown to be associated with different responses to inflammatory mediators such as LPS or susceptibility to certain disease states, including certain infectious diseases (Jacob et al. 1990;Wilson et al. 1994). Since very high levels of TNF-α were thought to be associated with severe disease, Westendorp and colleagues looked at *ex vivo* production of TNF-α from whole blood in survivors of MD, at least 6 months after recovery (Westendorp et al. 1995). The finding from their study is completely at odds with the notion that high pro-inflammatory cytokine levels were associated with worse prognosis. They found that TNF-α release was higher in those who had "experienced moderately severe disease" compared with those who had experienced mild disease. Intriguingly, patients who had survived fulminant disease, who had predicted mortality risk of 50%, the TNF-α response were low. This raised the intriguing possibility that in the patients who had the highest risk of mortality from SMD, actually produced low levels of TNF-α in response to LPS.

Two further reports then used a novel approach to explore these apparently counter intuitive results. Westendorp and workers studied first degree relatives of patients with SMD, looking at both their TNF-α and the anti-inflammatory cytokine IL-10 production in whole blood stimulated with LPS *ex vivo* (Westendorp et al. 1997). They found that relatives of non-survivors had a

highly significantly lower production of TNF-α and higher production of IL-10 compared to relatives of survivors. The conclusion drawn was that innate susceptibility to mortality from SMD could be due to a genetically determined capacity to produce an overall anti-inflammatory cytokine profile in response to bacterial stimuli. It was proposed that such a cytokine profile resulted in reduced clearance of invading organisms. Another study, this time looking at the outcome in patients admitted with a febrile illness (all causes, but mainly infectious), reported that a high ratio of IL-10 to TNF-α was associated with an adverse outcome to infectious disease (van Dissel et al. 1998).

The implications for this are important. As has been described above, earlier studies found high levels of pro-inflammatory cytokines, particularly TNF-α, positively correlated with the severity of meningococcal disease in children (Girardin et al. 1988). However, as poor outcome is also associated with very high levels of endotoxin activity (Brandtzaeg et al. 1989a;van Deuren et al. 1995), it may be that high levels of endotoxin, which are related to bacterial load (Heyderman et al. 1997), result in high levels of TNF-α production, even in 'low' responders (Westendorp et al. 1997). It would appear that that an innate ability to produce a brisk pro-inflammatory response might be beneficial, by effectively clearing infection in the early stages of bacteraemia before there is marked proliferation of meningococci. Although compelling, the notion that low TNF-α prodcution may be a risk factor for poor outcome in SMD conflicts with other studies. For example, it has been shown that there were more deaths from MD in children who were heterozygous for the TNF2 allele, which is thought to be associated with higher inducible levels of TNF-α (Nadel et al 1996). However, in the Westendorp study, there was

no difference in risk of death in people who possessed either the more common TNF1 allele compared to the less common TNF2 allele (Westendorp et al. 1997).

These findings have lead some people to propose new models for pathogenesis of the SIRS, particularly in response to systemic infection. In initial phases of infection, local production of cytokines and inflammatory mediators are required to mount an effective immune response to the invading organisms. It has been proposed that a compensatory, systemic anti-inflammatory reaction is also mounted. In most cases, these two types of immune response are 'in balance'. If the infection becomes systemic, and proliferation of bacteria is not contained, then these two types of mediators become unbalanced, resulting in what has been termed as 'immunological dissonance' (Bone 1996a). An unbalanced pro-inflammatory reaction induces an excessive inflammatory response, such as coagulopathy, whereas an unbalanced anti-inflammatory reaction induces immunosupression and anergy.

1.3.5 The Vascular endothelium and the inflammatory response

1.3.5.1. Introduction

The response of the vascular endothelium to insult, either due to direct action of bacteria or host inflammatory stimuli such as cytokines, or through tissue injury caused by trauma, irradiation, heat or oxidative stress is central to pathophysiologcal processes that occur in many disease states. The term endothelial-cell activation applies to the specific and co-ordinated events that take place when the vascular endothelium is exposed to a stimulus (Pober 1988). This process appears to occur in two functionally distinct phases. The first phase, type I activation, is immediate and results in release of molecules such as Von Willebrand factor and P-selectin, from pre-formed granules in the cytoplasm. Type II activation requires *de novo* protein synthesis as a result of signal transduction

mechanisms in response to specific stimuli. The nature of this type II activation can be further subdivided; expression of leukocyte adhesion molecules, changes to the pro-thrombotic phenotype, production of cytokines and chemokines, alterations in vascular status of endothelium, and up-regulation of HLA molecules (Hunt & Jurd 1997). The nature of the stimulus is critical in determining the response, and may fundamentally effect the tissue specificity and populations of different leukocytes that predominate in certain inflammatory situations (Springer 1994).

1.3.5.2. Leukocyte adhesion, emigration and recirculation is a multistep process

Emigration of leukocytes from the circulation to sites of inflammation via the endothelium is a multistep phenomenon and a critical part of the inflammatory response to infection and tissue injury (Carlos & Harlan 1994;Springer 1994). Selectivity of leukocyte populations, which may be important in different stages of the immune response may depend not only on specific ligand-ligand interactions, but also "combinational diversity" of signals elicited both by leukocytes themselves and the vascular endothelium (Springer 1994). The expression of vascular endothelial cell adhesion molecules is a major determinant of leukocyte adhesion, activation and transmigration.

1.3.5.3. Selectin family of vascular cell adhesion molecules supports initial attachment and rolling of leukocytes along endothelium

In vivo and *in vitro* flow models of leukocyte traffic along activated vascular endothelium clearly demonstrates that this process occurs in several functionally distinct phases, as shown in Fig 1.5. In the first stage, leukocytes can be observed to slow down and begin rolling along the endothelial wall. This initial tethering and

rolling process is mediated by the selectin family of adhesion molecules (Tedder et al. 1995). The three best-studied selectins are P-selectin, which is expressed on platelets and endothelium, L selectin, expressed constitutively on leukocytes and CD62E (E selectin), which is expressed solely by the vascular endothelium. All these molecules have an amino-terminal C like lectin domain, an epidermal growth factor-like domain, and a variable number of short consensus repeat units similar to sequences found in complement binding proteins (Tedder et al. 1995).

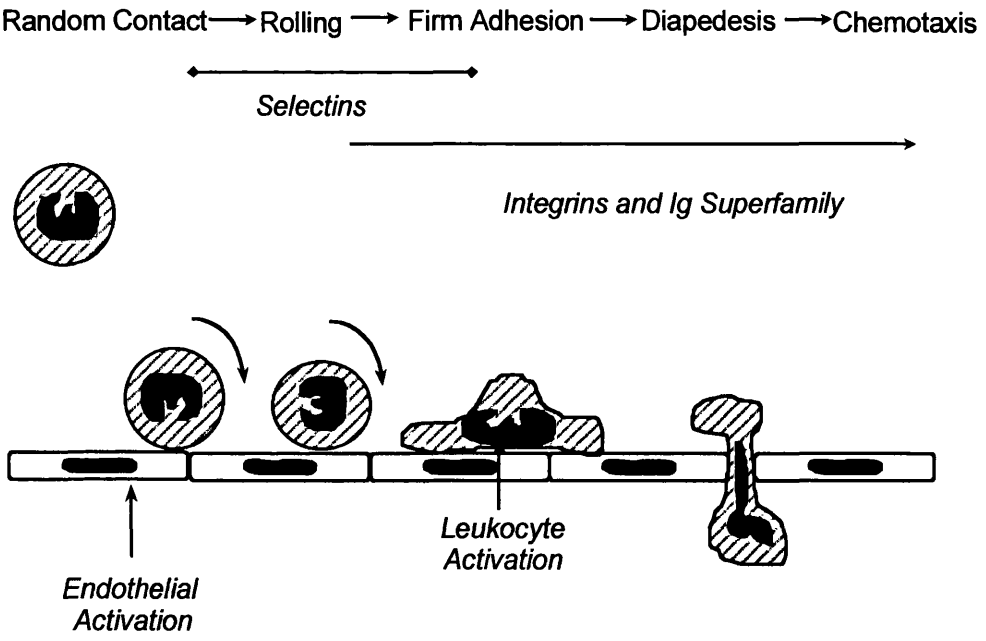

Figure 1.5. Leukocyte adhesion and transmigration under flow.

Under tubular flow, some leukocytes will randomly come into contact with endothelial wall. When exposed to activated endothelium, leukocytes can be seen to slow down and begin rolling along the wall. This initial phase is thought to be under control of selectin adhesion molecules on both leukocytes and endothelia cells. Integrins and members of the immunoglobulin superfamily mediate firm adhesion. Adapted from (Carlos & Harlan 1994).

Characterisation of the selectin ligands, in terms of which are biologically and functionally relevant has been far from easy. The amino terminal C-type lectin domain of the selectins can bind to a wide number of carbohydrate bearing molecules with varying avidity *in vitro*. The specificity and avidity of interactions of

the selectins are critically determined by the glycoslylation and sulfation status of these ligands (Varki 1997). CD62E, for example, forms low avidity interactions with fucosylated and sialylated oligosaccharides such as sialyl Lewis^x (Sle^x) (reviewed in (Tedder et al. 1995)). The glycoprotein E-selectin ligand 1 (ESL-1) mediates stronger affinity interactions with CD62E when decorated with these sialylated fucosylated oligosaccharide residues such as Sle^x (Zollner & Vestweber 1996). However, there are still doubts as to whether ESL-1 is functionally relevant in man *in vivo* (Varki 1997). P-selectin glycoprotein ligand-1 (PSGL-1) is considered to fulfil many of the criteria of real ligand for P selectin (Varki 1997). Interestingly, there is seems to be some functional redundancy and overlap in recognition of other selectins, in that PSGL-1 can act as a ligand for L-selectin (Varki 1997). Further studies are needed to elucidate the importance of these ligands *in vivo.*

Whatever the real nature of selectin interactions and their ligands, they are critical to leukocyte adhesion and recruitment in inflammatory and infective states (Zimmerman, Prescott, & McIntyre 1992). Mice with targeted gene deletions of all three selectins have severely impaired neutrophil recruitment, whereas single gene knockouts have variable defects, indicating, at least in mice, that each selectin can support leukocyte recruitment (Jung & Ley 1999). L selectin, which is present only on leukocytes, is rapidly shed once activated (Jutila et al. 1989). Interestingly, this is now thought to be a key event in regulating leukocyte rolling *in vivo,* by decreasing rolling velocity along endothelial wall (Hafezi & Ley 1999). It appears that L selectin may be most effective at initial capture of leukocytes under flow conditions (Lawrence, Bainton, & Springer 1994). CD62E, on the other hand, mediates rolling once leukocytes are captured. P selectin appears to be effective in both the initiation and maintenance of leukocyte rolling (Lawrence & Springer 1991;Luscinskas et al. 1991).

Expression of P-selectin by vascular endothelium is functionally different from that of CD62E. It is contained in Wiebel-Palade granules in endothelial cells and is rapidly externalised on stimulation with thrombin, histamine (Bonfanti et al. 1989), probably with the co-operation of platelet activating factor (PAF) (reviewed in (Zimmerman, Prescott, & McIntyre 1992)). P-selectin is also re-internalised providing a mechanism for reversible adhesion of leukocytes (in particular neutrophils) to endothelium, and is mirrored by neutrophil adhesion to endothelium activated with such agonists over short time periods. In the model proposed by Zimmerman et al, sustained adhesion of neutrophils is dependent on expression of CD62E that is transcriptionally up regulated in response to pro-inflammatory cytokines like IL-1 and TNF-α and LPS (Zimmerman, Prescott, & McIntyre 1992). CD62E is expressed solely on vascular endothelium, and has a significant role in recruitment of both neutrophils and monocytes in the early phase of the endothelial inflammatory response (Lawrence, Bainton, & Springer 1994;Lawrence & Springer 1993). This is consistent with the time course of surface CD62E expression in response to inflammatory stimuli *in vitro*. It appears at around 1 hour, peaks at 4 to 6 hours and disappears by 24 hours (Bevilacqua et al. 1987). Anti-bodies to CD62E partially inhibit neutrophil adhesion to activated endothelium *in vitro,* and can support neutrophil rolling in the absence of both activation and function of β_2-integrin (Zimmerman, Prescott, & McIntyre 1992), which mediate firm adhesion as detailed below.

1.3.5.4. Leukocyte activation

Leukocytes then undergo a critical activation stage, involving both alterations in shape such as flattening of the apposing surface, and also changes in both expression and conformation of cell adhesion molecules., particularly the leukocyte integrins (described in next section). For example, activation of

leukocyte integrins triggers conformational changes that converts them to an active state resulting in high avidity binding to their endothelial surface ligands. This "avidity modulation" is critical to the process of leukocyte-endothelial interactions (reviewed in (Smyth, Joneckis, & Parise 1993)). Underlying this is a process of "inside-out" signalling, in which signalling events occurring on intracellular cytoplasmic domains influence conformational changes and avidity of ligand binding of the extracellular domain of the integrin (Ginsberg, Du, & Plow 1992). Action of chemoattractants and chemokines, and cross-linking of other surface receptors can influence this process.

Chemoattractants such as C5a and chemokines IL-8, PAF and MCP-1 are critical to leukocyte transmigration. They impart a gradient that directs leukocyte movement (Huber et al. 1991). Different chemokine receptors present on different populations of leukocytes may contribute to tissue specificity of leukocyte traffic (reviewed in (Springer 1994)). Chemokines may regulate leukocyte-endothelial interactions by augmenting integrin-mediated adhesion. The chemokine IL-8 is produced by endothelium and can promote neutrophil adherence (Rot 1992). Whilst endothelial derived IL-8 may increase β2 integrins on neutrophils (Detmers et al. 1990) it can also decrease neutrophil adhesiveness to EC's expressing CD62E. This is thought to be due to action of IL-8 as a leukocyte adhesion inhibitor (Gimbrone-MA et al. 1989). Platelet activating factor (PAF), expressed rapidly on cell surface of endothelium when stimulated with thrombin, histamines and other agonists, induces inside-out signalling by β_2 integrins on neutrophils, especially when tethered by P-selectin and its ligands (reviewed in (Zimmerman, McIntyre, & Prescott 1996;Zimmerman, Prescott, & McIntyre 1992)).

1.3.5.5. Firm adhesion of leukocytes is mediated by the Integrin cell adhesion molecules

Integrins are a diverse and important group of adhesion molecules that mediate interactions between leukocytes and surface expressed vascular cell adhesion molecules and extracellular matrix proteins. Integrins are composed of two, non-covalently linked α and β chains. At least 8 β chains and 15 α chains have been reported, allowing for considerable combinatory diversity. They are transmembrane proteins that interact with cytoskeletal proteins (reviewed in (Carlos & Harlan 1994)). The best characterised of these are the β2 integrins CD11a/CD18 (LFA-1/$\alpha_L\beta_2$) and CD11b/CD18 (MAC-1/$\alpha_M\beta_2$), and the β1 integrin VLA-4 ($\alpha_4\beta_7$), which interact with endothelial adhesion molecules of the immunoglobulin super-family, ICAM-1 and VCAM-1, respectively (Springer 1990). In addition, CD11a/CD18 interacts with ICAM-2. CD11c/CD18 ($\alpha_x\beta_2$) is also expressed on leukocytes, but appears to have a minor role in neutrophil or monocyte adhesion (Carlos & Harlan 1994).

Firm adhesion involves cross talk between leukocyte integrins, endothelial ligands including selectins and locally released or surface expressed chemokines and chemoattractants. Binding of CD62E to neutrophils augments CD11b/CD18 adhesion to endothelial cells (Lo et al. 1991). In contrast, T cell activation is not affected by purified CD62E, but is by ICAM-1 and VCAM-1 (van et al. 1991). This is consistent with time course of leukocyte extravasation in response to experimentally administered LPS, with neutrophils being the early inflammatory cells, followed by monocytes and finally lymphocytes (Issekutz & Movat 1980). CD11b/CD18 mediated adhesion of monocytes to endothelium augments TNF-α

production, thus further increasing and prolonging expression of vascular cell adhesion molecules (Fan & Edgington 1993).

Other strong evidence that CD11/CD18 interactions are critical to adhesion and transmigration of neutrophils (and monocytes) is seen in patients with leukocyte adhesion deficiency syndrome (LAD type I) (Harlan 1993). This is a result of point mutations in regions in β_2 subunit critical to maintenance of tertiary and heterodimeric structure. Neutrophils in particular, and to some extent monocytes, fail to migrate into inflammatory sites with disastrous consequences for wound healing and response to infection. However, migration of lymphocytes is not affected. In addition, neutrophils from LAD type I patients are able to roll along but fail to adhere to endothelium *in vitro* (von Andrian 1993). Monoclonal antibodies to CD18 block neutrophil adhesion but not rolling (Arfors et al. 1987;von et al. 1991), which underpins the importance of CD11/CD18 interaction with cognate ligand on endothelium as being critical to firm adhesion. CD11b/CD18 can bind to iC3b deposited on endothelium during complement activation (Marks, Todd, & Ward 1989). In addition, there are a number of soluble molecules, such as fibrinogen, which can promote leukocyte adhesion to endothelium by bridging ligands expressed on both cell types (Languino et al. 1993).

1.3.5.6. Leukocyte transmigration

The term transmigration describes the process by which adherent leukocytes crawl over endothelial surface, then "squeeze" between the intercellular junction and enter the extravascular space (Carlos & Harlan 1994) (see figure 1.5). The term diapedesis applies only to the process of squeezing between intercellular junctions. This process is governed by complex interactions between vascular adhesion molecules, leukocyte integrins (especially modulation of avidity) and

chemokines. The platelet endothelial cell adhesion moleclue-1 (PECAM-1/CD31) is highly expressed on endothelial intercellular junctions (Muller et al. 1989), and also on many leukocytes. Monoclonal antibodies to PECAM-1 inhibit transendothelial migration, although the mechanism by which PECAM-1 influence leukocyte behaviour is not fully understood (Muller et al. 1993). Both chemoattractants and chemokines are involved in both adhesion and transmigration of leukocytes across endothelium. It is likely that intracellular signalling induced in neutrophils by adhesion receptor activation (probably integrin) and the action of stimulatory chemokines, particularly PAF (Zimmerman, Prescott, & McIntyre 1992) is critical to migration and subsequent priming of neutrophil inflammatory responses (Huang et al. 1993).

The role of vascular adhesion molecule ICAM-1 and its interactions with leukocyte integrins and the functional consequences of this on leukocyte transmigration are complex. ICAM-1 forms ligands with both CD11a/CD18 (LFA-1) and CD11b/CD18 (MAC-1/CR3) (Smith et al. 1989a). Blocking of ICAM-1 by monoclonal antibodies only partially inhibits neutrophil migration (Furie, Tancinco, & Smith 1991), which is enhanced when combined with antibodies to either CD62E or to CD11b/CD18 (Luscinskas et al. 1991). This is important, because it shows that selectin-ligand interactions and those between integrins and immunoglobulin family, although sequential, may overlap and functionally interact. It is very likely that ICAM-1 independent adhesion by means of other CD11b/CD18 endothelial ligands occur, including fibrinogen (Wright et al. 1988) and extracellular endothelial matrix proteins containing "RGD" sequence motifs found in a number of these molecules (Ruoslahti & Pierschbacher 1987).

1.3.5.7. Phenotype of activated endothelial determines selectivity of leukocyte adhesion

The importance of endothelial cell adhesion molecules on leukocyte adhesion and transmigration has been already discussed. The nature of the inflammatory signal is a critical determinant of the pattern and kinetics of cell adhesion molecule expression. For instance, the response of endothelium to pro-inflammatory cytokines such as TNF-α and IL-1 or bacterial LPS is quite different from that seen in response to IL-4 and IFN-γ derived from activated T cells (Shimizu et al. 1992). For example, TNF-α, IL-1 and LPS up-regulate CD62E, ICAM-1 and VCAM-1 (Bevilacqua et al. 1987;Dustin & Springer 1988;Karmann et al. 1996;Pober et al. 1986). IFN-γ stimulates up-regulation of VCAM-1, but not ICAM-1 or CD62E, whereas IL-4 stimulates VCAM-1, but not CD62E or ICAM-1 (Kotowicz et al. 1996). A consequence of this may be that in chronic inflammatory situations, where the production of the latter two cytokines may predominate, recruitment of monocytes and lymphocytes may be favoured over neutrophils. It has been shown, for example, that IL-4 increases adhesion of T lymphocytes but not neutrophils to endothelium (Thornhill, Kyan Aung, & Haskard 1990).

Phorbol esters, such as PMA, which activate protein kinase C, induce ICAM-1 and CD62E but VCAM-1 only weakly (Lane, Lamkin, & Wancewicz 1989). Furthermore, combinations of different cytokines, lymphokines or other inflammatory mediators significantly modulate the endothelial response (Carlos & Harlan 1994). Co-stimulation of IFN-γ with TNF-α or LPS, but not IL-1, increases and prolongs CD62E expression on endothelium (Doukas & Pober 1990). This appears to occur through post translational events (Doukas & Pober

1990). IL-4 suppresses TNF-α induced CD62E induction but augments VCAM-1 expression (Bennett et al. 1997;Thornhill & Haskard 1990). Thrombin, generated in DIC, potentiates TNF-α induction of CD62E (Anrather et al. 1997).

1.3.5.8 Endothelial activation and the systemic inflammatory response *in vivo*

Widespread endothelial activation is one of the key pathophysiological processes occurring in the systemic inflammatory response syndrome (SIRS) such as that seen in severe meningococcal disease. The direct action of bacterial LPS either associated with or released from meningococci is likely to be central to this process. LPS stimulates production of powerful pro-inflammatory cytokines TNF-α and IL-1 from monocytes that in turn can induce endothelial activation. Adherent, activated neutrophils, monocytes and platelets will then act in concert with these mediators to increase endothelial activation through adhesion and signalling, as has been described in the previous section. The plethora of inflammatory mediators generated in the septicaemia, such as thrombin, endothelial-derived chemokines IL-8 and PAF, complement activation products such as C5a and vasoactive molecules like histamine and adenosine diphosphate (ADP) and will also contribute. Alterations in blood flow within vessels are important. Blood vessel dilation is due in part to effects of endogenously released nitric oxide (NO). In addition, the presence of microthrombi within small vessels may lead to turbulent flow, or even occlusion leading to tissue hypoxia (Brandtzaeg 1995). All these processes will affect leukocyte behaviour, decreasing transit times, increasing likelihood of further contact and adhesion of leukocytes to vascular endothelium.

Comparison of data from experiments that investigate endothelial response to inflammatory stimuli *in vitro* and *in vivo* reveals some conflicting findings. For example, both LPS and cytokine stimulation of endothelium *in vitro* is sufficient to induce both neutrophil adhesion and transmigration to sub-endothelial space (Glauser et al. 1991a;Shibata, Metzger, & Myrvik 1997;Smith et al. 1989b). In contrast, in systemic administration of LPS *in vivo,* although both endothelial activation and neutrophil adhesion can be demonstrated, there is little or no neutrophil transmigration (Schleiffenbaum et al. 1998). It has been postulated that *in vivo*, there is an inhibitor of neutrophil transmigration produced in response to systemic inflammatory response, although it was shown that this was not IL-8 (Schleiffenbaum et al. 1998). The effects of IL-8 on neutrophil transmigration are complex and somewhat controversial. It has been shown that IL-8 both supports neutrophil transmigration when producing chemotactic gradient (Smith et al. 1991) and inhibits (Takahashi et al. 1995a) neutrophil transmigration when added exogenously. It is likely that such considerations are important when comparing the findings from either *in vitro* studies of cultured endothelium or those from *in vivo* experiments utilising administered inflammatory mediators.

What accounts for these differences is not clear. Both pro and anti-inflammatory molecules are generated in the systemic inflammatory response that may have opposing actions. It may be that there are specific inhibitor (s) of neutrophil transmigration that are generated *in vivo* and this is supported by the finding that pre-treatment of mice with LPS totally inhibited local neutrophil emigration (Schleiffenbaum et al. 1998). This occurred even though *ex vivo* activation of neutrophils from mice sensitised by LPS was normal and local endothelial cells were responsive to LPS challenge.

1.3.5.9. Mechanisms of endothelial injury

Vascular damage and dysfunction is a key component of the pathophysiology of the systemic inflammatory response syndrome. Leukocytes, particularly neutrophils, have capacity to induce endothelial damage that is consequent on both their activation and adhesion to the endothelium and release of powerful inflammatory mediators, as discussed in section 1.3.4.8. Part of this neutrophil mediated vascular damage involves degradation of extra-cellular matrix proteins that are essential for maintaining vascular integrity. These include the sulphated glycosaminoglycans (GAGS) and fibronectin (Kjellen & Lindahl 1991;Ruoslahti 1988). GAG's are important in the regulation of vascular permeability, maintenance of thromboresistance and anchoring to extracellular matrix proteins (Lindahl & Hook 1978;Marcum & Rosenberg 1984). Fibronectin is an important extracellular matrix protein that can bind to a number of different integrin molecules (reviewed in (Wahl, Feldman, & McCarthy 1996)). Proteolysis of fibronectin caused release of degradation products that further stimulate neutrophil adhesion (Vercellotti et al. 1983) and degranulation (Wachtfogel et al. 1988), providing a clear mechanism for pathophysiological cascade of damage. Stimulation of endothelial cells *in vitro* with bacterial LPS alone increases adherence of neutrophils to endothelium and degradation of GAG's and disruption of fibronectin when potent neutrophil activator FMLP is added as well (Klein et al. 1993). Neutrophils release soluble activators of matrix metalloproteinases, which have a central role in the proteolytic turnover of number or extra cellular matrix proteins, such as fibronectin, collagens and gelatins (Schwartz et al. 1998). Figure 1.6 summarises mechanisms of neutrophil mediated endothelial injury.

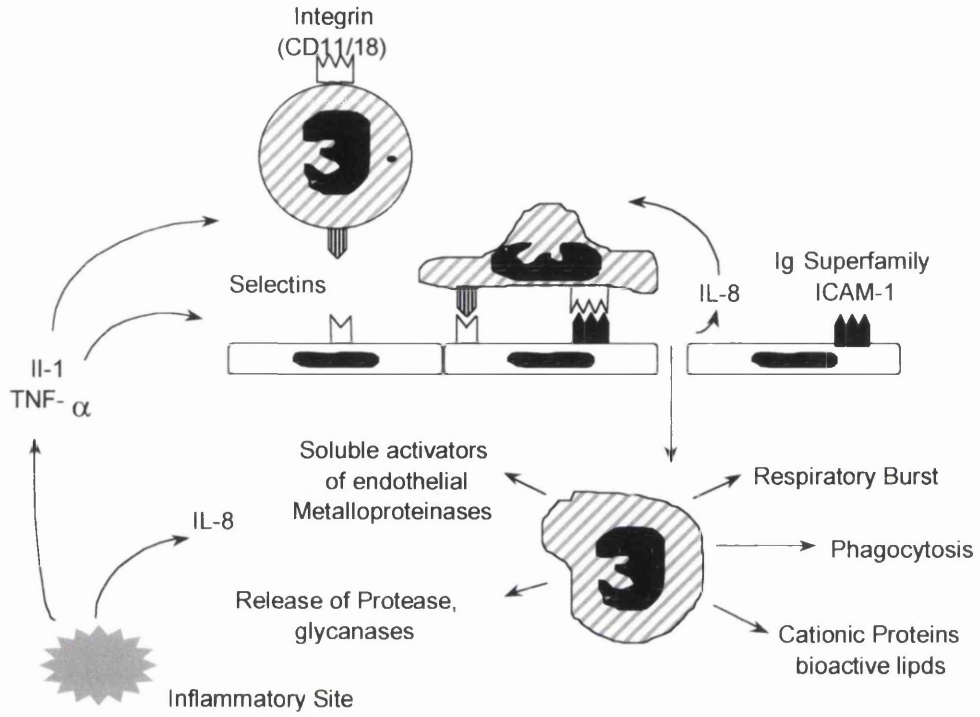

Figure 1.6 Mechanisms of Neutrophil mediated injury of activated endothelium.

This figure illustrates some of the critical processes in neutrophil interactions with activated endothelium. Expression and function of vascular cell adhesion molecules, such as CD62E/E-selectin and ICAM-1 is central component of this process. Neutrophil degranulation and release of potent inflammatory mediators are thought to contribute to vascular injury.

1.3.5.10. Interactions of Vascular endothelium and *N. meningitidis*

1.3.5.10.1 Histological studies.

Netter and Salanier first reported in 1917 that meningococci could be found, and cultured from the skin lesions of patients suffering from SMD (Netter & Salanier 1917). Hill and Kenney in 1947 demonstrated organisms in blood and histological skin specimens of children who died from meningococcal disease (Hill 1947). Indeed, skin biopsy and needle aspiration is still a useful, rapid diagnostic test for meningococcal infection (van Deuren et al. 1993). In 1976, Sotto and

workers conducted in depth study of the histological, immunological and inflammatory features of skin lesions from patients with FMS by light and electron microscopy (Sotto et al. 1976). Meningococci were "seen inside endothelial cells, inside the vascular lumina, and phagocytosed by neutrophils." "Marked vascular damage was observed, represented by necrosis of the endothelial cells and other elements of the vascular wall". Additionally, "Thrombi made up of fibrin and platelets" were seen. This, the authors state, is indicative of a local Schwartzman reaction. They also found deposits of immunoglobulin and complement in the vascular wall, indicating that host immunological factors contribute to the pathology. A model proposed by Evans et al, that in the case of rapid bacterial proliferation, both direct bacterial invasion and released endotoxin lead to vasculitis, thrombosis and haemorrhage (Evans et al. 1969). In tissue where there are few meningococci, damage may be triggered by released bacterial endotoxin, or by immunological factors such as complement

These findings demonstrate a number of critical processes that may contribute to the vascular damage characteristic of meningococcal disease. Firstly, meningococci associate with and invade endothelial cells *in vivo*. Secondly, there is a dense inflammatory infiltrate of neutrophils seen within the vasculitic lesions. Thirdly, platelets and thrombi are frequently seen in these lesions. Given that in the most severe cases circulating neutrophils and platelets are almost always reduced especially in the early stages of the disease, the postulate that this due to their sequestration in the inflammatory lesions is compelling. What is not demonstrable from this histological investigation is what is the primary initiating event that results in the pathological picture so graphically illustrated. Is it direct bacterial contact and endothelium, or by activated neutrophils that have already ingested organisms or the presence of large amount of endotoxin contained within

blebs released by meningococci? The fact that live meningococci can be cultured from purpuric lesions would lead one to speculate that direct contact between organisms and endothelium is a critical event

1.3.5.10.2. Interactions between meningococci and human vascular endothelium in vitro

In addition to the histological evidence described above, meningococci have been shown to adhere to and invade human vascular endothelial cells *in vitro.* A number of bacterial factors that effect the both adhesion and invasion have been described. Encapsulated meningococci adhere to endothelial cells mainly via either class 1 or class II filamentous pili, although non-piliated organisms are still able to associate with endothelial cells (Virji et al. 1991;Virji et al. 1992b). Pili themselves are complex structures, made up of pilin (PilE) subunits and associated tip located PilC adhesins, of at least two types, which are thought to be the essential component of adherence of meningococci to host cells (Rudel, Scheurerpflug, & Meyer 1995;Taha, Giorgini, & Nassif 1996). There is considerable structural diversity, including both primary amino acid sequence and glycoslylation status of pilin subunits between and sometimes within strains, which may alter adherence to both epithelial and endothelial cells (Virji et al. 1993b). As both pilin and PilC are subject to phase variation, it provides further mechanisms by which meningococci can alter their ability to adhere to cells depending on both host and environmental factors (Taha, Giorgini, & Nassif 1996).

Whilst pili are critical to adhesion of encapsulated meningococci to endothelial cells, other adhesins seem to be responsible for interactions of unencapsulated bacteria and endothelial cells. Work by Virji et al have shown that the class 5 outer membrane protein Opc facilitates both adhesion and phagocytic internalisation of meningococci to human endothelial cells (Virji et al. 1992a).

Unencapsulated mutants display high degree of internalisation into endothelial cells compared to encapsulated strains (Virji et al. 1992a). Further studies by Virji et al have shown that bacteria that express Opc adhere to and invade endothelial cells far more effectively than those bacteria than do not express this adhesin. Opc interactions with endothelial cells are inhibited by the presence of sialylated LPS in unencapsulated organism (Virji et al. 1993a). It has been shown that for the group B strain MC58 expressing the sialylated LPS immunotype, pili were critical interactions with epithelial and endothelial cells whether capsulated or not. Opc mediated invasion is only seen in unencapsulated organisms with a truncated, non-sialylated LPS and that co-expression of pili and Opc results in increased invasion (Virji et al. 1995). In addition, this and another study by the same group have demonstrated that Opc interactions with endothelium was via a trimolecular complex involving a serum factor, thought to be soluble vitronectin or a related molecule, and its cellular receptor, the $\alpha v\beta 3$ integrin (Virji, Makepeace, & Moxon 1994).

Other class 5 outer membrane proteins can mediate attachment of meningococci to endothelium, although not as avidly as that seen with Opc (Virji et al. 1993a). Nonetheless, the interplay between expression of virulence factors such as capsule, LPS sialylation and structure, pili and outer membrane proteins may be critical to the understanding of how pathogenic meningococci use host cellular systems to adhere to and invade endothelial cells. There may be differences between strains but this is equally the case with meningococcal interactions with epithelial cells and phagocytic cells like neutrophils.

1.3.5.10.3. Influence of capsule and LPS structure in meningococcal endothelial interactions in vivo

As has been stated, meningococci isolated from patients with invasive disease are predominantly encapsulated, have sialylated LPS and express pili and opacity proteins Opa and Opc (DeVoe & Gilchrist 1975;Mandrell & Zollinger 1977). This finding does not preclude the importance of unencapsulated organisms in natural infections. Organisms isolated from blood and CSF may have this phenotype because of the resistance these confer on the bacterium to host immune attack, either from opsonic or non-opsonic phagocytosis by neutrophils or complement-mediated attack (Hammerschmidt et al. 1996b;Jack et al. 1998;Jarvis & Vedros 1987b). The ability of meningococci to reversibly switch genes involved both capsule synthesis and sialylation of LPS has already been mentioned. This provides a theoretical mechanism whereby meningococcal adherence to and invasion of both epithelial and endothelial cells *in vivo* could be enhanced (Hammerschmidt et al. 1996a). Rapid and reversible switching on and off of capsule synthesis by the excision/insertion sequence element IS*1301* (described in section 1.3.2.3) has been shown to correlate both with invasion of epithelial cells *in vitro* and be associated with an outbreak of meningococcal disease (Hammerschmidt et al. 1996b). Capsule deficient mutants which can reversibly switch on and off capsule production, albeit at a low rate, were able to cause invasive disease in infant rats, whereas the stable isogenic mutant, unable to synthesise capsule, could not (Vogel, Hammerschmidt, & Frosch 1996). In addition, rapidly proliferating meningococci have been shown to be relatively deficient in capsule formation (Masson & Holbein 1985). Although lack of capsule may make meningococci more vulnerable to host attack, in permissive environments it may mean that meningococcal adherence to and invasion of

endothelial cells is increased. This process is critically dependent on variations in bacterial structure, which is likely to be a dynamic process occurring throughout the course of the disease.

In vitro studies thus described demonstrate that unencapsulated organisms are internalised to a much greater degree than encapsulated strains. It must be pointed out, however, that capsule expression was not determined in the *in vivo* histological studies showing meningococci internalisation within vascular endothelium. This is clearly an important area for further study. The current state of knowledge of the inflammatory response of vascular endothelium to meningococci will be discussed in Chapter 3.

1.4. Aims of the Thesis

The introduction to this thesis has attempted to give an overview of the current understanding of meningococcal disease, especially in terms of factors that may contribute to the severity of meningococcal septicaemia. The principal reason for undertaking this work was to explore a pathophysiologically relevant aspect of host-bacterial interaction; namely that between meningococci and vascular endothelium. Although the reason why this is a valuable question to address has been mentioned, a summary of the argument for the plausibility of the investigative approach would be useful. Systemic meningococcal disease occurs when there is failure to contain local mucosal invasion of bacteria and there is proliferation systemically. Uncontained proliferation of meningococci results in high levels of bacteria and associated inflammatory products such as LPS. Either as a result of direct damage by bacteria, or effects of host derived inflammatory mediators, a state of systemic inflammatory response ensues. Vascular damage and dysfunction is a critical component of this response in general and is characteristic of fulminant disease. Meningococci can adhere to and invade endothelium *in vitro*. *In vivo,* vasculitic lesions are associated with an inflammatory infiltrate, especially neutrophils. The expression and function of vascular adhesion molecules regulates leukocyte endothelial interactions. It is therefore biologically plausible that bacterial contact with vascular endothelium is a primary initiator of endothelial activation in terms of cell adhesion molecule expression and leukocyte adhesion and activation, and thus may significantly contribute to the pathogenesis of this condition.

To date there are no published studies that investigate how meningococci directly influence the expression of vascular cell adhesion molecules. The following

rationale for the experimental approach used in the following chapters are detailed below.

Chapter 3 explores the pattern of endothelial cell adhesion molecule expression of cultured human umbilical vein cells (HUVEC) in response to a clinical isolate of *N. meningitidis* and isogenic mutants that are deficient in either capsule expression or LPS sialylation. It has already been stated that variations in surface expression of these two important bacterial components may significantly influence the capacity of meningococci to adhere to and invade a number of cell types, including endothelial cells *in vitro,* in addition to be associated with invasive disease. The results suggest that capsulation and LPS structure had a significant influence on the capacity of meningococci to influence the degree of cell adhesion molecule expression on HUVEC. In addition, the pattern observed in response to meningococci was different to that seen in response to purified LPS. In particular, purified LPS was less potent at inducing expression of CD62E than meningococci, especially unencapsulated mutants. Whereas the effects on ICAM-1 and VCAM-1 expression were more comparable. A possible explanation for this is that components other than LPS are able to influence cell adhesion molecule expression, and that the effect of these may vary depending on the ligand under investigation.

Chapter 4 is a logical progression from the results obtained in chapter 3. Two experimental approaches were used to evaluate the relative role that meningococcal LPS plays in endothelial adhesion molecule expression in response to meningococci. Firstly, the response to an isogenic mutant that is entirely deficient in LPS was compared to the parent strain and meningococcal LPS. The second approach used a modified recombinant form of a naturally occurring antagonist of LPS, the bactericidal/permeability increasing protein (rBPI), in an

attempt to block LPS induced activation of endothelial cells. LPS has historically been considered the primary inflammatory component of gram-negative bacteria, including meningococci. For this reason, therapeutic approaches designed to influence the inflammatory response in this condition have centred on using compounds that have anti-LPS properties. During the time of the thesis, a large multi-centre trial that used rBPI as an adjunctive treatment in cases of severe meningococcal disease in children was undertaken. Since there were no *in vitro* studies that evaluated the effect rBPI had on endothelial activation in response to intact meningococci, this project appeared both interesting and pathophysiologically relevant.

In Chapter 5 a different investigative approach was used to further explore the observed potency of meningococci to induce expression of CD62E as compared to purified LPS. Differences in signal pathways, induced by bacteria or purified LPS might be detectable by examining the transcription factors that are critical to the transcriptional regulation of the CD62E gene. The electrophoretic mobility shift assay was used to detect activated transcription factors in nuclear extracts of HUVEC stimulated with either purified LPS or meningococci that were capsulated, unencapsulated or deficient in LPS.

Chapter 6 is different from the previous studies in that it explores the response of human dendritic cells to meningococci. This was undertaken for a number of reasons. Dendritic cells from a crucial bridge between the innate and adaptive immune systems. In addition to their capacity as antigen processing cells, they also provide signals that direct lymphocyte differentiation. The nature of these signals may depend in part, on how the dendritic cell senses the complex array of inflammatory components present in meningococci. They are likely to have very important roles in the immune response to whole bacteria during the

course of natural disease and to vaccines designed to elicit protective immune responses. From a conceptual point of view, the study was complementary to the previous ones in that it allowed comparisons of the host response to *N. meningitidis* using a different cellular system, in order to evaluate the influence of bacteria structure on host inflammatory and immune responses in a broader context. The same family of innate immune mechanisms that enable dendritic cells to recognise and respond to complex inflammatory signals in meningococci and induce profound activation pathways in vascular endothelium may in part determine the biological response to this organism.

Chapter 2

General Methods

2.1 Introduction

The following chapter describes those methods that are common to a number of sections. The list of reagents applies to all those used throughout the experimental work. Composition of specialised buffers is listed in the appropriate sections.

2.2 Reagents and Materials

Name	Company	Product Code
Accugel 19:1 Polyacrylamide: bisacrylamide	National Diagnostics	
Adenosine Triphosphate. ATP [γ-^{32}P] 10mCi/ml, 3000Ci/mmol specific activity at reference date.	Amersham pharmacia biotech	PB10168
AEBSF HCl. 4-(2Aminoethyl)benzenesulfonylfluoride	Calbiochem	101500
Ammonium persulphate (AMPS)	Sigma	A-9164
Bovine serum albumin (low endotoxin)	Sigma	A9543
Brefeldin A	Sigma	B4521
Bromophenol blue(3',3",5',5" tetrabromophenolsulfo-nephthalein), sodium salt, $C_{19}H_9Br_4O_5Sna$	Sigma	B5525
Cellfix (1% formaldehyde, 0.1% sodium azide	Becton Dickinson	340181
Chloramphenicol	Sigma	C 7795
Chromaspin 10 columns	Clontech	K1300-2
Collagenase Type II	GIBCOBRL	17101-015

Complete-proteinase inhibitor cocktail	Boehringer Mannheim	1 697 498
Dithiothretiol (DTT)	BDH Merck	44385 2A
DMSO (Dimethylsulphoxide)	Sigma	D-8918
Dulbecco's Modified Eagle Medium, with L-glutamine, D-glucose	GIBCOBRL	41965-039
EDTA (Ethenediaminetetraacetic acid, disodium salt)	Sigma	E7889
Endothelial cell attachment factor	Sigma	E9705
Endothelial Growth Medium supplement singlequots (see pp)	Clonetics	CC-4176
Ethanol (C_2H_5OH)	Hayman	
Fetal calf serum	PAA laboratories	
Fetal calf serum (endotoxin low)	Globepharm	F11962
Film, autoradiography, 8*10 inch, Kodak BioMax MR	Sigma	Z35,846-0
Film, autoradiography, 8*10 inch, Kodak BioMax MS	Sigma	Z36,300-6
Film, autoradiography, 8*10 inch, Kodak X-OMAT AR	Sigma	F 5513
Formaldehyde	Sigma	
Gentamycin	Roussel	Hospital stores
Glycerol, molecular biology grade	BDH Merck	44448 5B
Gonococcal agar	Difco	0289-17-3
Granulocyte-Monocyte Colony stimulating factor (GM-CSF)	Schering-Plough	
Hank's balanced salt solution, without Phenol red.	GIBCOBRL	14025-050

HEPES (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid])	GIBCOBRL	15630-056
Interleukin-4 (IL-4)	Schering-Plough	
Kanamycin	Sigma	K0879
Lipopolysaccharide, *E.coli* O111B:4, gel purified	Sigma	L3012
Lymphoprep	Nycomed	
Magnesium Chloride Hexahydtrate ($MgCl_2.6H_2O$)	BDH Merck	43699 2S
2-mercaptoethanol	GIBCOBRL	
MCDB 131 medium	GIBCOBRL	10372-019
Oligonucleotide sequences, customised	Sigma-Genosys	Special order
Paraformaldehyde $(CH_2O)_n$	Sigma	
Penicillin/Streptomycin	GIBCOBRL	15140-114
Phosphate buffered saline tablets	Oxoid	BR14a
Polydeoxyinosinic-deoxycytidylic acid (poly[dI-dC].poly[dI-dC]).	Sigma	P 4929
Potassium Chloride, KCl	BDH Merck	43702 3F
Protein Assay kit (Bradford method)	Biorad	5000116
RPMI 1640 basal medium	GIBCOBRL	51800-035
RPMI 1640 medium with 25mM HEPES and 10mM L-glutamine	GIBCOBRL	52400-025
RPMI 1640 medium without phenol red	GIBCOBRL	32404-022
Saponin	Sigma	S-4521
Sodium Azide (NaN_3)	BDH Merck	10369
Sodium Chloride, NaCl	BDH Merck	44382 4T
Sodium Hydroxide, NaOH	BDH Merck	10252 5P

T4 Polynucleotide kinase and reaction buffer	Promega	M4103
TEMED (N',N',N",N" tetramethylethylenediamine)	Sigma	T-7024
Tissue culture flasks +, 175cm^2	Sarstedt	831812-382
Tissue culture flasks, 25cm^2, Primaria	Becton Dickinson/ Falcon	35-3813
Tissue culture flasks, 75cm^2, Primaria	Becton Dickinson/ Falcon	35-3824
Tissue culture plates, 24 wells	Costar	3524
Tissue culture plates, 48 wells	Costar	3548
Tris ([hydroxymethyl]aminomethane) Borate EDTA buffer (10M)	Sigma	T4415
Tris([hydroxymethyl]aminomethane)-HCl (1M), pH 7.6	Sigma	T2788
Triton X-100 (iso-Octylphenoxypolyethoxyethanol)	BDH Merck	43700 2A
Trypsin-EDTA	GIBCOBRL	
Tumour Necrosis Factor-alpha (human recombinant TNF-α)	Not known	
VITOX	Oxoid	SR090A

2.3. General buffers		
Puck's A saline	KCl	0.4 g/L
	NaCl	8.00g/L
	$NaHCO_3$	0.35 g/L
	glucose	1.0 g/L
	EDTA	0.2%
	FCS	10%
	phenol red	0.005g/L
Phosphate buffered saline	NaCl	140.0mM
	KCl	2.7mM
	Na_2HPO_4	8.0mM
	KH_2PO_4	1.5mM
One tablet of PBS to 100ml of ddH_2O and autoclaved		
FACS washing buffer	1 times PBS	
	0.02% Sodium azide	
	5% FCS	

2.4 Antibodies

Name and isotype and species	Company/ Source	Product Code
anti-human CD14, mouse IgG2b. Clone HB246.	Prof. P.C.L. Beverley, Jenner Institute, UK	N/A
anti-human CD19, mouse IgG1. Clone BU12	D. Hardie, Birmingham	N/A
anti-human CD1a, mouse IgG2a. Clone NA1/34	Prof.A. McMichael, Oxford	N/A
anti-human CD25, mouse IgG1.	Dako	M0731
anti-human CD3, mouse IgG1. Clone UCH-T1	Prof. P.C.L. Beverley, Jenner Institute, UK	N/A
anti-human CD31, mouse IgG1. Clone WM59	Serotec	MCA1738
anti-human CD40, mouse IgG1. Clone LOB7/6	Serotec	MCA1590
anti-human CD62E, mouse IgG1. Clone 12.B6	Serotec	MCA883
anti-human CD83, mouse IgG2b. Clone HB15A	Serotec	MCA1582
anti-human CD86, mouse IgG1. Clone BU63.	D. Hardie, Birmingham	N/A
anti-human HLA-ABC, mouse IgG1. Clone W6/32	Serotec	MCA81
anti-human HLA-DQa, mouse IgG1. Clone L2	ICRF, London	N/A
anti-human ICAM-1, mouse IgG1. Clone 84H10	Serotec	MCA532

anti-human VCAM-1, mouse IgG1. Clone 1G11B1	Serotec	MCA907
anti-rat $IgG2_c$ heavy chain, mouse IgG1. Clone MARG 2c-3	Serotec	MCA 196
FITC conjugated anti-mouse $F(ab)_2$ goat polyclonal.	Dako	F0479
FITC conjugated anti-mouse IgG1 rabbit polyclonal.	Dako	F0261
FITC conjugated anti-mouse $F(ab)_2$ goat polyclonal.	Dako	R0480

Name, isotype and species	Company/ Source	Product code
PE conjugated anti-human IL-1a, mouse IgG1. Clone 364-3B3-14.	Pharmingen	18935A
PE conjugated anti-human IL-8, mouse IgG2b. Clone G265-8	Pharmingen	20795A
PE conjugated anti-human IL-12 (p40/p70). Clone C11.5	Pharmingen	18995A
PE conjugated anti-human IL-6, mouse IgG1. Clone AS12	Becton Dickinson	340527
PE conjugated anti-human TNF-α, mouse IgG1. Clone 6402.31	R & D systems	IC210P
PE conjugated normal mouse IgG1 control. Clone 11711.11	R & D systems	IC002P
PE conjugated normal mouse IgG2b control. Clone 20116.11	R & D systems	IC004P

2.5 Endothelial Cell Culture

2.5.1. Background

Endothelial cells can be isolated and cultured from a wide variety of sources, including different anatomical sites and different species. Each type requires specific techniques for isolation and growth. They may require various growth factors and special media. In general, endothelial cells require media which are rich in amino acids, and various sugar moieties, and also require high quality fetal calf serum at high concentrations (20%).

Human umbilical vein cells (HUVEC) are an ideal source of endothelial cells since they are derived from a plentiful renewable resource that would otherwise be discarded. The basic method employed is as described in (Gimbrone-MA, Cotran, & Folkman 1974). The method has been modified considerably, and the one detailed below is based on the method perfected principally by Dr. Karloena Kotovicz (Kotowicz et al. 1996).

2.5.2 HUVEC Culture media

The media used are listed in section 2.2. RPMI 1640 medium, containing 10mM L-glutamine, 80μg/ml gentamicin, 100 units penicillin/streptomycin was used for collection and storage of umbilical cords, and kept at 4^0 C in autoclaved polypropylene bottles. Washing of umbilical cords was performed with RPMI 1640 with same supplements as above but containing 5% fetal calf serum (FCS) (HUVEC wash medium). 1% Collagenase type II solution comprised of 1g collagenase dissolved in 1L of DMEM which was then passed through 0.2 micron tissue culture filter and stored in aliquots in -20^0 C until required.

Primary and secondary cultures used a specialised media for growth of endothelial cells, MCDB 131, supplemented with 10mM L-glutamine, 100 u/ml

penicillin/streptomycin and 20% fetal calf serum. In some instances, where high yields of endothelial cells were required, media was supplemented with a number of growth factors and supplements. MCDB 131 (with 10 mM L-glutamine and antibiotics) was supplemented with hydrocortisone (1μg/ml), epithelial growth factor (EGF) 10μg/ml and bovine brain extract (BBE)/endothelial cell growth factor (ECGF) 12μg/ml to produce 'Supergrow Medium'. 'Magic Medium' comprised of MCDB 131 (with 10mM L-glutamine and antibiotics) supplemented with hydrocortisone (1μg/ml), human fibroblast growth factor-β (hFGF-β, 1μg/ml), vascular endothelial growth factor (VEGF, 0.5ml), long R^3 Insulin-like growth factor-1 (R^3-IGF-1), Ascorbic acid (0.5ml), human epithelial growth factor (hEGF-1μg/ml) and heparin (1μg/ml).

2.5.3 Fetal calf serum

The quality and batch of FCS was found to be a critical determinant of yield of HUVEC from either primary culture or growth in secondary cultures. For this reason, batches of FCS were tested for quality. Specific batches, which proved to be most effective at sustaining growth of HUVECS, were ordered in large quantities and were used solely for HUVEC culture. Fetal calf serum was heat inactivated at 56^0 C for 60 minutes and stored at -20^0 C in aliquots until required.

2.5.4 Protocol for Isolation of HUVEC

Only fresh, intact cords were used, usually within 72 hours of collection. Cords that were heavily meconium stained were discarded. All the procedures described below were performed in Class II safety cabinet using aseptic technique. Autoclaved glassware was used. All metal instruments were sprayed with 70% IMS after thorough cleaning. Culture and wash media and enzymes were warmed to 37^0 C in a water bath.

1. The cords were inspected for damage such as cuts, needle punctures from cord blood sampling. Suitable cords were then sprayed with 70% IMS, and blood expressed into collection pot. Washed cords were then placed in a sterile Duran bottle containing RPMI 1640 medium with added antibiotics as described above.
2. Single cords were then cut at least 1cm from both ends. One end was clamped with an artery forceps. The other end was then inspected and the vein identified. The vein was dilated with blunt forceps, and cannulated with sterile plastic filling tube. This filling tube was secured with cotton thread and finally a small artery forceps.
3. The vein was then infused with warmed RPMI 1640 medium with 5% FCS (HUVEC wash medium). Sites of leakage were clamped as necessary.
4. This process was repeated for maximum of four cords at one time.
5. The vein was flushed with wash medium to remove excess blood, and then filled with 1% Collagenase II solution.
6. The cord was then incubated at 37^0 C in 5% CO_2 for 10 to 20 minutes.
7. After incubation, the digest was removed from cord into sterile Duran bottle, and flushed through with equal volume of wash medium. This was transferred to a sterile, 50ml conical tube.
8. The digest was centrifuged at 200g for 7 minutes at room temperature. The supernatant was discarded, and the pellet resuspended in MCDB 131 medium with 20 % FCS (HUVEC culture medium).

2.5.5. HUVEC primary culture

The digest was transferred to tissue culture flasks. These were always surface modified, polystyrene flasks designed for culture of adherent cells. 25, 75 or 175 cm^2 flasks were used, depending on requirements. In general, digest from a

large cord (enough to take 20 to 30 ml of collagenase solution) was resuspended into 10ml of culture medium into 25cm^2 flask. These were incubated at 37^0 C and 5% CO_2 Cells were washed in fresh culture media the following day and inspected under phase contrast microscopy.

HUVECS could be identified as small clusters of oval adherent cells. Cultures were inspected each day for growth. When cells were approaching confluence, which usually occurred after 72 hours culture, they were subcultured. Cells that grew poorly, or were contaminated with smooth muscle or fibroblastic cell overgrowth were discarded.

2.5.6 HUVEC subculture

HUVEC that were near confluent were then washed three times in warmed PBS, or HBSS to remove non-adherent cells and protein in FCS. Cells were washed once in 0.5 ml Trypsin-EDTA solution (per 25 cm^2 flask), removed and a further 0.5-ml Trypsin-EDTA solution added.

Cells were inspected under phase contrast microscopy, and when cells were rounding and becoming dislodged (usually after 30 seconds), the flask was tapped against bench top to aid removal. The cells were quickly resuspended in warmed HUVEC culture medium and transferred to 50-ml sterile conical tube. These were then seeded into 48 or 24 well flat bottom tissue culture plates which had been surface treated with endothelial cell attachment factor. Tissue culture plates were then incubated at 37^0 C in 5% CO_2.

2.5.7 Use of HUVEC in experiments

Details of specific conditions used to grow HUVECS for various experimental procedures are mentioned in the relevant sections in subsequent chapters. HUVEC morphology and phenotype, especially in terms of induction of cell adhesion molecules in response to pro-inflammatory stimuli alter dramatically

after serial passages. The cells used throughout this study were always after the first subculture passage. Generally, cells were used within one week of passaging. Medium was replaced every 3 days.

2.5.8 Identification of HUVEC

Endothelial cells were inspected under phase contrast microscopy prior to use for characteristic cobblestone appearance. In particular, care was taken to avoid cultures where there was significant overgrowth of smooth muscle like cells, which are characteristically spindle shaped and can be seen to arch over endothelial monolayers. Additionally, cells were identified by presence of high levels of CD31 by flow cytometry, as shown in Figure 2.1.

2.5.9 Protocol for detection of cell adhesion molecule expression by Flow cytometry.

Details of stimuli of HUVEC by organisms or purified LPS are given in the relevant chapter. Method given below is general protocol used throughout Chapters 3 and 4.

1. After stimulation, each medium from well from either 24 or 48 well plate was removed and discarded.
2. Each well was washed once in warmed Puck's A saline (see Materials and Methods) and incubated in 0.5ml Puck's for 15 minutes at 37^0 C on a rocking platform if available.
3. Cells were removed by mechanical scraping with a Pasteur pipette, and transferred to 12 × 75mm FACS tubes in 2mls of FACS wash (see Materials and Methods).
4. Cells were divided into appropriate number of separate tubes (one tube per stain), and centrifuged at 200g for 5 minutes.

5. Each sample was incubated with monoclonal antibody to CD62E, VCAM-1, and ICAM-1 for 15 minutes at room temperature. An isotype matched control to was included to control for non-specific binding of antibodies.
6. Cells were washed in 2mls wash, and centrifuged at 200g for 5 minutes.
7. Samples incubated with goat anti-mouse F $(ab)_2$ phycoerythrin (PE) or Fluoroscein isothiocyanate (FITC) conjugate antibody and incubated for 15 minutes in dark at room temperature.
8. After final centrifugation, cells were resuspended in 0.3 ml Cellfix solution until flow cytometry was performed.

2.5.10 Flow Cytometry

Fixed endothelial cells were analysed on FACScalibur flow cytometer using CellQuest software. Typical size and granularity characteristics and also presence of CD31 as shown in figure 2.1 identified endothelial cells. 5000 events within endothelial gate were collected. Typical instrument settings for endothelial cells are given in the Appendix I.

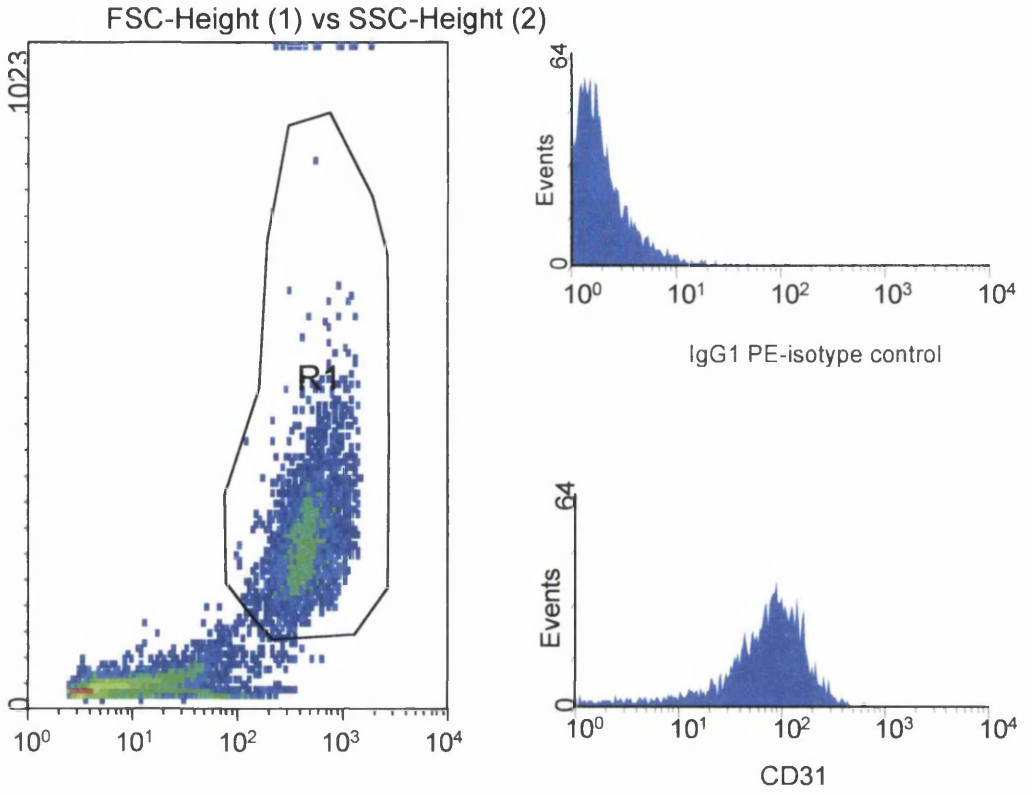

Figure 2.1 Flow cytometric characteristics of HUVEC.

Endothelial cells were identified by forward and side scatter characteristics and gated accordingly. Staining of irrelevant isotype matched control and antibody raised against the endothelial specific surface marker CD31 (PECAM-1) are shown

2.6 Microbiological Techniques

2.6.1. Introduction

The majority of the work presented in this thesis is based on the use of a unique set of isogenic mutants of two Group B *Neisseria meningitidis* strains. The two parent strains are disease causing organisms and clinical isolates. They are well characterised in terms of growth conditions, behaviour *in vitro* cultures, and phenotypic characteristics, as detailed below. Isogenic mutants are particularly useful tools for exploring pathogenesis of bacterial infections because they are stably transfected and do not revert to wild type spontaneously.

N. meningitidis is classified as a category 2 pathogen. In Great Ormond Street Hospital, it is handled as a category 3 pathogen, which has a number of

significant implications for their use in experimental situations. Preparation of live organisms required the use of a Class I safety cabinet that is housed in a dedicated safety room that meets a number of stringent safety criteria. All manipulations involving live meningococci in solution were performed within such a Cabinet. However, in a number of experiments, meningococci were killed either by heat inactivation or more commonly, fixation in 0.5% paraformaldehyde. In this situation, it was safe to transfer killed organisms to the tissue culture laboratory.

2.6.2. Bacterial strains.

The isogenic mutants and the parent organism from which they were derived were from two sources and will be described separately. The mutants derived from serogroup B *N. meningitidis* B1940 were from Professor Matthias Frosch, Institute of Hygiene and Microbiology, Wurzburg, Germany. Their characterisation has been described previously (Hammerschmidt et al. 1994). The parent organism B1940 (sero (sub) type NT: P1.3.6.15) expresses capsule and possesses a lipopolysaccharide (LPS) of immunotype L3, 7,9 that can be fully sialylated (figure 2.2). The *siaD*- mutant was constructed by insertional inactivation of the polysialytransferase gene, which is required for capsule synthesis and therefore is unencapsulated, but possesses a normal LPS. The *cpsD*- mutant is capsulated, but has an inactive *galE* gene, which codes for the enzyme UDP-galactose epimerase. This mutant cannot add a galactose moiety to glucose at the start of α-oligosaccharide chain of LPS. The resultant LPS is therefore truncated, and does not have the galactose acceptor site for sialic acid, and is therefore non-sialylated (see Figure 2.3). The *cps*- mutant misses the entire *cps*- locus, which normally contains both *siaD* and *galE* genes, and therefore is both unencapsulated and possesses a truncated LPS. Despite these changes, all the organisms express pili and class V outer membrane protein Opa and Opc (Hammerschmidt et al. 1996a).

The procedure of insertional inactivation of the genes involved a chloramphenicol resistance cassette. Mutants were grown in the presence of chloramphenicol when making new stocks of organisms to ensure purity.

Matthias Frosch and Ulrich Vogel also constructed two new mutants. The *lst-* of B1940, has a deleted α-2,3-sialyltransferase gene, and cannot sialylate terminal lacto-N-neotetraose of its LPS (Vogel et al. 1997). The double mutant, *lst-/siaD-* is unencapsulated in addition to being unable to sialylate its LPS (Dr. Ulrich Vogel, personal communication). These were constructed using a kanamycin resistance cassette and were grown in the presence of this antibiotic to ensure purity. The structure of the LPS of *lst-* mutant is represented schematically in Figure 2.3. The overall structure of all these mutants is shown schematically in Figure 2.2.

The isogenic mutant *lpxA-* and parent strain serogroup B H44/76 were from Professor Peter van der Ley, RIVM, Bilthoven, Holland. The serogroup B *N. meningitidis* strain 44/76 (sero (sub)-type (B: 15:P1.7, 16), ET-5 complex) was isolated from a case of fatal septicaemia in Norway (Andersen et al. 1995). The construction of the viable LPS deficient, isogenic mutant *lpxA-* has been described (Steeghs et al. 1998). This was constructed by insertional inactivation of *lpxA* gene with a kanamycin resistance cassette. The protein product of *lpxA* is the first committed step in lipid A biosynthesis was cloned from *E. coli* (Anderson & Raetz 1987). Absence of endotoxin activity in the mutant was determined by Limulus amebocyte lysate assay (LAL). In addition, absence of LPS was demonstrated by whole cell ELISA with LPS specific monoclonal antibody binding and gas chromatography /mass spectrometry analysis. The mutant strain showed similar binding pattern to monoclonal antibodies outer membrane proteins to the parent

strain (Steeghs et al. 1998). Purity of the *lpxA*- mutant was maintained by culturing on agar plates containing kanamycin (100μg/ml, Sigma, UK).

2.6.3. Growth and Preparation of *Neisseria meningitidis*

All the strains were stored in aliquots at -70^0 C in Mueller-Hinton broth and 15% glycerol. Bacteria were plated from these directly onto gonococcal agar plates supplemented with Vitox, and incubated at 36^0 C in 6% CO_2. Organisms were sub-cultured at least once before use. Prior to experimentation, organisms were confirmed to be gram negative dipplococci and oxidase positive. Gram' s staining involved air drying a small sample from plate onto a glass slide. Crystal violet was added to the slide for 30 seconds, rinsed in water, and then Lugol's iodine added. The slide was then washed in acetone, followed by water, counter stained by neutral red and visualised by oil immersion light microscopy. Oxidase positively was demonstrated by placing a colony from a plate against oxidase paper, which turns purple in presence of oxidase.

Organisms were used only in stationary phase after 16 to 18 hours after sub culturing in the studies presented in this thesis. Colonies were streaked from plates with sterile cotton wool swabs into RPMI 1640 medium (without phenol red). The optical density (OD) of ten-fold dilution of bacterial suspension was determined in spectrophotometer at 540nm. The bacterial suspension was then adjusted to an OD of 1.0.

Concentration of organisms in each suspension at OD of 1.0 was determined by ten-fold serial dilutions and plating and viability counts. Extensive repetitions of this procedure showed that a suspension of organisms in stationary phase adjusted to an OD of 1.0 contained around 10^9 colony forming units/ml (cfu/ml). The growth of the *lpxA*- mutant was known to be slower in log phase

than the parent organism (Steeghs et al. 1998). In viability counts, it was found to contain 2/3rds of the number of viable CFU/ml compared to the parent strain. However, flow cytometric analysis of fixed, propidium iodide organisms demonstrated that number of actual particles contained in a suspension was very similar between the *lpxA*- and parent strain. It is likely that the slightly reduced viability counts of the *lpxA*- mutant are due to its increased fragility as compared to the parent strain (Dr. Svein Andersen, personal communication).

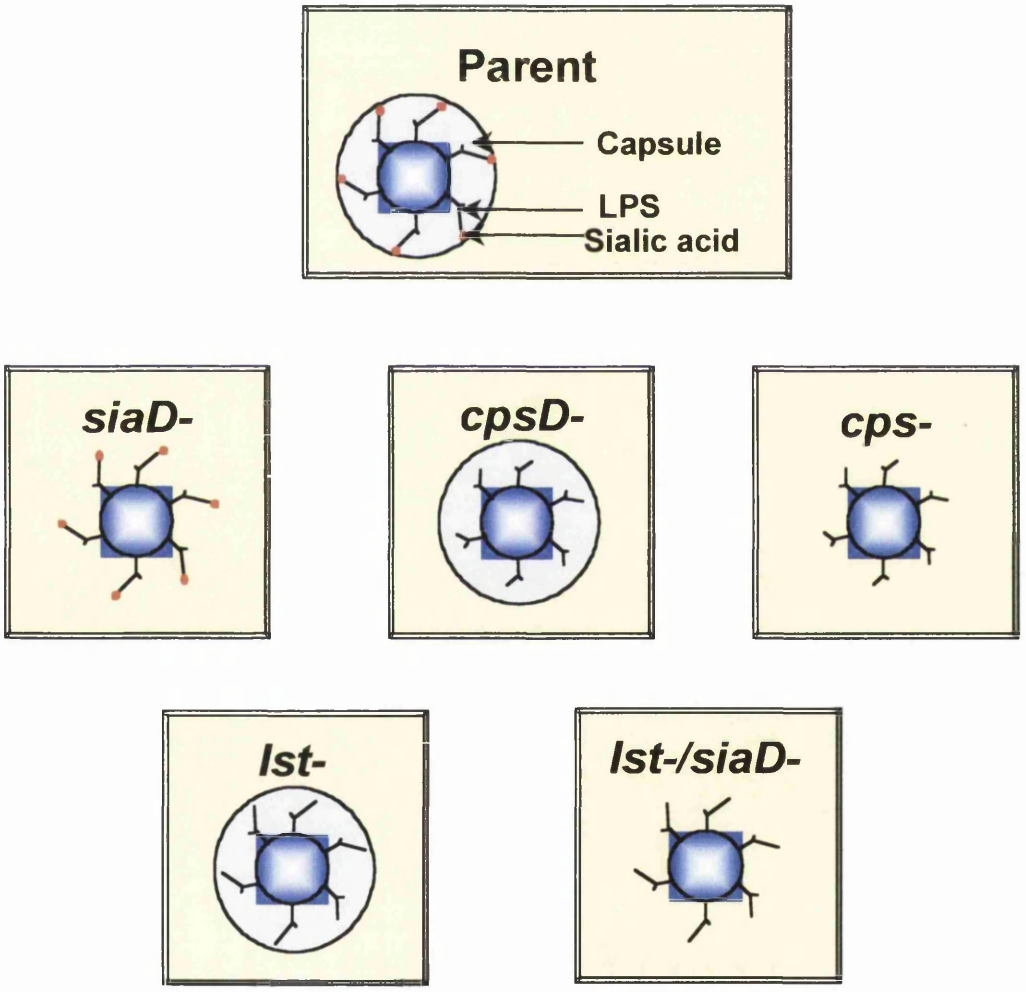

Figure 2-2 *N. meningitidis* B1940 parent strain and five derived isogenic mutants.

The parent organism possesses capsule and sialylated LPS. The *siaD*- mutant is capsule deficient; the *cpsD*- is capsulated, but possesses a truncated LPS that cannot be sialylated; the *cps*- lacks both capsule and has a truncated, non-sialylated LPS; the *lst*- is capsulated, but lacks the enzyme α-2,3-sialyltransferase, and cannot add sialic acid to terminal lacto-neo-tetraose; the *lst*/*siaD*- mutant is the double mutant that is non-sialylated and unencapsulated. Adapted from (Jack 1998).

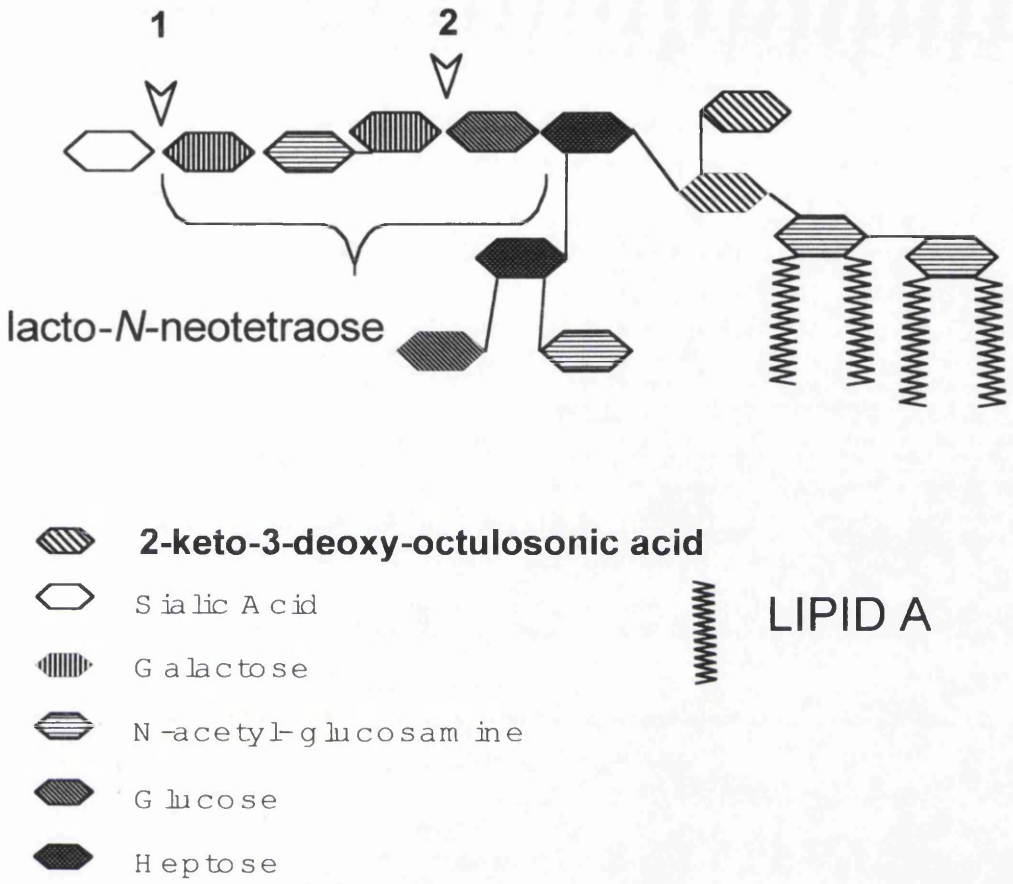

Figure 2-3 Schematic Representation of meningococcal L3 LPS

Meningococcal Lipopolysaccharide sometimes called Lipooligosaccharide, due to its relatively short, branched carbohydrate component. This is attached to 2-keto-3-deoxy-octulosonic (KDO) acid moieties that link to lipid A (Pavliak et al. 1993). The arrows indicate the truncation sites for the *lst-* (and *lst-/siaD-*) (1) and both the *cpsD-* and *cps-* strains (2). Adapted from (Jack 1998 and Schneider 1991)

Chapter Three

Induction of endothelial cell adhesion molecules by *Neisseria meningitidis*

3.1. Introduction

3.1.1 Background

Vascular injury is responsible for many of the clinical manifestations and pathophysiological processes characteristic of severe meningococcal disease. Although there is some evidence that meningococci can directly injure endothelial cells, at least *in vitro* (Virji et al. 1991), other work suggests that endothelial damage induced by meningococci is also mediated by activated neutrophils (Klein et al. 1996). The expression and function of vascular cell adhesion molecules is critical to the regulation of leukocyte endothelial interactions. Whilst much is known about the expression and function of adhesion molecules in response to either pro-inflammatory cytokines or purified bacterial LPS, little is known about how meningococci directly influence these receptors. This chapter addresses the role played by meningococci in endothelial adhesion molecule expression. This response was compared to that seen with purified LPS, which is a potent inducer of endothelial activation *in vitro* and *in vivo*.

3.1.2 Induction of vascular endothelial cell adhesion molecules by lipopolysaccharide

Bacterial lipopolysaccharide (LPS) is the major inflammatory component of gram-negative bacteria such as *N. meningitidis*. Due to its importance in the pathophysiology of gram negative septic shock (Parrillo 1993), there is a large amount of experimental data on the host inflammatory response to this molecule (reviewed in (Raetz et al. 1991)). The pattern of endothelial activation seen in response to bacterial LPS, usually derived from *E. coli* has been described (Bevilacqua et al. 1987;Dustin & Springer 1988;Marui et al. 1993;Schleimer & Rutledge 1986). LPS induces all three cell adhesion molecules. The response

appears similar, in terms of kinetics and potency, to that seen in response to IL-1, which is not surprising given the similarities in signalling pathways. The kinetics of cell adhesion molecule up-regulation on cultured endothelial cells *in vitro* stimulated by pro-inflammatory cytokines TNF-α and IL-1 have been described (Karmann et al. 1996). CD62E (E-selectin) is rapidly up regulated within 1 to 2 hours, peaks between 4 to 6 hours and returns to baseline by 24 hours. VCAM-1 expression commences at 3 to 4 hours, is maximal between 8 to 24 hours and gradually declines. ICAM-1 rises from 3 hours, is maximal at 24 hours and is still high at 72 hours post-stimulation (Karmann et al. 1996).

3.1.3 Structure of meningococcal and *E. coli* LPS

The majority of experiments investigating endothelial activation by LPS have been performed with *E.coli* LPS. However, there are a number of significant structural differences between meningococcal LPS and that derived from *E. coli.* Firstly, they differ in their carbohydrate structure. Meningococcal LPS does not possess an O antigen like *E. coli*, but rather has a short outer core, formed from branched oligosaccharide chains attached to a conserved inner core (Andersen 1997). The structure of these oligosaccharide chains varies amongst the eleven-meningococcal immunotypes (Mandrell & Zollinger 1977). The immunotype associated with the majority of pathogenic meningococci is L3,7,9 (see fig 2.2). Importantly, the terminal galactose of the α–oligosaccharide can be reversibly sialylated in a number, but not all meningococcal LPS immunotypes, the consequence of which will be discussed in detail later.

The lipid A portion also varies between *E. coli* and meningococcal LPS in terms of nature and location of fatty acids and the substitution of various phosphate containing moieties attached to the backbone (Kulshin et al. 1992). The functional consequences of structural differences in lipid A moiety, in terms of

endotoxin activities, are currently not well understood. Nonetheless, such differences may be important, especially in terms of interactions with host LPS recognition receptors.

3.1.4. Activation of endothelium by *N. meningitidis*

Widespread vascular endothelial injury is a characteristic feature of meningococcal sepsis (Mercier et al. 1988). As mentioned in the introduction, cutaneous lesions contain large numbers of organisms that are associated with both endothelium and infiltrating neutrophils (Hill 1947). Since meningococci have the capacity to bind endothelial cells in a receptor-ligand specific fashion (Virji et al. 1992a;Virji et al. 1993a), bacterial-endothelial contact may itself be critical in mediating the vascular injury seen in this disease (Sotto et al. 1976). There is limited information on either endothelial activation in general and endothelial adhesion molecule induction specifically in response to live meningococci *in vitro*. Indirect evidence for the effect of induction of cell adhesion molecule expression by meningococci can be seen from studies that investigated how *N. meningitidis* B1940, a clinical isolate, and isogenic mutants with defined structural differences, mediate injury to cultured endothelial cells.

Klein et al (1996) demonstrated that the isogenic mutants differed in their propensity to adhere to cultured vascular endothelial cells. Mutants that were encapsulated but possessed a truncated LPS (*cpsD-*) were more adherent to vascular endothelium, whereas unencapsulated mutant with normal LPS (*siaD-*) was more adherent still. The unencapsulated mutant with a truncated LPS (*cps-*) was the most adherent. Preincubation of HUVEC with the unencapsulated mutants resulted in greater numbers of neutrophils adhering to endothelium resulting in greater damage, as measured by degradation of the heparan sulphate, than the less adherent parent strain (Klein et al. 1996). However, despite the large

differences in bacterial adherence to endothelium, no damage was observed in the absence of neutrophils. Moreover, in addition to the difference in adhesion to endothelial cells, the unencapsulated mutants also caused more shedding of CD62L and increase in CD11b/CD18 on neutrophils than the parent strain. As mentioned in the introduction, an important component of the coagulation cascade, tissue factor, is up regulated to a greater extent by the unencapsulated *siaD*- mutant of B1940 than by the parent strain (Heyderman et al. 1997). This difference was most pronounced when organisms were exposed to the endothelium for short time periods.

This chapter describes a set of studies designed to investigate how bacterial structure affects the expression of the vascular cell adhesion molecules on cultured human umbilical endothelial cells. Binding of bacteria to vascular cells takes place under conditions of flow *in vivo*. In order to simulate these *in vitro*, non-adherent bacteria were removed by washing HUVEC after exposure to either purified LPS or organisms. These experiments use isogenic mutants of group B meningococcus B1940, that vary in expression of both capsule and lipopolysaccharide structure. The bacterial strains, and their structural mutations, are described fully in Material and Methods section.

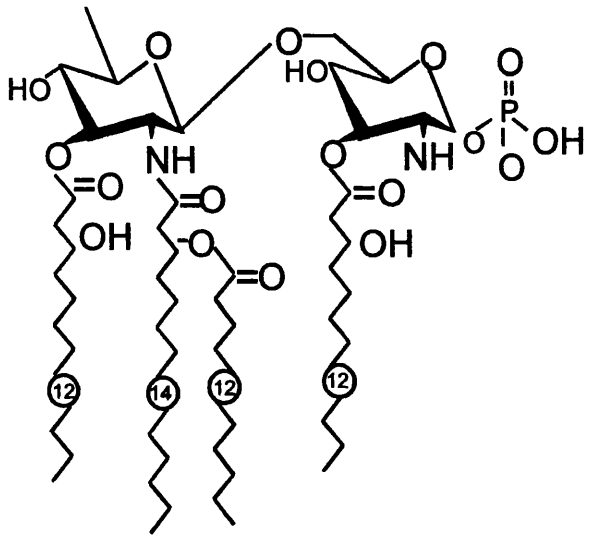

Figure 3.1 Chemical structure of Meningococcal Lipid A.

Lipid A is covalently linked to a two KDO and the oligosaccharide chain depicted in Figure 2.2. Removal of acyl groups from lipid A backbone completely abolishes endotoxin activity.

3.2 Methods

3.2.1. Preparation of organisms.

The growth and preparation of the organisms used in this set of experiments and the phenotypic characteristics of the organisms is described in detail in Chapter 2.

3.2.2. Culture of HUVEC.

The condition for the isolation, culture and maintenance of HUVEC has been described in Chapter 2. In general, 24 well tissue culture plates were used, each containing 1ml of culture media. Care was taken to use good quality endothelial cells with absence of contaminating smooth muscle cells or leukocytes. HUVEC were used 2 to 7 days after achieving confluence. Medium was changed to RPMI 1640 with 10mM HEPES and 20% FCS at least 24 hours prior to experimentation. This final medium contained no antibiotics when live organisms were being used.

3.2.3 Stimulation of HUVEC by LPS or *N. meningitidis*

Initial experiments with organisms used either heat killed (60^0 C for 1 hour) or fixed in 1% formaldehyde. In later experiments, the fixation method preferred was with 0.5% paraformaldehyde. This was added to suspensions of organisms, which were then washed three times in fresh RPMI 1640 medium. Experiments using live organisms were conducted within a category I safety cabinet within a category 3 facility.

In experiments investigating the effect of duration of exposure of either LPS or bacteria, medium was removed from tissue culture wells by sterile Pasteur pipette after a specified time period, and each well was washed in fresh medium three times. In order to avoid discrepancies between the first and last well tested, medium was removed from all wells quickly, and washing steps performed in the same order.

3.2.4 Assays for detection of cell adhesion molecules by flow cytometry.

The protocol for flow cytometric detection of cell adhesion molecule expression of HUVEC is given in detail in Chapter 2. Flow cytometry was performed on a FACSCalibur flow cytometer using CellQuest software. The instrument settings are given in Appendix I. 5000 events were collected within a gate corresponding to size and granularity characteristics of endothelial cells, and bright staining with CD31.

3.2.5 Data representation and Statistical analyses.

All experiments were performed at least three times. In general, data is represented as median fluorescence intensity. Differences in cell adhesion molecule expression induced by different strains of bacteria after brief exposure of organisms to HUVEC was analysed by Kruskall-Wallis H test.

3.3 Results

3.3.1. Flow cytometric analysis of CD62E, VCAM-1 and ICAM-1 expression on HUVEC

After 5 hours incubation with purified *E coli* 0111:B4 LPS, an increase in CD62E, VCAM-1 and ICAM-1 staining could be detected. Figure 3.2 shows a typical cell adhesion molecule profile detected in the gated endothelial window. After stimulation, CD62E was first detected by 1 to 2 hours, and reached maximum expression between 4 to 6 hours. Expression had decreased by 12 to 18 hours and virtually disappeared by 24 hours. VCAM-1 expression could be detected at 4 hours, increased at 6 hours, was still increasing at 12 hours and remained high after 24 hours incubation. An increase in ICAM-1 expression could be detected after 4 hours incubation, and was still increasing at 12 hours and remained high at 24 hours. The pattern and kinetics of cell adhesion molecule expression in response to heat killed, or paraformaldehyde fixed meningococci was similar to that seen with purified LPS (data not shown).

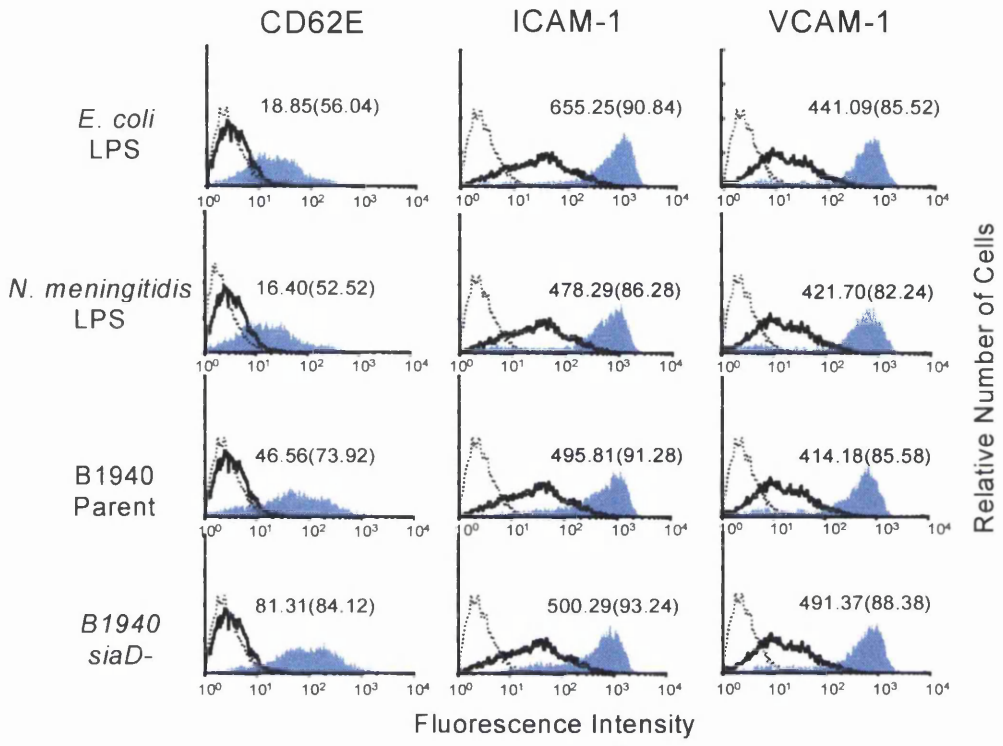

Figure 3.2 Flow cytometric profiles of cell adhesion molecule expression in response to purified LPS and *N. meningitidis* B1940.

HUVEC were stimulated with 100ng/ml of *E. coli* 0111:B4 and meningococcal H44/76 LPS and 10^7 cfu/ml B1940 and *siaD*- strains. Shaded areas represent cell adhesion molecule staining in response to stimuli, solid line lines staining in unstimulated cells, and dotted lines staining with an irrelevant isotype matched control. Median fluorescence intensities, and percent positive events (bracketed) are given. Data presented is representative of three experiments yielding similar results.

3.3.2. Duration of exposure of LPS on expression of CD62E, ICAM-1 and VCAM-1

When 10ng/ml purified LPS was added to HUVEC which were then washed in fresh medium with 20% FCS at various time points, and incubated for a further 4 to 6 hours, the pattern of cell adhesion molecule expression shown in Figure 3.3 was observed. After 15 minutes exposure to HUVEC, LPS induced small increases in the expression of all cell adhesion molecules measured. Increasing the length of exposure resulted in an incremental increase in cell

adhesion molecule expression. The maximum expression for all three cell adhesion molecules was detected after continuous exposure to LPS (fig 3-3).

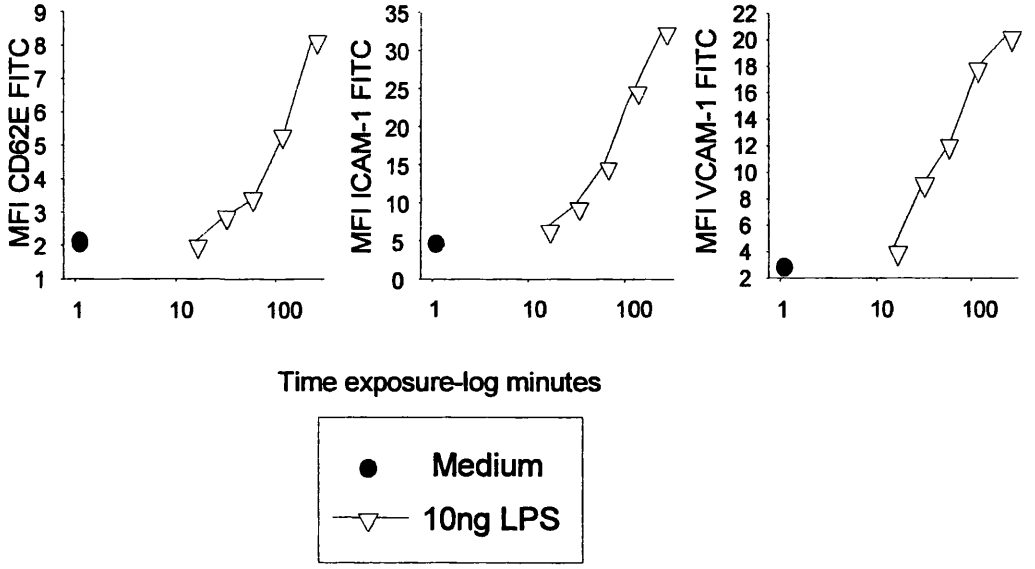

Figure 3.3 Effect of duration of exposure of LPS to HUVEC on cell adhesion molecule induction.

HUVEC were exposed to 10ng/ml of *E. coli* 0111:B4 LPS for various lengths of time prior to washing cells in fresh medium. HUVEC were then incubated for a further 5 hours and cell adhesion molecule expression determined by flow cytometry. Filled circle represents expression in unstimulated cells, empty triangles in LPS stimulated cells. Data is representative of three separate experiments.

3.3.3. Duration exposure of LPS and *N. meningitidis* on expression of CD62E, ICAM-1 on HUVEC

When the unencapsulated *siaD*- mutant was compared to purified LPS, the duration of exposure required to induce CD62E, ICAM-1 expression was markedly reduced (Fig 3-4). As little as 5 minutes exposure of the *siaD*- organism was sufficient to induce an increase in expression in both CD62E and ICAM-1, although the extent of the increase induced at these very brief time points varied between experiments. However, the pattern seen in response to LPS over these time points was very similar to that seen in fig 3-3, insofar as continuous exposure resulted in maximum expression of both cell adhesion molecules. This was not observed in response to the *siaD*- organism. The maximum expression of either

CD62E or ICAM-1 was not always at the longest time exposure. This varied according to experiment and was dependent on the cell adhesion molecule measured. For example, the maximum CD62E expression seen in response to *siaD*- organism varied between 60 and 120 minutes or continuous exposure (fig 3-4.). Taken together, when the response of duration of exposure to LPS and *siaD*- organism was compared, the bacteria induced greater levels of both cell adhesion molecules at short time exposures (5 to 180 min) with the most marked differences after 15 to 120 minutes' exposure. Additionally, maximum expression of CD62E induced by the bacteria was always greater than that induced by LPS alone. In contrast, the maximal levels achieved were similar for ICAM-1 expression.

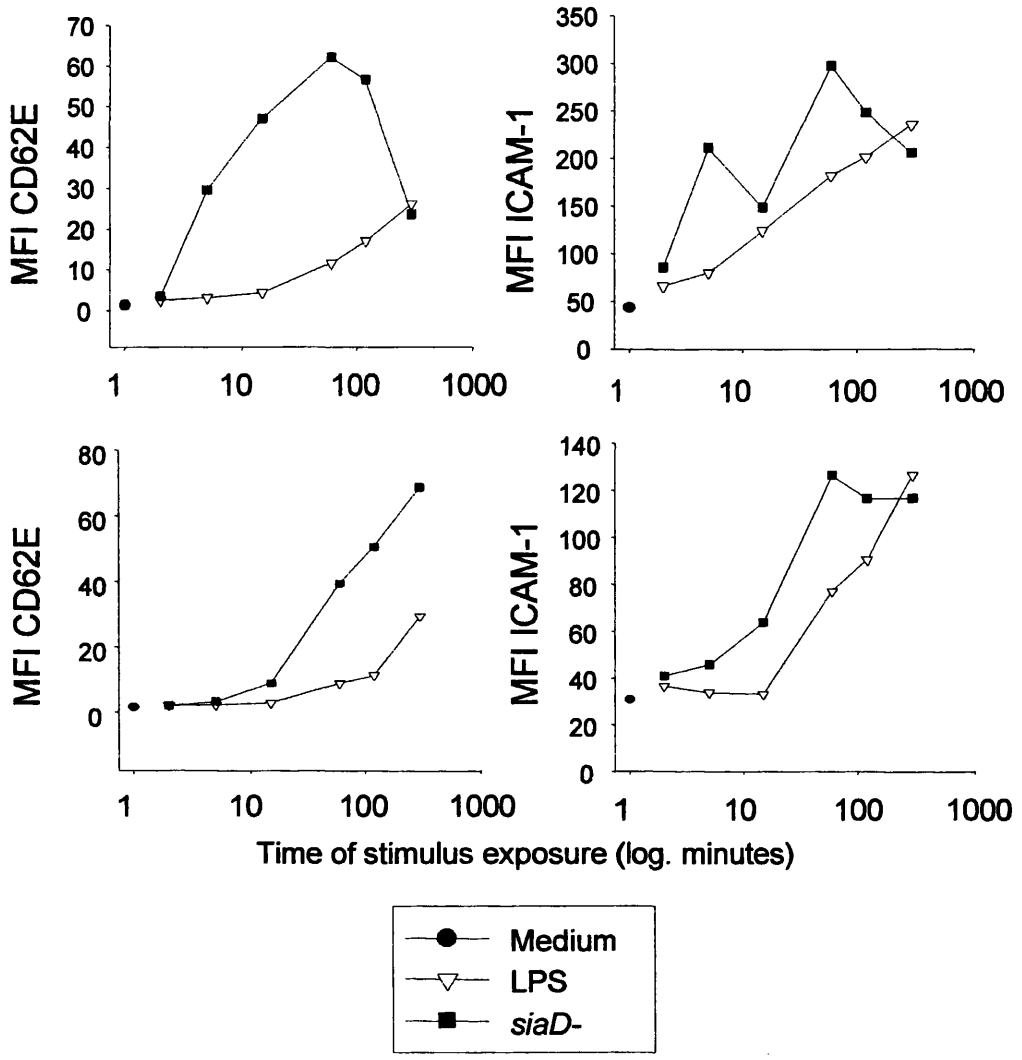

Figure 3.4 Effect of duration of exposure to purified LPS and *N. meningitidis* B1940 *siaD-* strain on expression of CD62E and ICAM-1 on HUVEC.

10ng/ml of purified LPS or *N. meningitidis* B1940 *siaD-* mutant at 10^6 organisms/ml were added to HUVEC, washed in fresh medium at 5, 60, 120 and 240 minutes and incubated for a total of 5 hours prior to determination of CD62E and ICAM-1 expression by flow cytometry. Results here are from two experiments, conducted on different days and on HUVEC derived from different umbilical cords.

3.3.4. Effect of capsulation and lipopolysaccharide structure on induction of adhesion molecule expression on HUVEC by *N. meningitidis* B1940

Having established the effect of duration of exposure for one meningococcal strain, the parent and three isogenic mutants were compared.

HUVEC were exposed to parent strain, and isogenic mutants *cpsD-*, *siaD-* and *cps-* for 15 minutes prior to washing in fresh medium. A number of observations can be made from the results depicted in Fig 3-5. The capacity of the different mutants to modulate all three cell adhesion molecules at this time period are ranked in the following order; parent<*cpsD-*<*siaD-* <*cps-*. This relationship was statistically significant and true for all three adhesion molecules assayed. In addition, the mutants that were most effective in influencing cell adhesion molecule expression under these conditions were unencapsulated (*siaD-* and *cps-*), but possession of a truncated, non-sialylated LPS had an additional effect. Thirdly, that there was a difference in the pattern of expression induced by purified LPS compared to the organisms. In these experiments, the expression of cell adhesion molecules induced by LPS was after continual exposure. Hence, the organisms, particularly those that are unencapsulated, were markedly more effective inducers of CD62E expression even after brief exposure to endothelium compared to continual exposure to purified LPS. In contrast, LPS appeared to be at least as effective as the bacteria at inducing both ICAM-1 and VCAM-1 on HUVEC under these conditions.

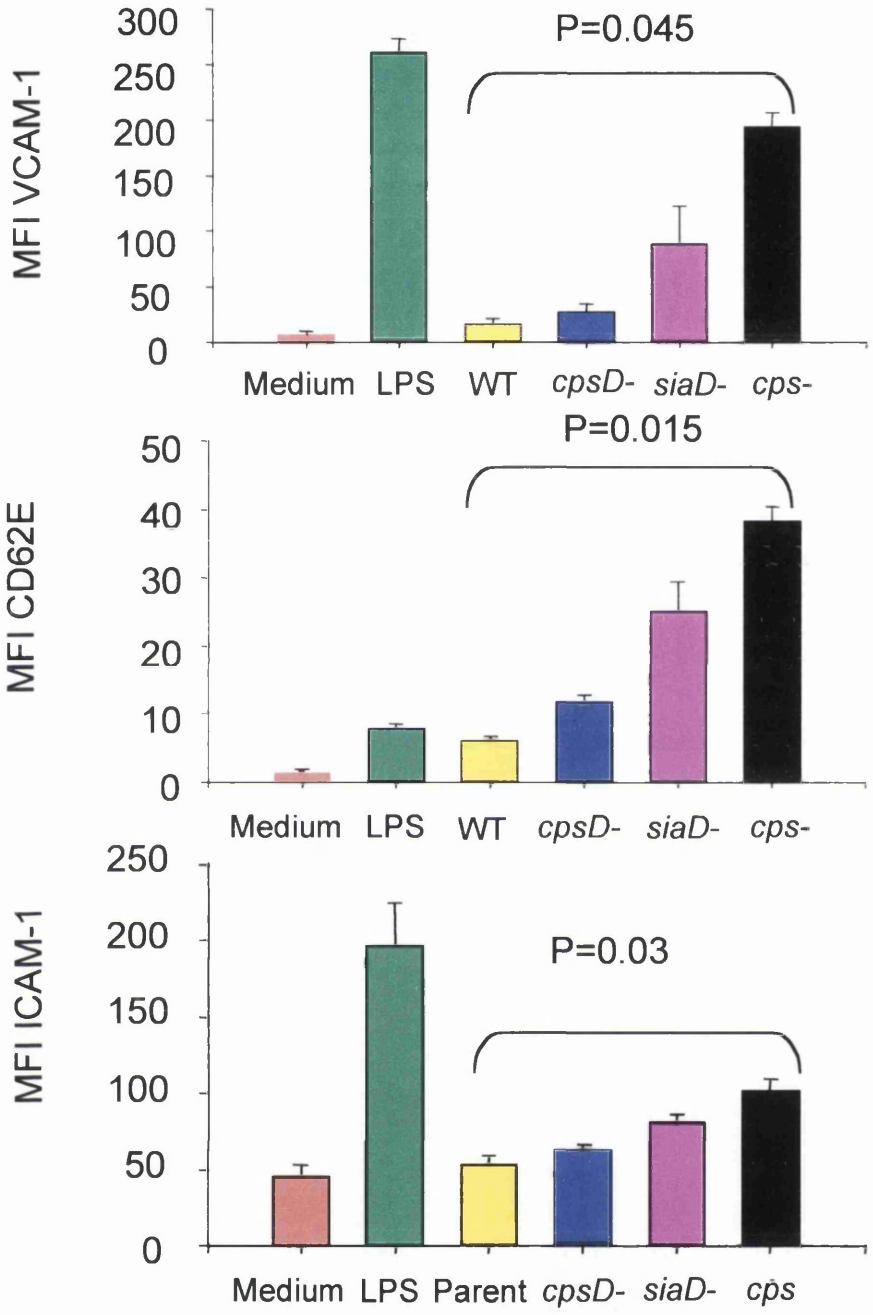

Figure 3.5. The effect of capsulation and LPS structure on capacity of *N. meningitidis* B1940 to induce CD62E, VCAM-1 and ICAM-1 expression after brief exposure to HUVEC.

HUVEC were exposed to 10^6 organisms/ml of *N. meningitidis* B1940 parent and *cps-*, *siaD-* and *cpsD-* mutants for 15 minutes, washed thoroughly in fresh medium and incubated for a total 5 hours prior to determination of cell adhesion molecule expression by flow cytometry. *E coli* LPS was added at 10ng/ml and incubated for 5 hours. Results here are expressed as mean and standard error of the mean. Capacity of parent and mutants to induce cell adhesion molecules was analysed by Kruskall-Wallis one way ANOVA.

Having established the pattern of cell adhesion molecule expression at 10^6 organisms/ml, the influence of a range of different bacterial concentrations was investigated. When a number of concentrations were to be evaluated, comparisons were only made between two or three of the bacterial strains, due to the technical difficulties of the experiment. Fig 3-6 shows that over a wide range of initial stimulating bacterial concentrations, the unencapsulated, *siaD-* mutant induced higher levels of CD62E and VCAM-1 than the parent organism. Hence the largest differences in induction of adhesion molecule expression observed between the two strains occurs over the 10^5 to 10^7 CFU's /ml. Fig 3.7 shows that over a range of initial stimulating concentrations, the same ranking order of CD62E and VCAM-1 expression was observed to that seen in Fig 3.6. In Fig 3.7 A and B, the parent, *cpsD-* and *siaD-* mutants were compared at 10^4 to 10^8 CFU's/ml and in Fig 3.7 C and D, all four strains were compared over10^5 to 10^7 CFU's /ml. Several observations can be made. The parent organism does not induce either CD62E or VCAM-1 expression after 15 minutes exposure unless the bacterial concentration is 10^7 CFU/ml. Unencapsulated mutants induced cell adhesion molecule expression at least a 2 log lower concentration than the parent strain. Additionally, the differences observed between the strains were less apparent at the highest stimulating dose. Although the unencapsulated mutants could induce cell adhesion molecule expression at low concentrations, the encapsulated *cpsD-* mutant, that has a truncated, non-sialylated LPS, always induced higher levels than the parent strain.

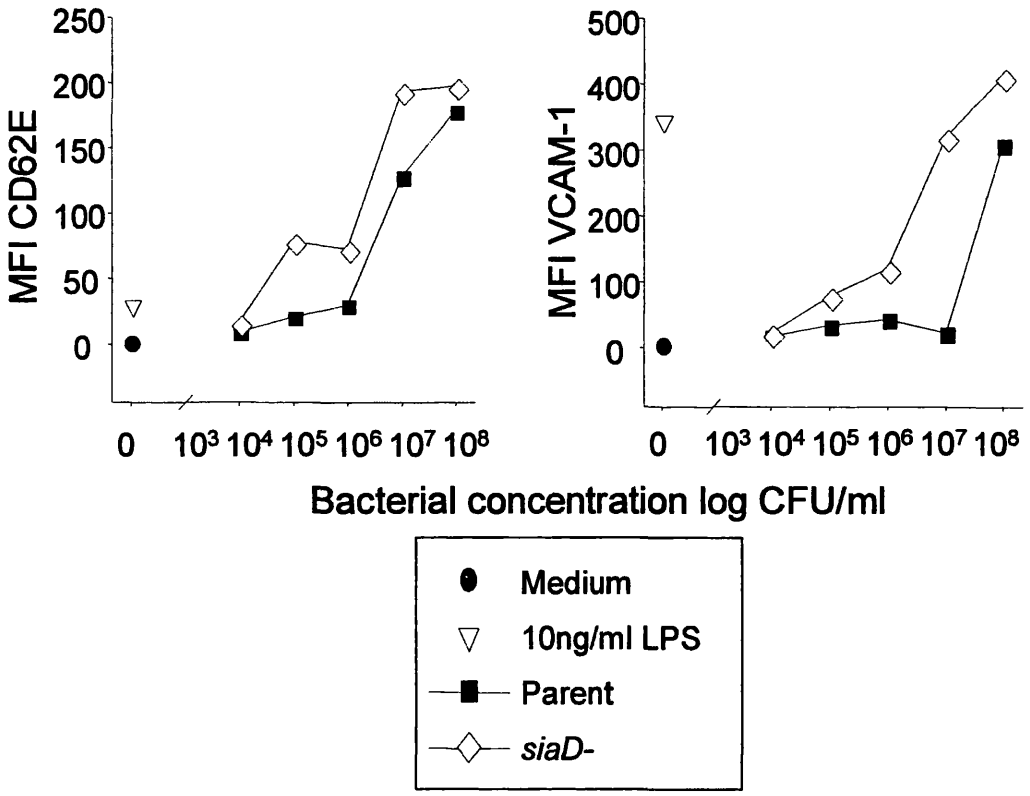

Figure 3.6. The effect bacterial concentration of B1940 parent and *siaD-* mutant on induction of CD62E and VCAM-1 expression after brief exposure times (15 minutes).

HUVEC were exposed to various concentrations of organisms, followed by washing in fresh medium and incubated for a further 5 hours prior to determination of cell adhesion molecule expression. LPS was continually exposed to HUVEC (open triangles). This is representative of 3 separate experiments which yielded similar results.

The effect of duration of exposure to meningococcal strains on HUVEC incubated over longer time periods was studied. Fig 3-8 shows the results when HUVEC were exposed to the strains for 15 minutes and incubated for a further 24 hours. Similar results were obtained to that seen at 5 hours in terms of ranking order. However, the increase in expression of ICAM-1 and VCAM-1 was only seen with all the mutants when the initial stimulating dose was at least 10^7 CFU/ml. No CD62E expression could be detected on HUVEC stimulated with either LPS or organisms after 24 hours incubation (data not shown).

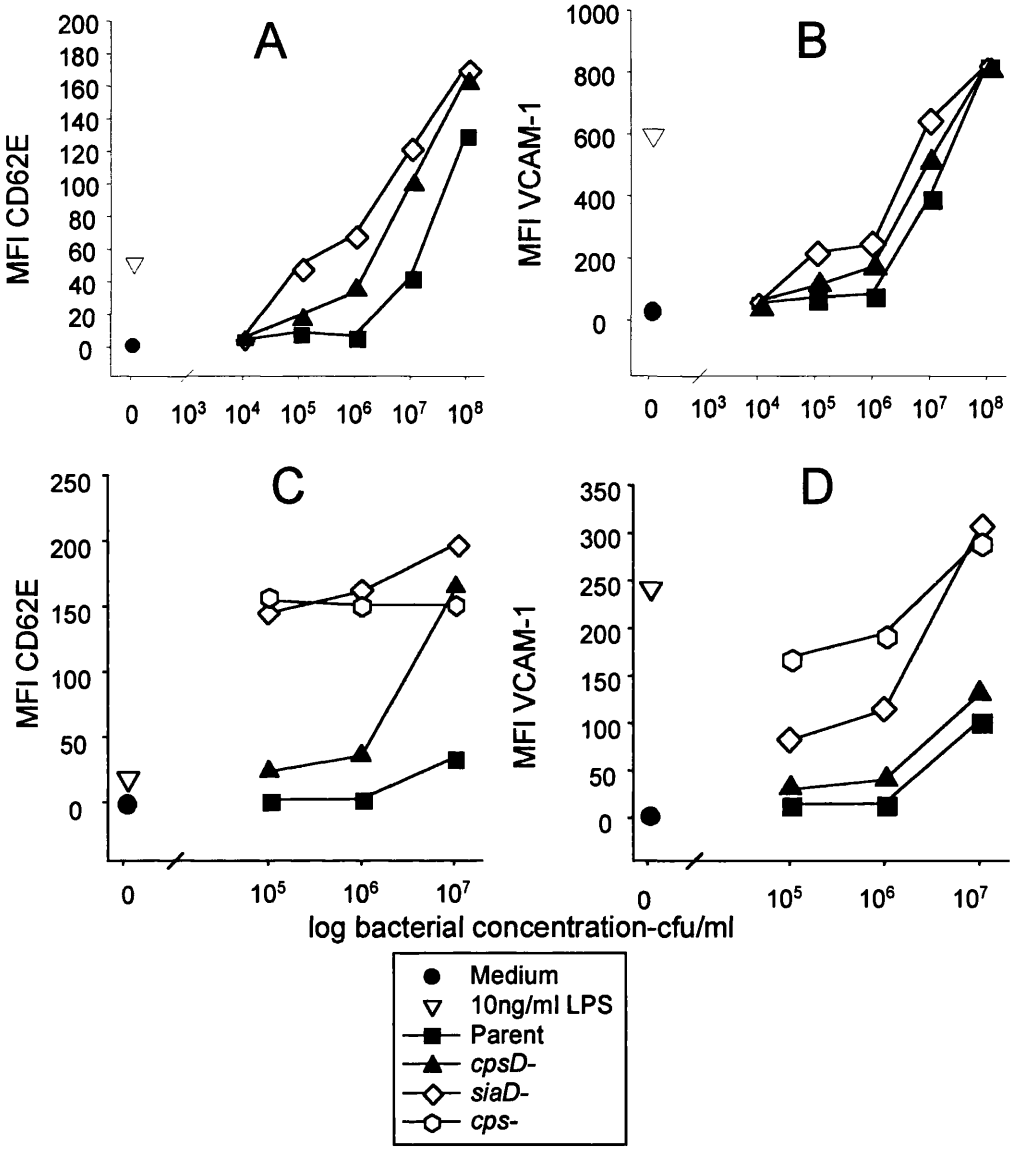

Figure 3.7. The effect bacterial concentration of B1940 parent and *siaD-*, *cps-* and *cpsD-* mutants on induction of CD62E and VCAM-1 expression after brief exposure times (15 minutes).

HUVEC were exposed to various concentrations of organisms, followed by washing in fresh medium and incubated for a further 5 hours prior to determination of cell adhesion molecule expression. LPS was continually exposed to HUVEC (open triangles). Graphs A and B, and graphs C and D are derived from separate experiments. Each experiment is representative of 3 separate experiments which yielded similar results.

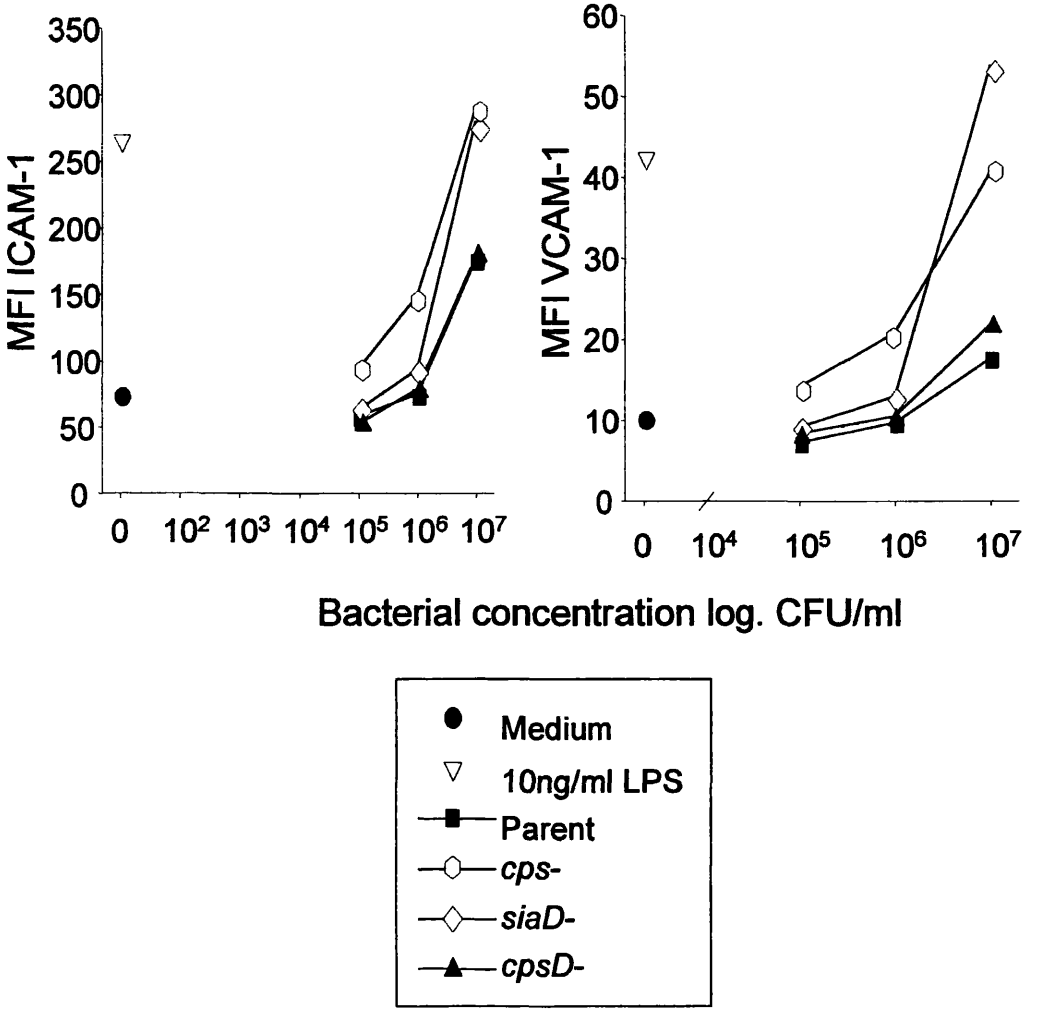

Figure 3.8. The effect bacterial concentration of B1940 parent and *siaD-*, *cps-* and *cpsD-* mutants on induction of ICAM-1 and VCAM-1 expression on HUVEC after brief exposure times (15 minutes), followed by incubation for 24 hours.

HUVEC were exposed to various concentrations of organisms, followed by washing in fresh medium and incubated for a further 24 hours prior to determination of cell adhesion molecule expression. LPS was continually exposed to HUVEC (open triangles). This is representative of 3 separate experiments which yielded similar results.

To explore further the effect of LPS sialylation and bacterial exposure to endothelium on the expression of cell adhesion molecules, the parent strain was compared to the unsialylated, encapsulated mutant *lst-*, the *siaD-* mutant and the *lst-/siaD-* mutant, which is both unsialylated and unencapsulated. There was little difference in the ability of the *lst-* mutant to induce cell adhesion molecule expression after 15-min duration compared to the parent. A small effect could be observed at 60-min duration (fig 3.9). However, the *lst-/siaD-* strain showed a

similar profile to that seen with the *cps-* mutant, in that it induce higher levels of both CD62E and VCAM-1 than the *siaD-* mutant. This indicates not only the importance of capsulation but also that the lack of terminal sialic acid can modulate endothelial cell adhesion molecule expression induce by the *lst-* mutant.

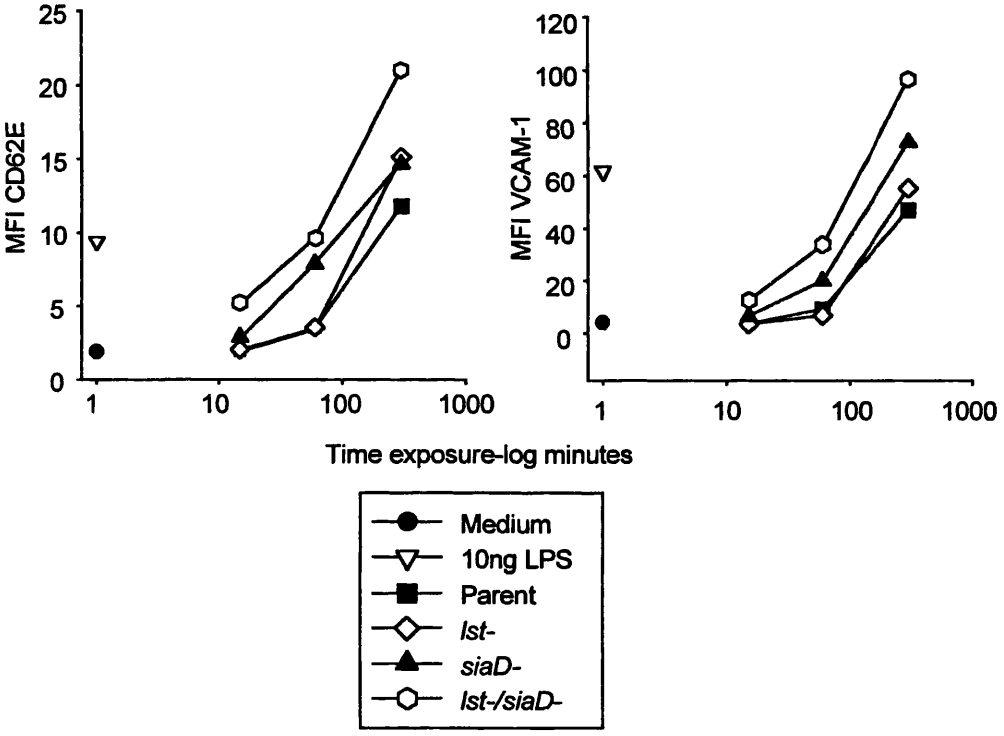

Figure 3.9. Effect of LPS sialylation and capsulation on capacity of *N-meningitidis* B1940 to induce cell adhesion molecule expression.

N. meningitidis B1940, *lst-*, *siaD-* and *lst-/siaD-* mutants at 10^6 organisms/ml were added to HUVEC, washed in fresh medium at 15, 60, and 240 minutes and incubated for a total of 5 hours prior to determination of CD62E and VCAM-1 expression by flow cytometry. Open triangles represent response to continual exposure to 10ng/ml of purified LPS.

Interestingly, similar results were observed when either heat-killed or paraformaldehyde fixed organisms were used in stead of live organisms. Fig 3.10 illustrates the pattern of three cell adhesion molecules in response to heat killed organisms that were exposed for 15 minutes to HUVEC, washed and incubated for a further 5 hours.

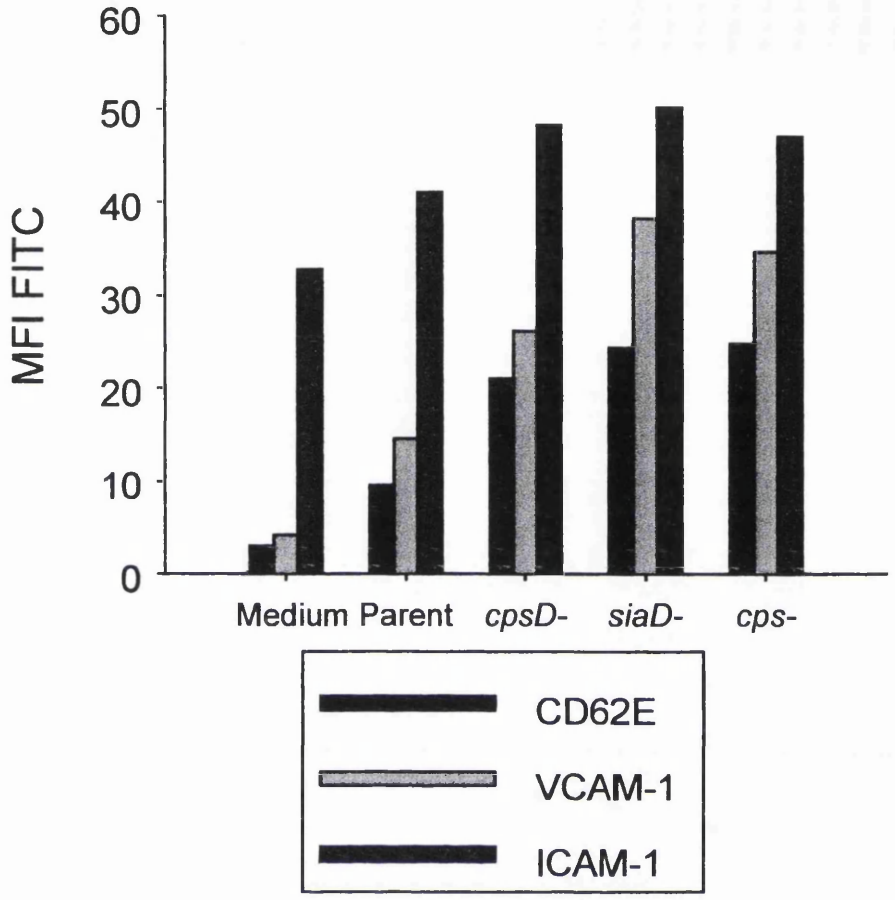

Figure 3.10. The effect of capsulation and LPS structure on capacity of heat killed *N. meningitidis* B1940 to induce CD62E, VCAM-1 and ICAM-1 expression after brief exposure to HUVEC.

HUVEC were exposed to 10^7 /cfu/ml of heat killed organisms, washed in fresh medium and incubated for further 5 hours prior to determination of cell adhesion molecule expression.

3.3.5. The patterns of vascular cell adhesion molecule expression in response to *N. meningitidis* B1940 and purified *E. coli* and meningococcal LPS

In all of the studies so far described, endothelial response to purified LPS utilised LPS purified from *E coli* 0111B: 4, since this preparation is available commercially, widely used and well standardised. There appeared to be some consistent and often striking differences in the pattern of endothelial molecule expression observed between purified LPS and the organisms. In order to

investigate whether this could be due to structural differences between *E. coli* and meningococcal LPS, the response of endothelium to both LPS species was performed. Meningococcal LPS appeared to be the more effective stimulus for all three cell adhesion molecules, especially at lower concentrations (0.1 to 10ng/ml) but that at the higher concentration (100ng/ml) they were equally effective (figure 3.11). Meningococcal LPS was no more effective than the *E coli* 0111B:4 LPS at inducing CD62E expression, and thus could not explain the greater capacity of the organisms to induce this cell adhesion molecule.

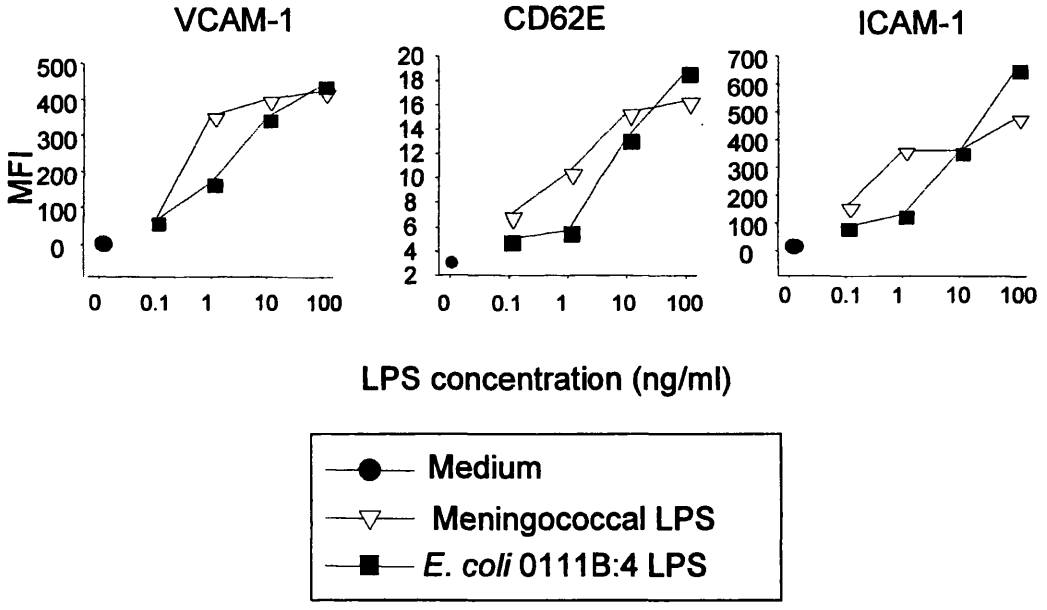

Figure 3.11. Expression of cell adhesion molecules on HUVEC in response to different concentrations of *E coli* 0111B:4 and meningococcal LPS.

HUVEC were stimulated with LPS at the indicated concentrations for 5 hours prior to determination of cell adhesion molecules. Results are representative of three experiments.

In addition, increasing LPS concentration to 1μg/ml from either species did not result in any further increase in cell adhesion molecule expression (data not shown). Under these experimental conditions, 100ng of LPS induced the maximal response from HUVEC.

Cell adhesion molecule expression in response to continual exposure of a range of meningococcal LPS and bacterial concentrations was then determined. Even at high doses of LPS (100ng/ml), CD62E expression was always lower than that seen with either the capsulated parent or unencapsulated *siaD*- strain at any of the concentrations tested. In contrast, the same dose of LPS induces a comparable level of expression to 10^6 to 10^7 CFU of organism for VCAM-1 expression and 10^7 to 10^8 CFU for ICAM-1 expression, consistent with the pattern seen in the previous results.

Comparison of the parent and the unencapsulated *siaD*- mutant over a range of concentrations revealed that when duration of exposure was not a factor, there were still some differences in the pattern of CD62E, ICAM-1 and VCAM-1 expression induced by these two strains. Dose of organism was an important factor. At high doses (10^7 to 10^8 CFU/ml) expression of both CD62E and VCAM-1 was greater in response to the unencapsulated mutant than the parent, whereas the profiles of ICAM-1 expression were remarkably similar, as shown in figure 3.12.

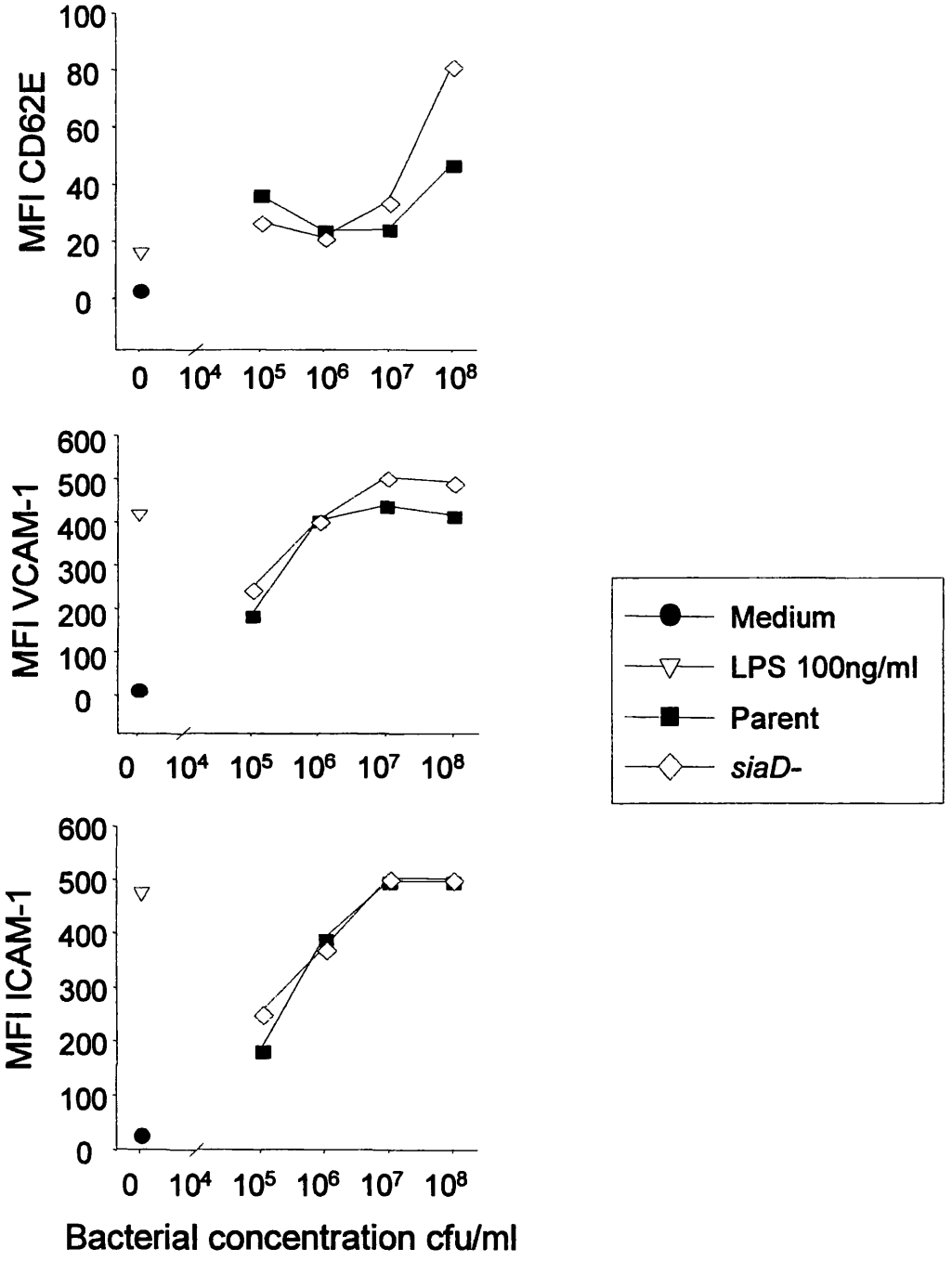

Figure 3.12. Expression of cell adhesion molecules on HUVEC in response to different concentrations of *N. meningitidis* B1940 parent and *siaD-* mutants

HUVEC were stimulated with meningococci at the indicated concentration for 5 hours and cell adhesion molecule expression determined by flow cytometry. Open triangles indicate level of expression in response to 100ng/ml of meningococcal LPS. This experiment was performed at least three times, of which this is figure is a representative.

3.4. Discussion

The rationale for this study started with the hypothesis that direct contact between meningococci and vascular endothelium is a primary initiator of inflammatory response that leads to the severe vascular damage seen in this condition. These results demonstrate that the group B meningococcus B1940 is a potent inducer of CD62E, ICAM-1 and VCAM-1 expression on HUVEC. Isogenic mutants derived from a single parent strain are useful for evaluating the effect that important structural variations such as encapsulation, LPS structure have on the activation of endothelial cells by meningococci.

These data show that both encapsulation and LPS structure are important meningococcal determinants of vascular cell adhesion molecule expression in HUVEC. Klein et al showed that ability of meningococci to adhere to vascular endothelium was also determined by these structural differences (Klein et al. 1996). It is interesting that the ranking order of adhesion with parent<*cpsD*-<*siaD*-<*cps*- is exactly the same as the ranking order found in this study for the adhesion molecules expression. Clearly, a reasonable explanation for this would be that the greater number of organisms bound to endothelial cells effectively provides a greater dose of bacteria (and hence inflammatory moieties such as LPS) to stimulate the HUVEC. In practical terms, what this implies is that under conditions of flow as occurs *in vivo,* the ability of meningococci to adhere to the endothelium could have important pathophsysiological consequences.

The results presented here suggest that brief exposure to as little as 10^5 CFU/ml of the unencapsulated mutants is sufficient to cause a large increase in expression of cell adhesion molecules. One could speculate that focal association of meningococci with endothelium might be a critical determinant of subsequent events. Up-regulation of cell adhesion molecules may support leukocyte adhesion,

activation and transmigration, most likely in the acute phase with neutrophils but also monocytes. Activated leukocytes would then lead to endothelial damage via degradation of GAGS (Klein et al. 1993;Klein et al. 1996), that in turn could affect ability of both inflammatory cells but also meningococci themselves to bind. In addition, increases in adhesion can lead to other changes, such as increase in tissue factor, which will alter the balance between pro and anti-thrombotic properties of vascular endothelium (Heyderman et al. 1997).

What could be the reasons for the observed differences in adhesion of meningococci to vascular endothelia *in vitro*? The isogenic mutants vary according to LPS structure and capsulation status. Previous studies have shown that encapsulation is the major determinant of meningococcal adhesion and internalisation to vascular endothelium *in vitro* (Klein et al. 1996;Virji et al. 1992a;Virji et al. 1993a). The class 5 OMP's, Opa and Opc probably are the main mediators of adhesion of acapsulated organisms to endothelium. It is also known that these interactions are inhibited in the presence of sialylated LPS, as are pilus-mediated interactions in the case of encapsulated bacteria (Virji et al. 1995). The *cpsD-* organism, which has a truncated, non-sialylated LPS, is both more adherent to endothelium (Klein et al. 1996) and induces greater expression of cell adhesion molecules as shown in the results from this study. This indicates that sialylation of LPS is the determinant of this effect. However, the *lst-* mutant, which lacks the ability to sialylate the terminal lacto-neo-tetraose, did not induce greater adhesion molecule expression than the parent strain at brief time exposures to endothelium. However, unencapsulated *lst-/siaD-* mutant induced greater cell adhesion molecule expression than the unencapsulated *siaD-* mutant. This suggests that terminal sialylation can modulate responses of vascular endothelium, especially in unencapsulated organisms.

The responses observed between the *cpsD-* and *lst-* strains are likely to be as result of their different LPS structures. The *cpsD-* mutant (*galE-*) lacks the complete lacto-neo-tetraose of the oligosaccharide chain whereas at the *lst-* lacks the enzyme that catalyses the addition of the terminal sialic acid to this structure. It is interesting to note that Mannose Binding Lectin (MBL) binds avidly to both the *cps-* and *cpsD-* mutants but not to sialylated parent or *siaD-* strains (Jack et al. 1998) but fails to bind to the *lst-* organism (D. Jack, personal communication, 1999). This indicates that host endothelial ligand interaction with carbohydrate motifs on meningococci could be determined by quite subtle alterations in LPS structure. In addition, interactions between other important meningococcal outer membrane structures, such as opacity proteins and pili will be modulated by such differences in LPS structure. Proving this would require detailed investigation of the three-dimensional interactions and steric mechanisms influencing meningococcal and endothelial ligand interactions.

The majority of the results presented in this chapter involved experiments using live meningococci in antibiotic free medium. The experiments were designed to explore the effect of the active process of host bacterial interactions occurring with living, intact bacteria. Killing organisms, either by heat inactivation, fixation with paraformaldehyde or the use of antibiotics may alter other bacterial properties in an unpredictable fashion. Nonetheless, it could be argued that in experiments using live meningococci, where there were greater numbers of bacteria adherent to endothelium, subsequent growth of microcolonies would amplify any effect seen due to greater numbers of bacteria and thus larger concentrations of LPS. However, the fact that a similar pattern of was observed with fixed organisms argues against this being the sole effect. Interestingly, there was less difference between the two unencapsulated mutants when using fixed

organisms. This could be an effect of the fixation process on the *cps-* mutant. Interestingly, it was often observed that after fixation, *cps-* bacteria to clump together making washing and resuspsension of this strain difficult.

Whilst the effective dose of LPS in bacteria in contact with the endothelium may be a critical determinant of the level of cell adhesion molecule expression in HUVEC, other findings from this study indicate that this is by no means the whole story. Two separate lines of evidence illustrate this. Firstly, that the pattern of cell adhesion molecule differs between purified LPS and meningococci and secondly, this pattern is also different between the strains themselves.

It was striking that in the vast majority of experiments, purified LPS appeared to be a much less potent stimulus for CD62E expression than meningococci. In contrast, for the same dose of LPS on the same sample of endothelial cells, LPS appeared an equally effective stimulus for VCAM-1 and ICAM-1 expression as meningococci. Direct comparison is difficult, because of the uncertainty of amount of LPS present in a given suspension of bacteria. There is evidence that approximately 10^5 CFU of *E. coli* contains 1ng of native LPS (Berry 1985). However, as has been stated in the introduction, it has been calculated that the LPS content of 10^7 meningococci is 8.6ng (M van Dueren, personal communication). In this case, the capacity of meningococci to induce expression of all three cell adhesion molecules is greater than an equivalent dose of meningococcal LPS, especially at the lower concentrations. However, this does alter conclusions drawn from the observation from dose response experiments that the maximal expression of VCAM-1 and ICAM-1 induced by either LPS or organisms was equivalent, whereas the CD62E response was different.

What is the explanation for the different dose responses between *E. coli* and meningococcal LPS? This is likely to be due to the fact that bioactive, lipid A component of a given concentration of purified LPS will vary greatly dependent on the molecular weight of species of LPS (S. Rune Andersen, personal communication). The molecular weight of *E coli* 0111B: 4 LPS is in the region of 10 to 20 kDA whilst that of L3, 7,9 LPS is around 3 to 4 kDA. The greater molecular weight of *E coli* 0111B:4 LPS is due to its large polysaccharide (including O antigen) component (Ulevitch & Tobias 1995) compared to the relatively small oligosaccharide component of meningococcal LPS (Pavliak et al. 1993). Hence, the amount of toxic lipid A in meningococcal LPS would be higher than that derived from *E coli.* Comparison of these two LPS preparations by dose response would support this view. Although molar ratios of lipid A may be an important difference between the two LPS species, polysaccharide structure may well play a role in the inflammatory response induced by purified LPS. There is evidence that polysaccharide chain length correlates with cytokine release in monocytes (Feist et al. 1989). Whether this is true for endothelial cells is unknown.

It was also interesting to note that the unencapsulated mutants appeared to cause a different profile of cell adhesion molecule expression on HUVEC than the encapsulated strains. In particular, levels of CD62E and to a lesser extent VCAM-1 induced by the *siaD-* mutant were greater than that observed in response the parent strain. In contrast, ICAM-1 profile looked strikingly similar between the two strains. The reasons why difference in bacterial structure should influence expression of some cell adhesion molecules and not others are not entirely clear. It is tempting to speculate that the cellular and molecular processes involved bacterial adhesion and invasion of endothelial cells, which can be affected by alterations in bacterial structure, also influence signal transduction pathways implicated in

control of expression of cell adhesion molecules. This will be addressed in chapter 5.

3.5 Concluding remarks

The results from this study clearly indicate that capsulation and LPS structure can affect the way meningococci interact with and activate human endothelial cells *in vitro*. This could have important consequences for the modulation of leukocyte influx into inflammatory sites. It also demonstrates that the profile of cell adhesion molecule expression induced by the major gram-negative inflammatory mediator LPS is quite different to that seen with organisms. Why do intact bacteria cause different patterns of endothelial adhesion molecule expression than that seen in response to purified LPS? One critical factor that may therefore explain the different capacities of purified LPS and meningococci to induce vascular adhesion molecule expression is the effect of bacterial components other than LPS. This will be addressed in the subsequent chapters.

Chapter Four

LPS dependent and independent endothelial activation by *N. meningitidis:*

4.1. Introduction

There is convincing evidence that bacterial endotoxin (lipopolysaccharide/LPS), plays a major role in the pathogenesis of meningococcal disease. Plasma levels of endotoxin correlate strongly with outcome (Brandtzaeg et al. 1989a). Patients with a fulminating course and persistent shock have high levels (>700ng/L) of circulating endotoxin as compared with patients with milder course and have a much poorer prognosis (Brandtzaeg et al. 1989a). It was assumed that much of the severe inflammatory response seen in meningococcal disease was due to the cascade of humoral and cellular inflammatory processes initiated by bacterial LPS, since it is the generally held view that the major inflammatory component of gram negative bacteria is LPS (Brandtzaeg 1995). Studies investigating the effects of LPS *in vitro,* in addition to experimental LPS induced sepsis, and from gram negative shock in humans supports this notion (reviewed in (Glauser et al. 1991a;Parrillo 1993)).

It is not surprising therefore that a great deal of attention has been given to develop methods to block the action of this most toxic of microbial products. Initial attempts using humanised mouse monoclonal antibodies against LPS in gram negative sepsis have been generally unsuccessful (Anon 1994;McCloskey et al. 1994;Ziegler et al. 1991). This failure was probably as a result of many complex reasons. For example, the HA-IA anti-LPS antibody used although able to bind and neutralise lipid A, is not effective in an *in vivo* model of LPS endotoxaemia (Marra et al. 1994). Additionally, there are some doubts about its efficacy *in vitro* (Helmerhorst, Maaskant, & Appelmelk 1998).

The existence of naturally occurring, cationic proteins, which have both antibacterial and anti-LPS properties, have been known for some time. Many of these are produced by polymorphonuclear leukocytes (Levy 1996). One such

protein is a 50-kDa molecule called Bactericidal/permeability increasing protein (BPI). BPI is a member of the same family of lipid binding proteins as LPS binding protein (LBP), which, as has been described, is critically involved in Lipopolysaccharide recognition and signalling (Elsbach 1998). BPI, in contrast to LBP, is antagonistic to LPS. The anti-LPS actions of BPI are considered to be due to its high affinity to lipid A, probably by competing for LBP (Gazzano et al. 1992;Marra et al. 1990). Additionally, BPI has anti-bacterial properties, due to binding and increasing the permeability of the bacterial cell membrane initially resulting in inhibition of growth followed by killing (Takahashi et al. 1995b). A recombinant, N-terminal fragment of BPI (rBPI) has been shown to kill gram negative bacteria and decrease TNF-α production induced by gram-negative bacteria or LPS in whole blood (Weiss et al. 1992). rBPI also binds to purified LPS and abrogates the degree of endothelial activation in terms of both IL-6 production and CD62E expression (Arditi et al. 1994). Interestingly, this same study demonstrated that monoclonal antibody HA-1A was not an effective inhibitor of LPS *in vitro*.

The evaluation of the contribution of non-LPS components in the induction of inflammatory response by meningococci would appear to be problematic for several reasons. LPS is an important structural component of gram-negative bacteria and although outer membrane components can be separated and purified by various methods (including LPS itself), this does not allow for their evaluation in the context of intact bacteria. In addition, since LPS is so potent an inflammatory mediator even at minute concentrations, contamination with LPS is a particular problem when assessing these components. In 1998, an LPS deficient meningococcal mutant was produced. This isogenic mutant of the group B meningococcus 44/76 lacks an essential enzyme *lpxA*-,

which is involved in the initial steps of lipid A biosynthesis (see Fig 4-1). Remarkably, this organism was found to be both viable and had the preserved structure of the parent, albeit with a reduced growth rate and smaller colonies on agar (Steeghs et al. 1998). Since the *lpxA-* mutant possesses the other outer membrane structures of the parent organism, it provides a unique tool with which to explore the non-LPS components of meningococci that may contribute to the host inflammatory response.

From the results presented in chapter 3 it was hypothesised that the differences between cell adhesion molecule expression induced by meningococci and LPS was due to non-LPS components in meningococci. In order to address this question, two approaches were used. Firstly was to examine the pattern of endothelial activation in response to the LPS deficient mutant, and compare it to the parent and purified LPS. Secondly, to attempt to inhibit LPS activity in organisms by use of anti-LPS protein, $rBPI_{21}$. This was doubly useful. rBPI has been used as adjunctive therapy in children with severe meningococcal disease (Giroir et al. 1997). However, there was relatively little information about its effect on endothelial activation in response to live meningococci *in vitro*.

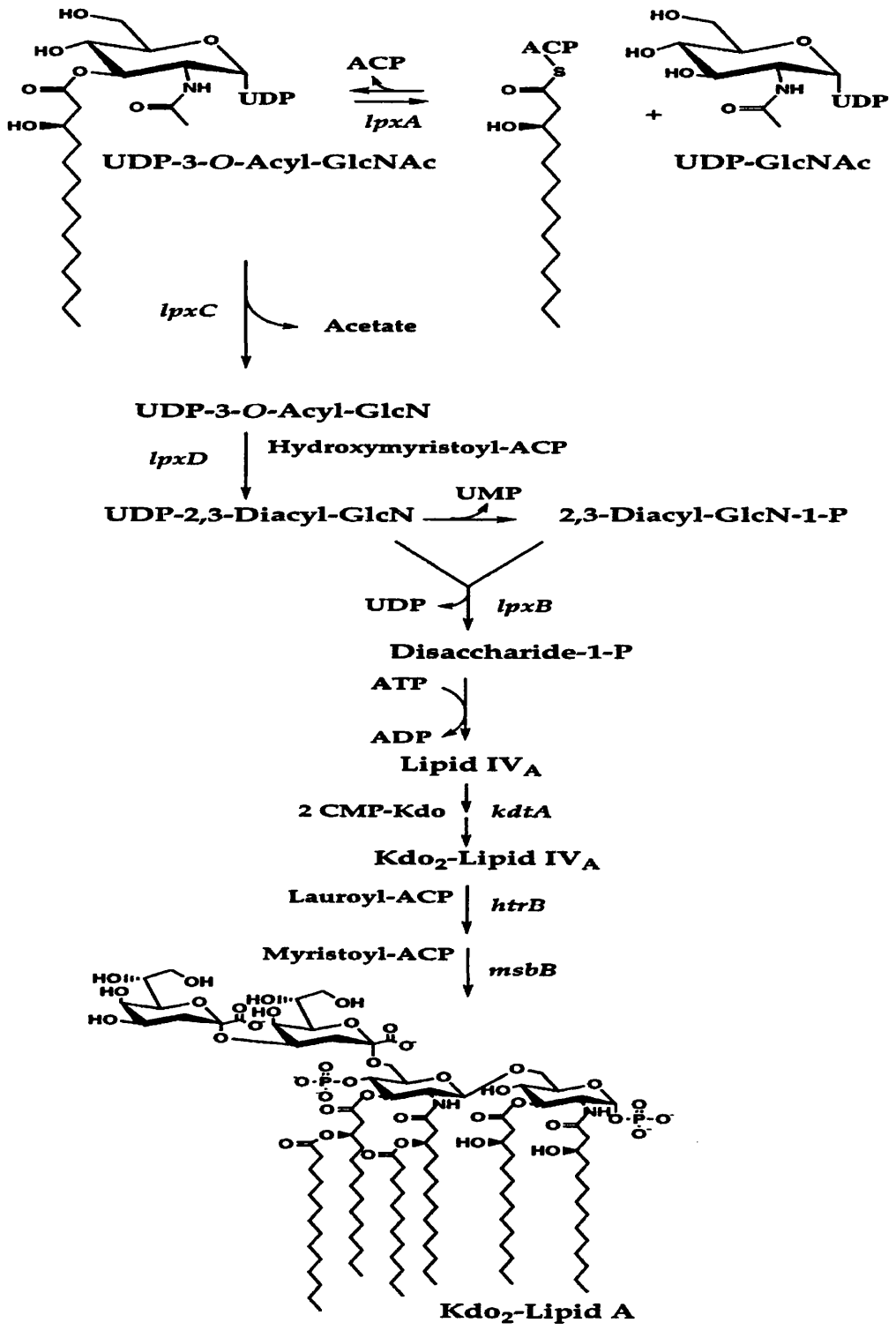

Figure 4.1. Biosynthetic pathway of *E. coli* LPS

The enzyme, *lpxA* is catalyses one of the initial committed steps in lipid A biosynthesis. The mutant of group B meningococcus, H44/76 has a deletion mutation of this gene, and thus produces no lipid A. Taken from (Odegaard et al. 1997).

4.2 Methods

4.2.1 Growth and preparation of organisms

The growth and preparation of the meningococci and isogenic mutants has been described in detail in chapter 2, section 2.6. As mentioned, the LPS deficient *lpxA*- mutant of H44/76 has a slower growth rate than the parent. Bacteria were only used in stationary phase (16 to 18 hours after subculture) Care was taken to keep the LPS deficient mutant free from contamination by culturing the bacteria on agar plates containing 100μg/ml kanamycin.

4.2.2 Materials

In addition to general materials used and described in Chapters 2, this set of experiments used the following two reagents.

Lipopolysaccharide from *N. meningitidis* serogroup B strain 44/76, was purified by Dr. Svein Andersen, Jenner Institute for Vaccine Research, Compton, Berkshire. Its preparation has been previously described (Andersen et al. 1996). Briefly, LPS was extracted by hot aqueous phenol extraction, ultra-centrifugation, gel filtration and cold ethanol/NaCl precipitation (Tsai et al. 1989) The final product contained <0.3% protein and was without detectable nucleic acids.

$rBPI_{21}$, a recombinant, modified amino-terminal fragment of bactericidal/permeability increasing factor, was a kind gift from Dr. Russ Dedrick, XOMA (US) LLC, Berkeley, California.

4.2.3 HUVEC culture

The isolation and culture of human umbilical vein cells has been described in detail in chapter 2, section 2.5.

4.2.4 Assays for effect of $rBPI_{21}$ on induction of cell adhesion molecules by purified LPS or *N. meningitidis*

The concentration of $rBPI_{21}$ used in these studies was between 5 and 20μg/ml. Initial experiments determined that 5μg/ml $rBPI_{21}$ was sufficient to completely inhibit the cell adhesion molecule induction on HUVEC in response to 10ng/ml of purified *E. coli* 0111:B4 LPS. This is in agreement with a previous study examining the inhibitory effects of rBPI on HUVEC IL-6 production (Arditi et al. 1994). In later experiments, 10μg/ml $rBPI_{21}$ was used when using LPS derived from meningococcal strain H44/76.

In most cases, $rBPI_{21}$ was added a few minutes prior to incubation with purified LPS or organisms. In experiments that investigated the effect of delaying the addition of $rBPI_{21}$ after incubation with meningococci, non-adherent organisms were removed after 15 minutes and then $rBPI_{21}$ added at the indicated time points.

Since $rBPI_{21}$ has both anti-LPS and bactericidal activities, some of the observed effects could be as a result of inhibiting of the growth of viable bacteria in addition to any LPS inhibition observed. In some experiments, paraformaldehyde fixed organisms was used to address this point.

4.2.5. Assays for detection of cell adhesion molecules by flow cytometry

The protocol for flow cytometric detection of cell adhesion molecule expression of HUVEC is given in detail in Chapter 2, sections 5. Flow cytometry was performed on a FACSCalibur flow cytometer using CellQuest software. The instrument settings are given in Appendix I. 5000 events were collected within gate

corresponding to size and granularity characteristics of endothelial cells, and bright staining with CD31.

4.2.6 Data representation and statistical analyses

All experiments of a single design were performed at least three times. In general, data is represented as median fluorescence intensity. However, in some experiments, both MFI and percentage positive increase in antibody staining is included where there were low levels of adhesion molecule expression.

4.3 Results

4.3.1 The effect of $rBPI_{21}$ on LPS induced expression of CD62E, ICAM-1 and VCAM-1 on HUVEC

When HUVEC were pre-incubated with 5 to 20μg/ml of $rBPI_{21}$ and then stimulated with 10ng of purified *E coli* LPS, expression of all three cell adhesion molecules were completely inhibited. $rBPI_{21}$ itself had no effect on unstimulated HUVEC (data not shown) and did not effect the response of HUVEC to TNF-α (Figure 4-2). This suggests that the effect observed was due to the specific action of $rBPI_{21}$ on LPS. Delaying the addition of $rBPI_{21}$ after addition of LPS resulted in partial inhibition of cell adhesion molecule induction.

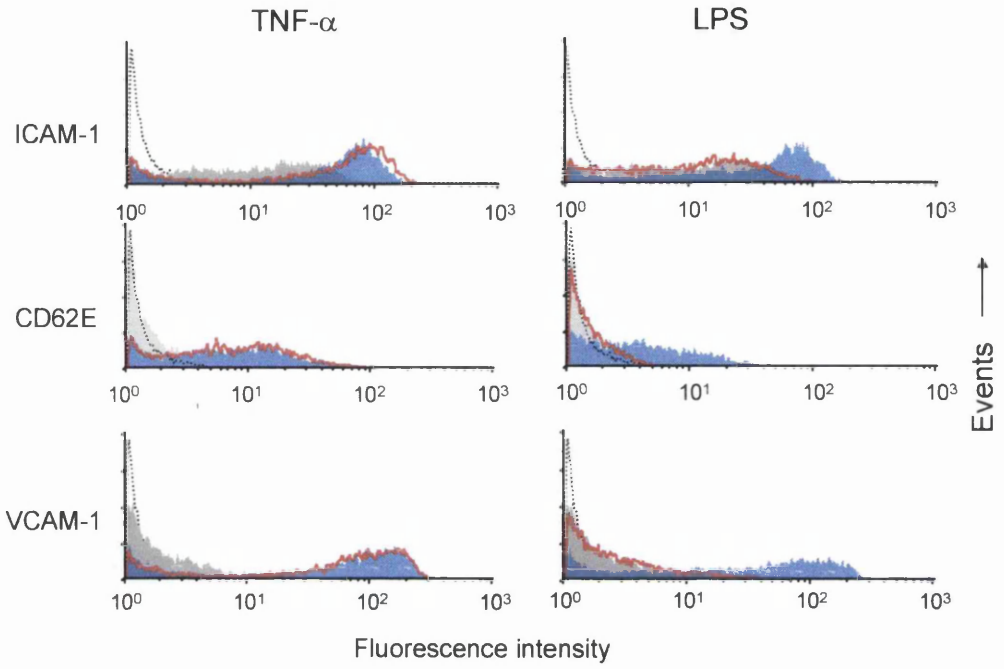

Figure 4.2. Effect of $rBPI_{21}$ on TNF-α and LPS induced cell adhesion molecule induction on HUVEC.

HUVEC were incubated with 10ng/ml of *E. coli* LPS or 10ng/ml TNF-α in presence and absence of 10μg/ml $rBPI_{21}$, incubated for a further 5 hours and ICAM-1, CD62E and VCAM-1 expression determined. Dotted line, isotype control staining; grey fill histogram, cell adhesion molecule expression in unstimulated cells; red line, expression in presence of $rBPI_{21}$; blue fill histogram, expression in presence of stimulus alone.

When HUVEC were pre-incubated with 10μg/ml $rBPI_{21}$, induction of CD62E and VCAM-1 in response to 100ng of *E. coli* LPS was inhibited. However, as Fig 4-3 demonstrates, this dose of $rBPI_{21}$ only partially inhibited 100ng of meningococcal LPS. However, the response to 10ng/ml of meningococcal LPS was almost completely inhibited by this dose of $rBPI_{21}$. Increasing the dose of $rBPI_{21}$ (up to 50μg/ml) resulted in a very small increase in inhibitory effect on 100ng/ml of meningococcal LPS (data not shown).

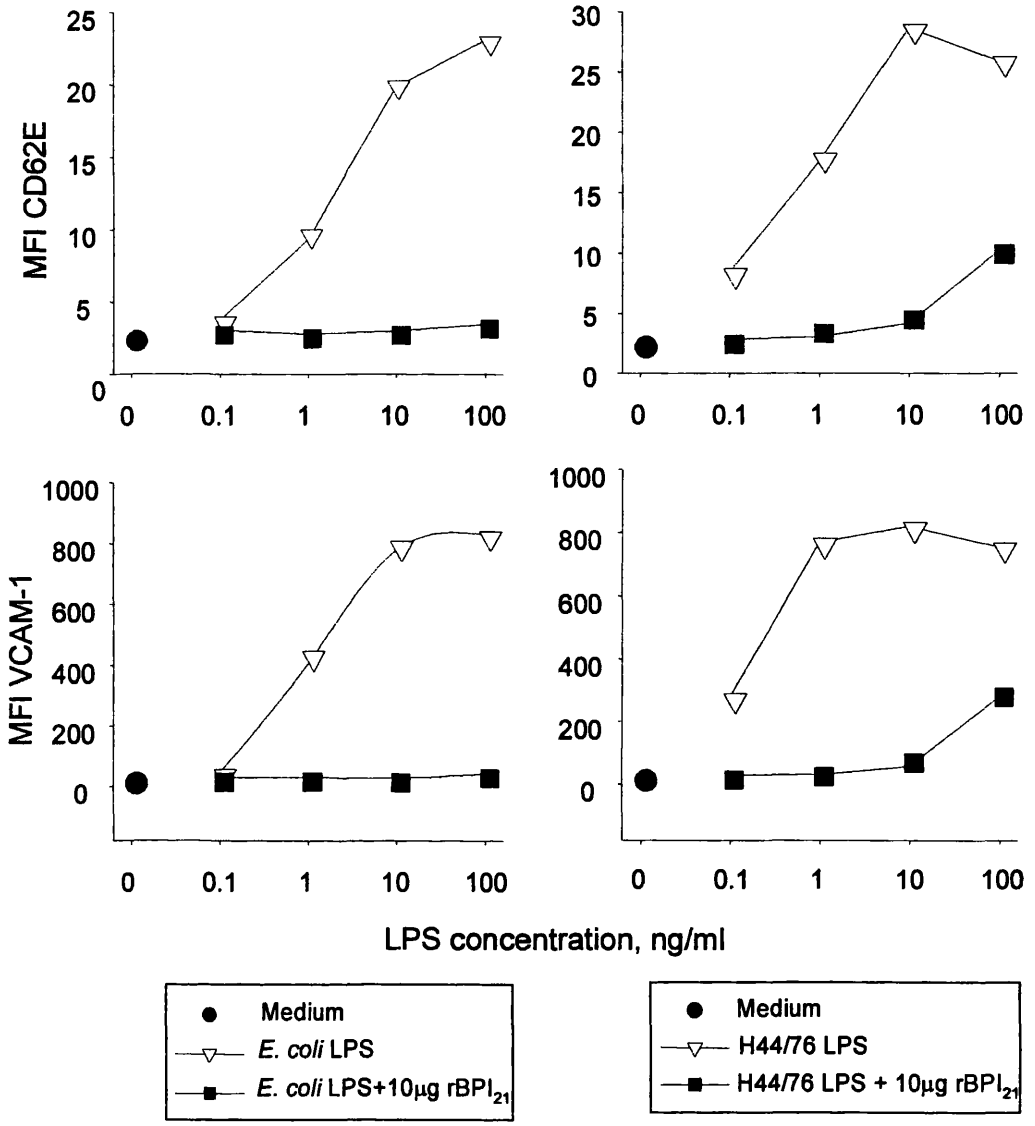

Figure 4.3. Effect of $rBPI_{21}$ on *E. coli* 0111B:4 and meningococcal LPS induction of cell adhesion molecule induction on HUVEC.

HUVEC were-incubated with various concentrations of *E. coli* and meningococcal LPS in the presence and absence of 10µg/ml $rBPI_{21}$ incubated for 5 hours and CD62E and VCAM-1 expression determined by flow cytometry

4.3.2. The effect of $rBPI_{21}$ on cell adhesion molecule induction on HUVEC in response to *N. meningitidis* B1940

HUVEC were incubated with 5 to 10µg/ml of $rBPI_{21}$ prior to the addition of the organisms. The results shown in chapter 3 suggested that the pattern of cell adhesion molecule expression be influenced by bacterial structure. For this reason,

the capsulated parent organism was compared to the unencapsulated *siaD*- mutant over a range of bacterial concentrations.

When 10μg/ml $rBPI_{21}$ was added to either the parent B1940 or *siaD*- mutant at a concentration of 10^4 organisms/ml, there was a marked reduction in CD62E expression. However, at a concentration of 10^5 organisms/ml, inhibition was between 20 and 50%. The effect observed when 10^6 organisms/ml were used was variable. There was either no inhibition, or occasionally an enhancement of cell adhesion molecule expression in the presence of $rBPI_{21}$, as shown in Fig 4-4. This was in marked contrast to the inhibitory effect of $rBPI_{21}$ on purified LPS as also illustrated in Fig 4-4. It is noteworthy that the same differential expression of cell adhesion molecule expression in response to the organisms as compared to purified LPS (Chapter 3) can be observed. Similar results were observed with VCAM-1 (Fig4.5).

Another feature of this set of experiments was that the inhibitory effect of $rBPI_{21}$ on either the parent or *siaD*- mutant was generally equivalent. Even if the *siaD*- mutant was the more potent inducer of CD62E or VCAM-1 expression, the degree of inhibition seen was similar. This indicated, at least under these experimental conditions, that the presence of capsule did not influence the ability of $rBPI_{21}$ to inhibit cell adhesion molecule induction by meningococci

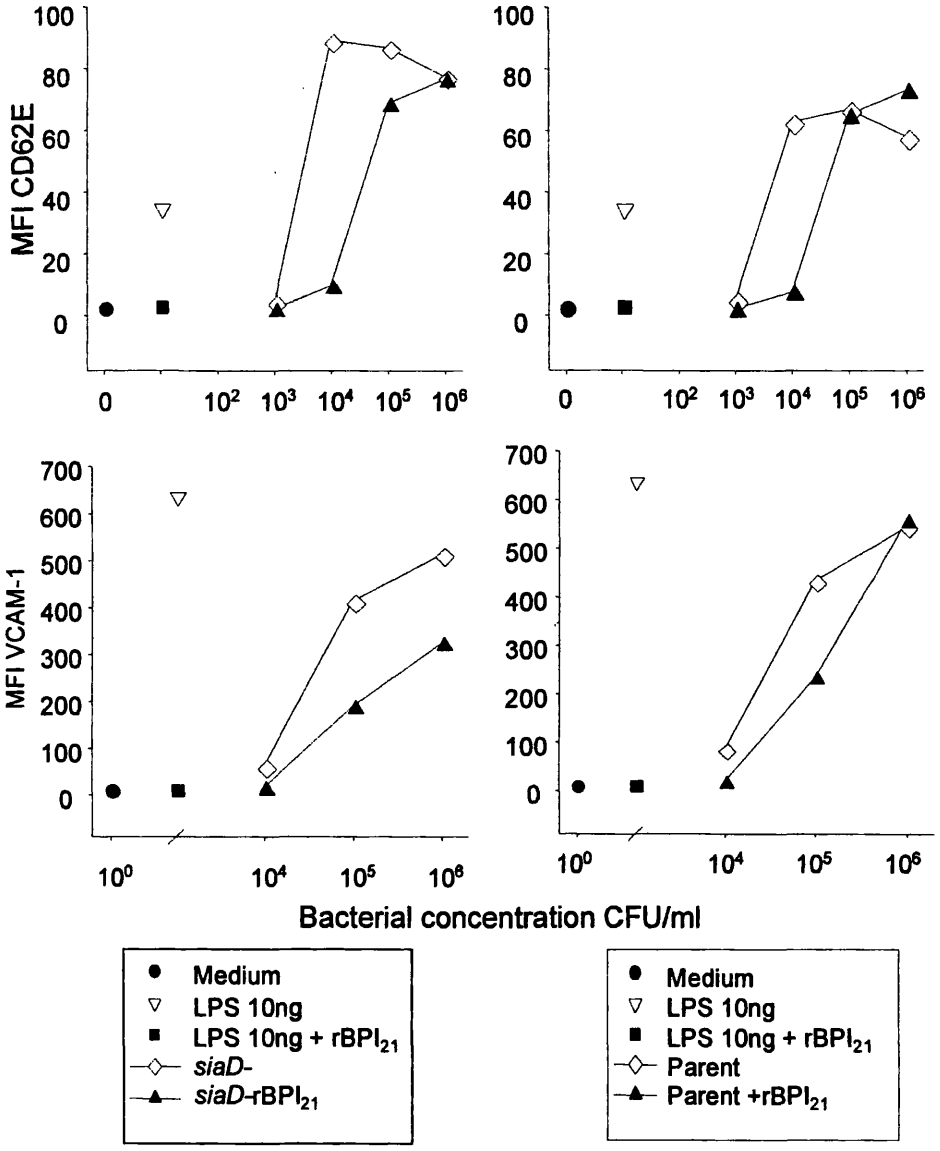

Figure 4.4. Effect of $rBPI_{21}$ on induction of CD62E and VCAM-1 expression by *N. meningitidis* B1940 parent and *siaD*- strains.

HUVEC were incubated with various concentrations of organisms in the presence or absence of 10μg/ml of $rBPI_{21}$ and incubated for 5 hours prior to determination of CD62E expression. Data shown is representative of 3 experiments which yielded similar results. CD62E and VCAM-1 data are extracted from different experiments. Note complete inhibition of bothCD62E and VCAM-1 expression induction by purified LPS in the presence of $rBPI_{21}$.

4.3.3. LPS structure and capsulation influence the inhibitory effect of $rBPI_{21}$ on the induction of HUVEC adhesion molecule expression by meningococci

The role that bacterial structure may play on the efficacy of $rBPI_{21}$ to inhibit endothelial cell adhesion molecule induction in response to meningococci was further explored. The previous experiments identified that a dose of 10μg/ml could inhibit the induction of both CD62E and VCAM-1 in response to 10^5 bacteria/ml of both parent and *siaD-* strains by between 20 and 50%. For this reason, this concentration was used to compare the parent, *cps-*, *cpsD-* and *siaD-* mutants.

In agreement with previous results, the induction of CD62E expression on HUVEC in response to 10ng/ml LPS was completely inhibited by $rBPI_{21}$. Fig 4-5 shows that induction of CD62E in response to both the parent and *siaD-* mutant was reduced by approximately 50%. In contrast, the response to the *cps-* and *cpsD-* mutants was reduced by 80 to 90%. It seemed therefore that the increased efficacy of $rBPI_{21}$ on these meningococcal strains was dependent on the structure of LPS, and not the presence of capsule. Both these mutants possess a truncated, non-sialylated LPS. It is possible that the effect observed was due to either the absence of sialic acid, the absence of other sugar residues or both. In order to test this further, separate experiments comparing the parent to the unsialylated *lst-* mutant were performed. Figure 4-5 B demonstrates that the degree of inhibition of CD62E expression in response to the *lst-* mutant is greater than its sialylated parent strain, at least at the concentration of organisms and $rBPI_{21}$ used in these experiments.

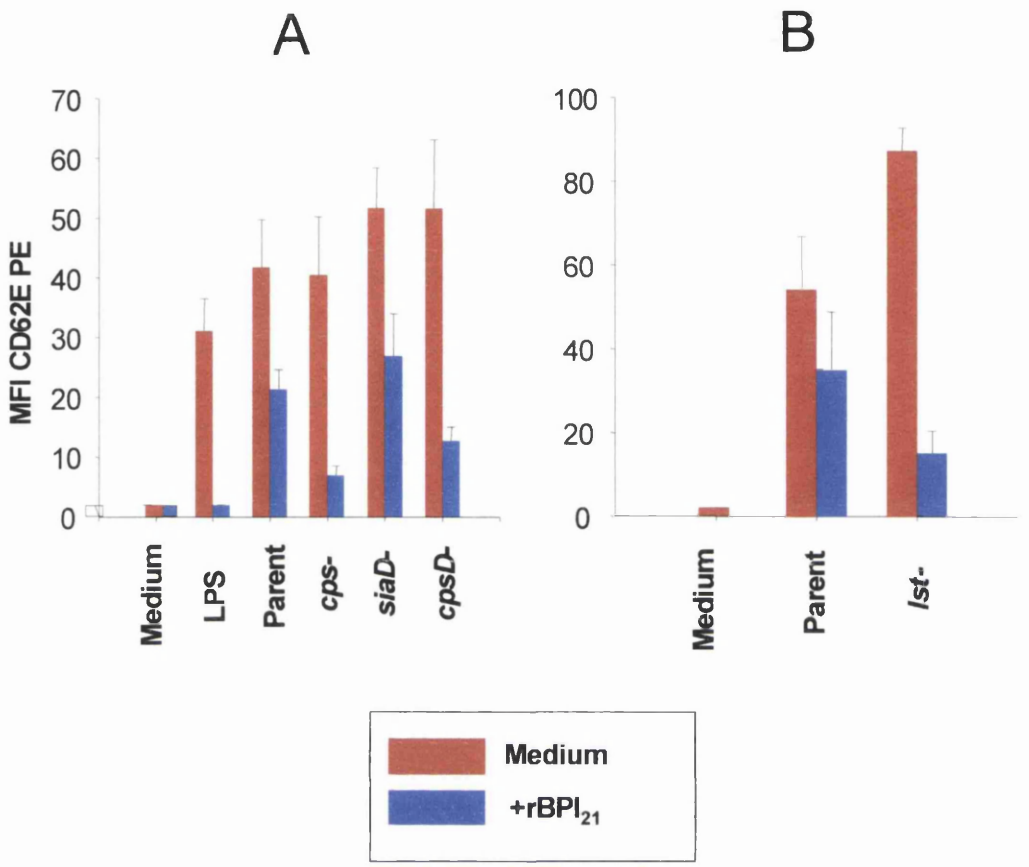

Figure 4.5. Differential inhibitory effect of $rBPI_{21}$ on *N. meningitidis* B1940 and isogenic mutants induction of CD62E on HUVEC

HUVEC were stimulated with 10^5 CFU/ml of B1940 parent, *cps-*, *siaD-*, and *cpsD-* (A) or parent and *lst-* in the presence of 10μg/ml $rBPI_{21}$. CD62E expression was determined after 5 hours incubation. Data is expressed as mean ±SEM, from 3 separate experiments, conducted on different days, using HUVEC pooled from several umbilical cords.

Delaying the addition of $rBPI_{21}$ by 15 minutes after stimulation resulted in reduced level of inhibition of cell adhesion molecule induction (Fig 4-6). Interestingly, the level of inhibition observed in response to all the stimuli was very similar when either 15 or 60 minutes delayed addition of $rBPI_{21}$. When $rBPI_{21}$ was delayed for 60 minutes, CD62E expression was inhibited by approximately 50% of that seen in the absence of rBPI in response to any stimuli. Even after 2 hours delay, some inhibition was observed. Interestingly, if administration of rBPI was delayed for 60 minutes after continual exposure to LPS, the degree of inhibition of ICAM-1 induction was less than that seen with CD62E (data not shown).

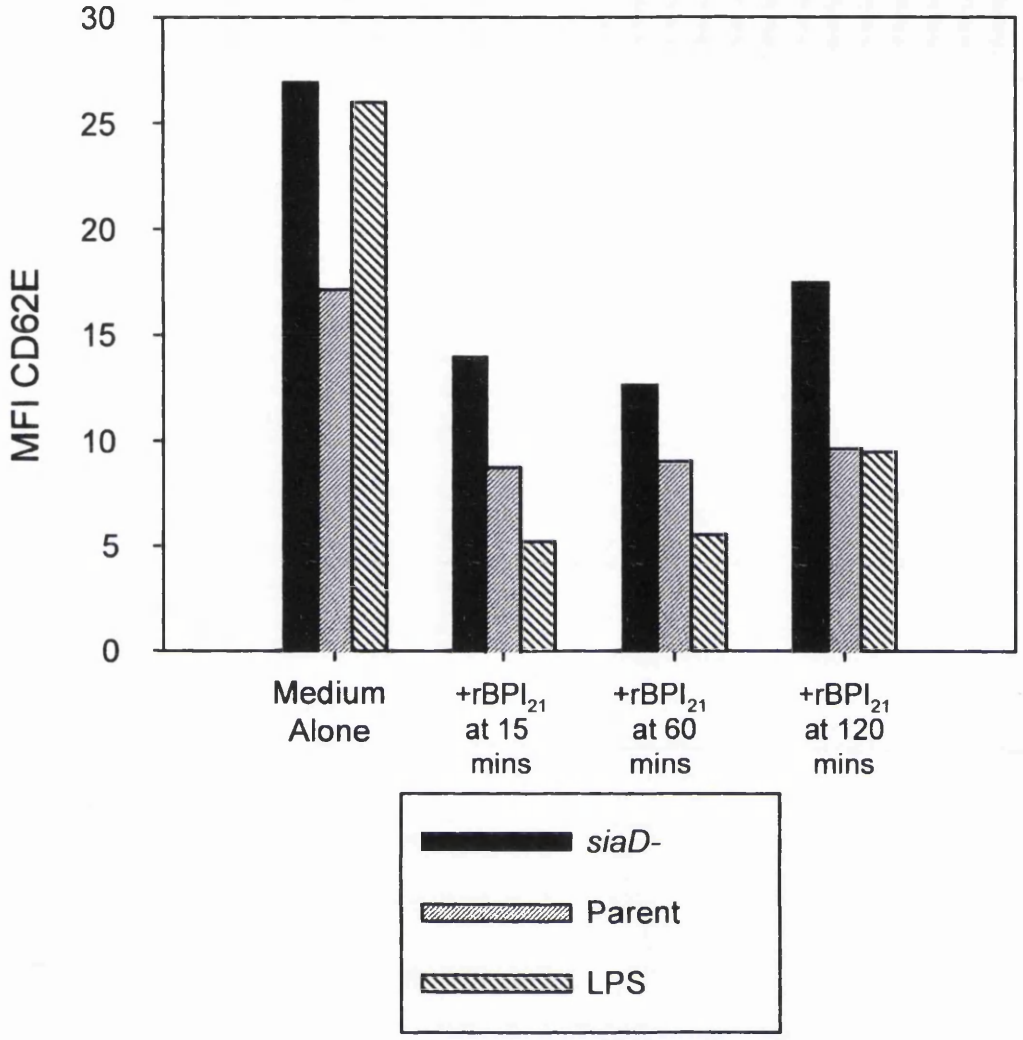

Figure 4.6. Effect of delay in addition of $rBPI_{21}$ to HUVEC stimulated with *N. meningitidis* parent and *siaD*- strains and LPS.

HUVEC were incubated with 10^6 CFU/ml organisms for 15 minutes and then non-adherent bacteria removed by washing wells containing fresh medium. $rBPI_{21}$ was added at the indicated time points. 10ng/ml LPS was added and $rBPI_{21}$ added at the indicated time points. Total incubation time 5 hours.

4.3.4. Cell adhesion molecule expression on HUVEC in response to a viable, LPS deficient strain (*lpxA*-) of *N. meningitidis* H44/76.

The *lpxA*- strain was used to investigate whether meningococci could induce cell adhesion molecule expression on HUVEC in the absence of LPS. The capacity of the *lpxA*- mutant to induce cell adhesion molecule expression was compared to its LPS sufficient parent strain, H44/76, as well as

meningococcal LPS. As fig4-7 shows, the *lpxA*- organism is able to induce CD62E on HUVEC.

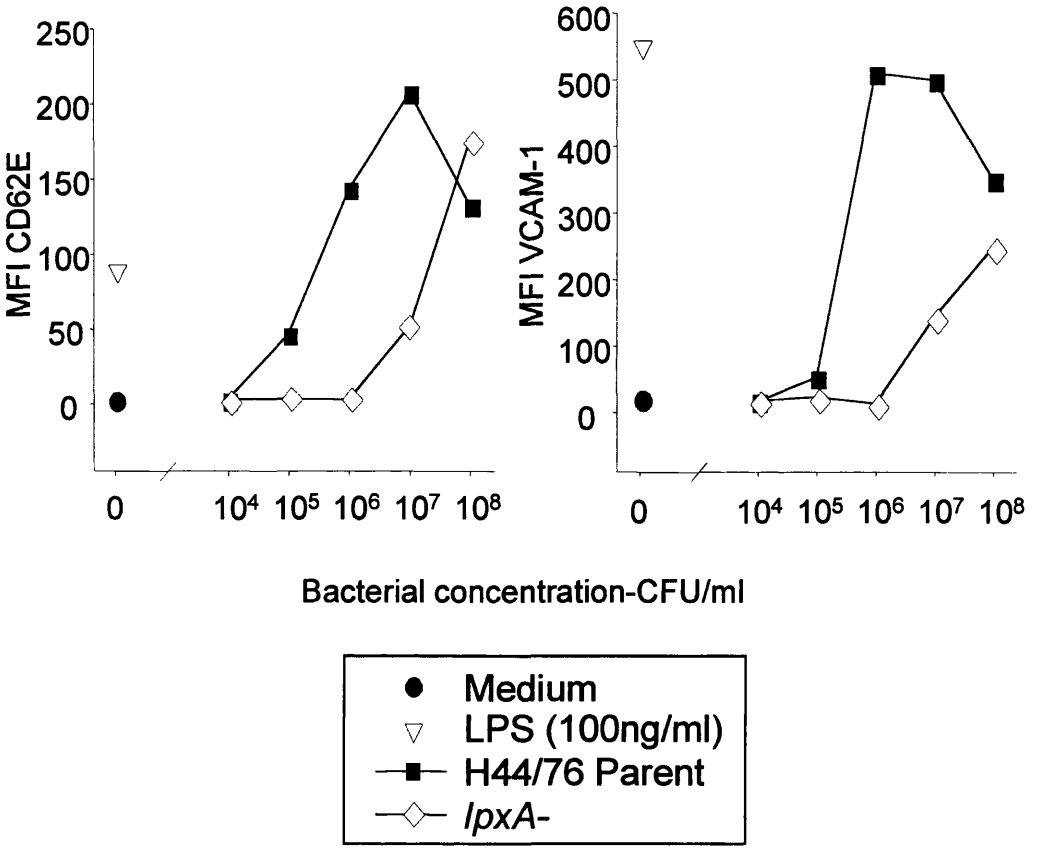

Figure 4.7. Induction of CD62E and VCAM-1 expression on HUVEC in response to H44/76 parent and LPS deficient *lpxA*- strains

HUVEC were incubated with various concentrations of organism and adhesion molecule expression determined after 5 hours incubation. Response to organisms is compared to maximum response to meningococcal LPS (100ng/ml). This is representative of three experiments that yielded similar results.

However, this only occurs to any extent at doses of 10^7 CFU's or above, which is an at least 2-log order greater of magnitude than the concentration required of the parent strain to produce a comparable response. A small increase in percent positive cells expressing CD62E was detected in response to 10^6 CFU's of the *lpxA*- mutant. At 10^8 CFU's/ml, the intensity of CD62E expression in response to the *lpxA*- mutant was very similar to that seen in response to the parent. Moreover, CD62E concentration at the highest dose of the *lpxA*- mutant was higher than the maximal level induced by purified LPS. Fascinatingly, a different pattern of response was observed with induction of VCAM-1. The maximum level

of VCAM-1, for example, induced by the *lpxA*- mutant was never as high as that seen with the either the parent or purified LPS (fig 4.7). Similar results to that observed with VCAM-1 were obtained for ICAM-1 expression (data not shown).

4.3.5. The effect of $rBPI_{21}$ on the induction of cell adhesion molecule expression by *lpxA*- strain

The effect of the anti-LPS protein on the capacity of the LPS deficient mutant to induce cell adhesion molecule induction was investigated for several reasons. Firstly, it was important to attempt to exclude the possibility that low levels of LPS that may be contaminating the *lpxA*- preparation. This was approached in several ways. The stocks of the *lpxA*- mutant were grown on agar plates containing kanamycin, as the insertional deletion contain a kanamycin resistant construct. This process would prevent any contamination by the parent strain. Second, $rBPI_{21}$ was used to block any low-level LPS contamination that might be present for other reasons, such as in media or on agar. Another reason for employing $rBPI_{21}$ was to investigate whether it could influence non-LPS components. From the results with the B1940 mutants, it was clear that $rBPI_{21}$ was only partially inhibiting endothelial response to meningococci. One contributory response for this could be the presence of non-LPS components.

Fig 4-8 shows that the effect of $rBPI_{21}$ on the H44/76 parent strain is similar to that seen with the B1940 parent. Induction of CD62E was inhibited by over 50% at 10^5 cfu/ml, whereas at10^6 and 10^7 cfu/ml, CD62E expression was, if anything increased in the presence of $rBPI_{21}$. When the *lpxA*- mutant was exposed to $rBPI_{21}$, CD62E expression was not inhibited. Indeed, expression was always enhanced in the presence of $rBPI_{21}$, sometimes quite dramatically (fig 4.8). This was most marked at the highest bacterial concentration (10^8 cfu/ml). Interestingly,

in contrast to the pattern seen with CD62E expression, VCAM-1 expression induced by *lpxA*- was slightly reduced when $rBPI_{21}$ was present.

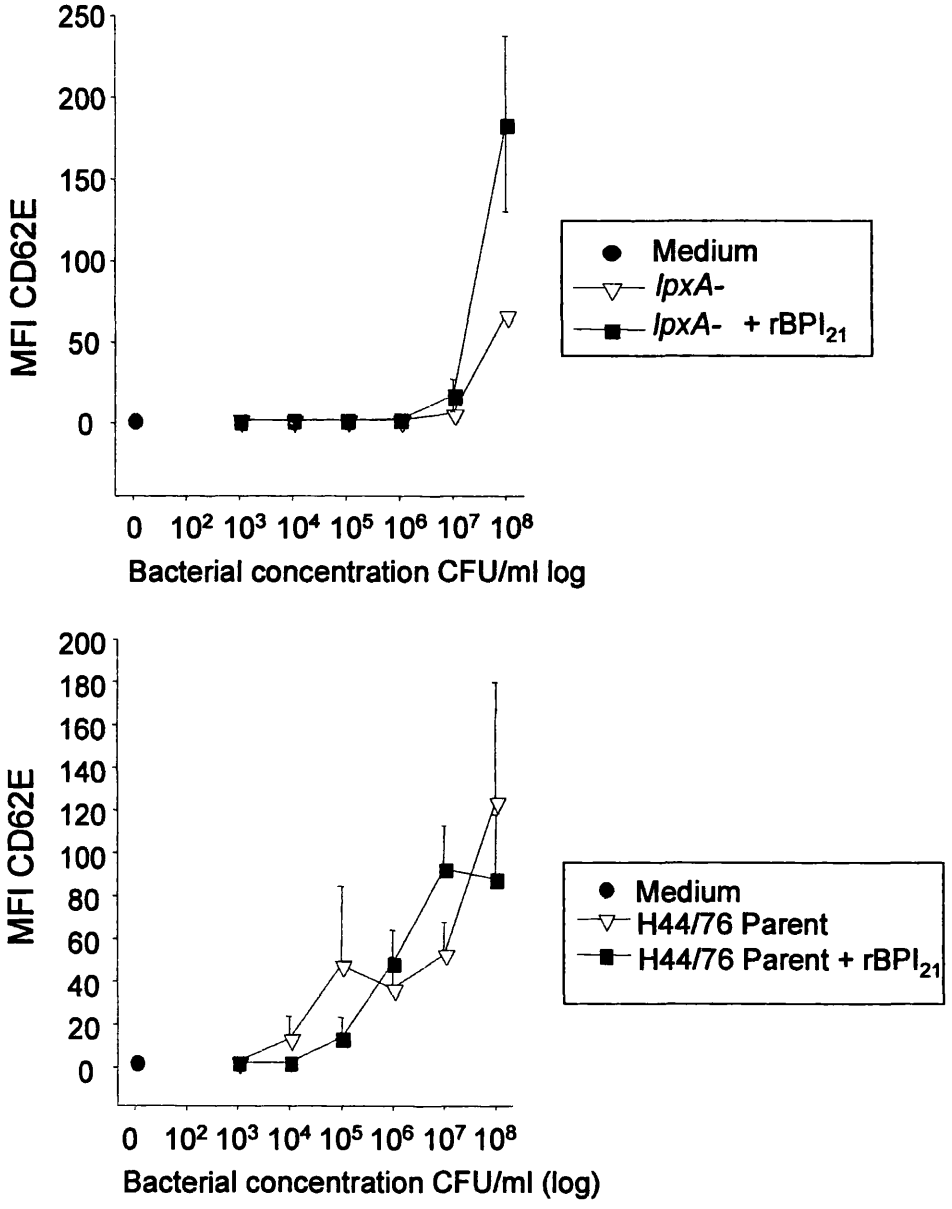

Figure 4.8. Effect of $rBPI_{21}$ on CD62E expression induced by H44/76 parent and *lpxA*- strains.

HUVEC were incubated with organisms at the indicated concentrations in the presence and absence of 10μg/ml $rBPI_{21}$ and CD62E expression determined after 5 hours. Results are expressed as mean MFI ± SEM from 3 separate experiments. Where no error bars are visible, they are contained within data point.

4.4. Discussion

The question posed at the start of this chapter is what is the contribution of LPS dependent and independent activation of endothelial cells by meningococci. Does the presence of non-LPS components explain the discrepancies between the endothelial response to purified LPS as compared to bacteria observed in chapter 3? The results presented here clearly show that meningococci without any native LPS are able to induce cell adhesion molecules expression on endothelial cells. As will be discussed below, the pattern of response observed gives some clues to the answer to the second part of the question. The second methodological approach, using an inhibitor of LPS, also gives some insight into the question posed above. In addition, the results presented reveal some hitherto unknown effects of the effects of anti-LPS protein $rBPI_{21}$ on the inflammatory response induced by meningococci, which deserves further comment.

These results demonstrate that $rBPI_{21}$ is a potent inhibitor of CD62E, ICAM-1 and VCAM-1 expression on HUVEC in response to purified meningococcal LPS. This is consistent with previously published work that showed that rBPI abrogates the release of IL-6 and expression of CD62E on HUVEC in response to *E. coli* LPS (Arditi et al. 1994;Huang et al. 1995). The concentration ratio of rBPI to LPS of 100:1 used in these experiments resulted in complete inhibition of cell adhesion molecule expression and was similar to that reported by Arditi et al (Arditi et al. 1994). They also reported that delaying addition of rBPI by 30 minutes resulted in inhibition of IL-6 release from endothelial cells of 45%. The results from this chapter show several new findings.

Firstly, that LPS-mediated induction of both VCAM-1 and ICAM-1 could also be inhibited by $rBPI_{21}$ in a similar fashion to that seen with CD62E. A delay

of up to 2 hours prior to the addition of rBPI could still inhibit the action of LPS to some degree. Secondly, it was interesting to note that the source and structure of purified LPS determined the effectiveness of $rBPI_{21}$. Whilst 100ng of *E coli* 0111:B4 LPS could be inhibited by 10μg/ml of $rBPI_{21}$, the same dose only inhibited expression of CD62E and VCAM-1 by 70% in response to meningococcal LPS. There may be a number of possible reasons for this difference. The action of rBPI seems to be due to its high affinity for lipid A and competing for LBP binding to soluble CD14 (Elsbach 1998). The stiochiometric ratio of rBPI to lipid A will clearly be a determinant of the ability to a given dose of rBPI to bind to and inhibit a given dose of LPS (Svein Andersen, personal communication). As has been mentioned, the lipid A content of meningococcal LPS for a given concentration is likely to be far higher than smooth *E. coli* LPS, because of the significantly smaller molecular weight of meningococcal LPS. The correct molar ratio of lipid A between different LPS preparations could be measured directly by mass spectrometry, or more indirectly by determination and equalising for KDO_2 (see section 1.3.2.4.2) content (M. van Deuren, personal communication). This would help determine what effect the different lipid A and carbohydrate structure of the two endotoxin species may have on interactions between rBPI and LPS.

The response of the organisms to the effects of $rBPI_{21}$ was quite different. Firstly, $rBPI_{21}$ only partially inhibited cell adhesion molecule induction in response to the organisms. At doses of 10^5 cfu/ml, the level of inhibition of cell adhesion molecule induction by either the parent or *siaD*- strain in the presence of $rBPI_{21}$ was between 20 to 50%. Even at 10^4 cfu/ml, very low levels of cell adhesion molecule expression could be detected in the presence of $rBPI_{21}$. Complete inhibition was only observed at 10^3 cfu/ml, at which concentration induction of

cell adhesion molecule expression by the organisms was very low. This is in contrast to the effect seen with even high doses of purified LPS, which was more effectively inhibited.

The failure of $rBPI_{21}$ to completely inhibit cell adhesion molecule induction in response to the organisms could be due to a number of reasons. The results indicate that it is not simply the stoichiometric ratio of $rBPI_{21}$ to LPS within or released by organisms. If that were the case then one might expect the same degree of inhibition of 10^5 or 10^6 cfu/ml as that seen with 10ng purified LPS. This was clearly not the case. Indeed, in the vast majority of experiments, cell adhesion molecule expression actually increased in the presence of rBPI when concentration of organisms was 10^6 cfu or above. One reason for the lack of effectiveness of $rBPI_{21}$ is that in order to exert its anti-LPS effect, $rBPI_{21}$ has to bind to lipid A embedded in the lipid bilayer. One could speculate that $rBPI_{21}$ is just not able to fully penetrate and bind to and neutralise lipid A. This would be more apparent at the higher concentrations because of the relatively larger amounts of LPS therein, which simply cannot be bound by $rBPI_{21}$.

The observation that bacterial structure effects the inhibitory capacity of $rBPI_{21}$ is therefore interesting. When all the mutants were compared, it was clear that some of them were more sensitive to the inhibitory effects of $rBPI_{21}$ than others. It was striking that those organisms that possessed either the truncated galE LPS (*cps*- and *cpsD*-) or non-sialylated LPS (*lst*-) were more sensitive to the inhibitory effects of rBPI, whether capsulated or not. The conclusion that may be drawn is that LPS structure is an important determinant of the inhibitory effects of rBPI. Close inspection of the degree of inhibition of CD62E in response to the organism suggests that that the unencapsulated *cps*- mutant appears more sensitive than the capsulated *cpsD*-, indicating that capsulation may play a role. This finding

has a number of implications. It demonstrates that simple dose of LPS is not the only determinant of the inhibitory effect of $rBPI_{21}$. At the same concentrations, the amount of LPS in the mutants is likely to be similar, as judged by the equal potency of cell adhesion molecule induction seen in the absence of $rBPI_{21}$. What then, could be the explanation of this effect?

The accessibility of native LPS (or lipid A) within organisms to cationic proteins such as rBPI maybe an important factor in this effect. It has been known for some time that the resistance of gram-negative bacteria to cationic leukocyte proteins like BPI is dependent on LPS structure (Capodici et al. 1994;Weiss, Beckerdite, & Elsbach 1980). Weiss et al showed that sensitivity to BPI increases with decreasing length of polysaccharide chain of endotoxin in *E. coli* and *S. typhimurium*. It was hypothesised that long carbohydrate chains shield negatively charged lipid A from positive charged cationic proteins like BPI (Weiss, Beckerdite, & Elsbach 1980). What is interesting from these experiments is that in meningococci, which have a short oligosaccharide structure, addition of a single sugar moiety (sialic acid) can significantly alter the sensitivity to the action of rBPI. It must be remembered that in addition to its anti-LPS activities, rBPI is also bactericidal, which is thought to be dependent on its binding to LPS in the bacterial membrane. (Elsbach 1998).

The apparent resistance that sialylation of LPS confer on meningococci to the inhibitory action of rBPI is interesting. It was assumed that the main effect of LPS sialylation that occurs in pathogenic meningococci is the resistance to serum bactericidal activity (Mandrell et al. 1991). It is interesting therefore that a number of molecules involved in host innate immunity may also be affected by sialylation of LPS. This includes the action of the acute phase protein, mannose-binding lectin (MBL) (Turner 1996). It would seem that carbohydrate structure of LPS is a

critical determinant of the binding of MBL to organisms and thus its biological activity. MBL binds more avidly to mutants that possess a truncated galE LPS (Jack et al. 1998), but interestingly not to the *lst-* mutant (D. Jack, personal communication). From the results presented here, LPS structure also influences the action cationic proteins such as rBPI. It maybe that pathogenic meningococci have evolved mechanisms of LPS sialylation in response to a number of different host innate immune processes designed to counteract invading bacteria.

Although conclusions drawn from the artificial situation of *in vitro* stimulation assays must be limited in terms of the processes occurring in a therapeutic scenario, a number of useful conclusions can be drawn. In very severe sepsis, with a large bacterial burden, the effectiveness of $rBPI_{21}$, in the face of very high amounts of LPS contained either within bacterial cell membrane or in blebs, may be limited. This reinforces the critical importance of limiting bacterial proliferation before such high levels of bacteraemia occur. Despite this, it was interesting to note that, even after two hours exposure to endothelium, there was some reduction in cell adhesion molecule expression in response to both purified LPS and organisms. This suggests that continuous exposure to either LPS or organisms result in ongoing signalling to endothelial cells, further increasing cell adhesion molecule expression, an also suggest that anti-LPS molecules can have a role after meningococci have come into contact with endothelial cells. In addition, since $rBPI_{21}$ is both bacteristatic and bactericidal, part of the differential effect on the mutants may be due to increase killing, or limitation of growth, due to the action of $rBPI_{21}$ on the more sensitive mutants. It is also clear that rBPI is a highly effective antagonist of purified LPS, and can influence the inflammatory response induced by meningococci on endothelial cells *in vitro*, at least at reasonably low bacterial concentrations. Together with results from studies, showing that $rBPI_{21}$

can partially inhibit neutrophil activation (Heyderman et al. 1999), this indicates a role for anti-LPS strategies after host cells have already been exposed to organisms

The results using the LPS deficient mutant *lpxA-* clearly demonstrate that vascular cell adhesion molecule induction by meningococci can occur independently of LPS. There are several important points to note. The *lpxA-* mutant is far less potent than its LPS-sufficient parent, as demonstrated by the different dose response curves. There seems to be a threshold concentration of 10^6cfu/ml at which detectable cell adhesion molecule expression is induced by the *lpxA-* strain, but is 10^4 cfu/ml for the parent strain. At 10^8 cfu/ml, the levels of CD62E expression induced by both organisms were very similar. This may indicate that at these high concentrations, non-LPS components may be very important activators of endothelial cells and might significantly contribute to endothelial activation and consequently damage, by the mechanisms discussed in previous chapter 1. The results also show these LPS independent mechanisms effect expression of cell adhesion molecules differentially. The maximum level of expression of CD62E induced by the LPS deficient mutant was always higher than the maximum level seen in response to purified LPS. However, the converse was observed for both VCAM-1 and ICAM-1 expression. This is a fascinating observation in light of the results from chapter 3 on increased effectiveness of meningococci to induce CD62E expression compared to LPS.

The difference in potency of parent meningococci compared to the LPS deficient mutant was striking, and must lead one to conclude that LPS is the major inflammatory mediator in meningococci. LPS activation of host cells involves CD14, LBP and the recently characterised cellular Toll-like receptors (Faure et al. 2000;Ulevitch & Tobias 1995;Yang et al. 1998). There is also evidence that CD14 is involved in recognition of non-LPS microbial molecules, such as bacterial

peptidoglycan (Gupta et al. 1996), polysaccharides such as poly-mannuronic acid (Jahr et al. 1997), and is involved in activation of endothelial cells by outer membrane components of *Staphylococcus aureus* (Kusunoki et al. 1995a), and *Bacteroides fragilis* (Sato et al. 1998). Interestingly, it has also been shown that gram negative bacteria induced CD62E and tissue factor expression and NF-κB translocation in cultured HUVEC, which required soluble CD14 (Noel-RF et al. 1995). In complete contrast, gram positive organisms, whether live or killed, did not effect these activation markers (Noel-RF et al. 1995). It would seem that gram negative bacteria are particularly potent activators of vascular endothelium.

There are a number of meningococcal components that could activate endothelial cells, including outer membrane proteins, porins, pili, and a number of other components common to most bacteria. Recent evidence suggests that bacterial lipoproteins and pedptidoglycans, which are contained in the cell wall of numerous species of bacteria, can stimulate innate immune responses via Tlr2 (Brightbill et al. 1999a;Schwandner et al. 1999). Interestingly, it has been shown recently that *Neisserial* Immunoglobulin A1 protease can induce cytokines in monocytes (Lorenzen et al. 1999). The relative contribution of all these potential inflammatory mediators on endothelial cell adhesion molecule expression induced by meningococci remains speculative, but from the results using the *lpxA*- mutant would suggest that LPS is the most potent of these. However, the *lpxA*- mutant may differ from the parent strain in ways other than lack of LPS. For example, outer membrane proteins are phase variable, and it is possible that these components will be expressed in different fashion than the parent.

Another feature of the results from this study deserves further comment. The level of CD62E expression induced by the *lpxA*- mutant was always higher in the presence $rBPI_{21}$. Why this should be is not entirely clear. $rBPI_{21}$ is clearly

having some effect on the *lpxA*- mutant. Components of the gram-positive bacterium *S. aureus* are able to activate monocytes via CD14 but is neither enhanced by LBP nor inhibited by rBPI (Kusunoki et al. 1995b). In contrast, polysaccharides such as mannuronic acid polymers derived from gram negative *Pseudomonas aeruginosa* activates monocytes via CD14, augmented by LBP and inhibited by rBPI (Jahr et al. 1997), which is analogous to LPS induced activation. It is likely therefore, that a proportion of bacterial molecules dependent on CD14 will be inhibited by rBPI whereas others will not. In any bacterium, there may be a number of these molecules and the net effect is likely to be a complex balance of either inhibition or no effect. It is interesting that even at low concentrations of the parent strain, cell adhesion molecule expression was never completely inhibited. This may be in part due to the effect of $rBPI_{21}$ on non-LPS components, in addition to the other mechanisms discussed above.

Chapter 5

The transcriptional regulatory elements induced in endothelial cells activated by *Neisseria meningitidis.*

5.1. Introduction

5.1.1 Background

Expression of the vascular endothelial cell adhesion molecules CD62E, ICAM-1 and VCAM-1 is tightly controlled at a transcriptional level (Collins et al. 1995). This is of great importance, because the modulation of leukocyte traffic has to be controlled in a time and tissue specific manner. The results shown in both Chapters 3 and 4 suggest that the pattern of expression induced by *N. meningitidis* is significantly different to that seen in response to purified LPS. The organisms, particularly those that are unencapsulated, induce much higher levels of CD62E than purified LPS. Additionally, the mutant that is totally deficient in LPS, induces much higher levels of CD62E than maximal dose of LPS, albeit at high concentrations of bacteria. One hypothesis for these observations is that on contact with endothelial cells, meningococci activate a number of signalling mechanisms in addition to LPS that may be either additive or synergistic in terms expression of CD62E expression. One method for exploring these signals is to examine the activation of nuclear transcription factors that control CD62E gene expression.

5.1.2 CD62E is coded by a transcriptionally regulated, early inflammatory response gene

CD62E is not expressed constitutively on resting endothelium, except in haemapoetic tissue (Schweitzer et al. 1996). CD62E expression is rapidly up-regulated on exposure of endothelial cells to inflammatory stimuli *in vitro* (Bevilacqua et al. 1987). On HUVEC, surface expression can be detected by 1 to 2 hours, reaching a peak by 4 to 6 hours and returns to baseline by 24 to 48 hours.

However, the pattern of CD62E expression varies depending on the inflammatory stimulus. There is a hierarchy of potency in response to the pro-inflammatory signals TNF-α, IL-1 and CD40 that is transcriptionally regulated (Karmann et al. 1995). The half-life of CD62E is short, which is probably a combination of transcriptional regulatory processes (Ghersa et al. 1997) and endocytic removal (von Asmuth1992). Interestingly, IFN-γ does not itself induce CD62E expression but prolongs expression in response to TNF-α and LPS (Doukas & Pober 1990), through mechanisms that may not involve transcriptional regulation. IL-4, by contrast, inhibits CD62E expression induced by TNF-α by transcriptional repression (Bennett et al. 1997), but augments VCAM-1 expression (Thornhill & Haskard 1990). There are clearly multiple levels of regulation that are required for correct recruitment of different leukocyte populations in specific inflammatory situations (Carlos & Harlan 1994).

Both LPS and gram-negative bacteria whether heat-killed or live, induce CD62E expression on HUVEC, which is dependent on soluble CD14 (Noel-RF et al. 1995;von Asmuth et al. 1993). As has been stated, gram-positive organisms are poor inducers of CD62E. One might conclude that LPS is the major inflammatory mediator of this process. *Staphylococcus aureus,* a gram-positive bacterium, induces both ICAM-1 and VCAM-1 expression and increased adhesion of monocytes and neutrophils to HUVEC, but fails to up-regulate CD62E (Beekhuizen et al. 1997). There is evidence that certain protein components of some gram-positive bacteria can induce CD62E on HUVEC, such as *Listeria monocytogenes* (Schwarzer et al. 1998), and *Streptococcus viridans* (Vernier et al. 1998). In addition, non-LPS components of gram-negative *Bacteroides fragilis* induce CD62E expression in a CD14 dependent manner (Sato et al. 1998). However, it is very interesting to note that these components only induce CD62E expression on

endothelium at high doses {milligram to microgram quantities compered to LPS which stimulates endothelial cells in picogram to nanogram concentrations).

5.1.3.Sustained expression of CD62E in disease states

Expression of CD62E is important in many inflammatory states, including SIRS, diabetes, and myocardial infarction and a host of other disorders (Bevilacqua et al. 1994). Additionally, plasma CD62E is often regarded as a marker of surface selectin expression in disease states, due to surface turnover, probably via endocytosis and proteolytic cleavage (Bird et al. 1997). CD62E expression can be prolonged in certain states (Cotran et al. 1986), but the cause and role of sustained expression of the selectins is yet to be fully determined. However, it is interesting to note that in septic shock due to meningococcal disease, soluble CD62E levels remain very high for some time after the initial insult, even up to 2 weeks. This may represents the degree of endothelial damage and repair, or continuing stimulus to expression. *In vitro,* CD62E expression is sustained on activated endothelium in the presence of adherent monocytes, but not to maximal doses of TNF-α (Noble et al. 1996). This suggests an important role for leukocyte adhesion in the sustained activation state of endothelium, and may play a role in continued ingress of inflammatory cells.

5.1.4. Signal transduction mechanisms involved in induction of cell adhesion molecules with special reference to CD62E (E selectin)

The key events in induction of cell adhesion molecules in response to pro-inflammatory stimuli, such as cytokines TNF-α, IL-1 and bacterial products like LPS involves elaborate signal transduction pathways that starts with ligand- ligand interactions with both humoral and membrane bound receptors. This is followed by complex intracellular cytoplasmic signalling, which ultimately leads to gene

transcription but also in addition induces alterations to cytoskeletal molecules resulting in morphological changes to cells. The resultant phenotypic changes are critical to the host response to tissue injury and infection. Activation of endothelial cells for example, involves *de novo* transcription of cell adhesion molecules required for leukocyte extravasation (Zimmerman, Prescott, & McIntyre 1992). Figure 5.1 illustrates the basic pathways involved in signalling by TNF-α, IL-1 and bacterial LPS. The majority of studies on transcriptional regulation of cell adhesion molecule genes have involved cytokine inducible mechanisms, reviewed in (Collins et al. 1995). Whilst the membrane associated receptors for TNF-α and IL-1 have been well characterised, the receptors and pathways involved in the recognition of bacterial LPS and the subsequent signalling have only just begun to be elucidated.

5.1.5. The role of Human Toll-like Receptors (TLR's) as pattern recognition receptors for microbial components including bacterial LPS

A key advance in recent years has been the identification of mechanisms by which the immune system discriminates self molecules from potentially dangerous, pathogenic microbial products (Medzhitov & Janeway-CA 1998). The ability to recognise and respond to bacterial LPS is a key step in host anti-microbial defence systems against gram-negative bacterial infection. This is graphically illustrated in the case of certain strains of mice that are hyporesponsive to LPS but also fail to clear gram negative bacteria, and are highly susceptible to gram negative sepsis (Eden, Shahin, & Briles 1988;Vogel et al. 1999b). One enduring puzzle has been how the host recognises LPS in the first place. LPS itself is not directly toxic, but delivers a so called 'warning signal' to the host to induce a battery of inflammatory molecules designed to clear invading gram-negative

organisms (Ulevitch 1999). However, during a systemic infection when the levels of bacteria and LPS are high, the process thus triggered can be disastrous, and lead to state of lethal septic shock (Natanson et al. 1994;Takahashi et al. 1995b). It has been known for some time that the recognition sequence involves CD14, either membrane bound or soluble, in combination with an opsonic protein LBP (Fenton & Golenbock 1998). However, CD14, assumed to be the receptor for LPS, is not a transmembrane protein and cannot by itself directly transduce cytoplasmic signals (Ulevitch & Tobias 1995). In addition, certain LPS hyporesponsive strains of mice are not deficient in CD14, and it was long suspected that these mice carried a defective '*lps*' gene. It is now known that the reason why such mice fail to response to endotoxin is because they have mutations in the toll like receptor gene 4(Poltorak et al. 1998).

The *toll* family of proteins is encoded by a highly evolutionarily conserved set of genes that controls dorsoventral development of the embryo of the fruit fly, *Drosophila melanogaster*. Fascinatingly, the *toll* gene product is an anti-fungal pattern recognition protein in adult fly (Belvin & Anderson 1996). A human homologue of the *Drosophila toll* gene, the human Toll-like receptor (TLR) gene, was discovered (Medzhitov, Preston, & Janeway-CA 1997). Six members of the TLR's have been cloned (Rock et al. 1998;Takeuchi et al. 1999a) and their number is expanding. TLR's are transmembrane receptors with an extracellular domain containing a leucine rich region (LRR), and cytoplasmic domain that has sequence homology to the IL-1 receptor, called the Toll/IL-1 receptor homologous domain (TIR) (reviewed in (Anderson 2000)). The extracellular domains are large with divergent between different TLR's and are likely to impart ligand specificity. Figure 5.1 summarises toll and toll-like receptors and pathways.

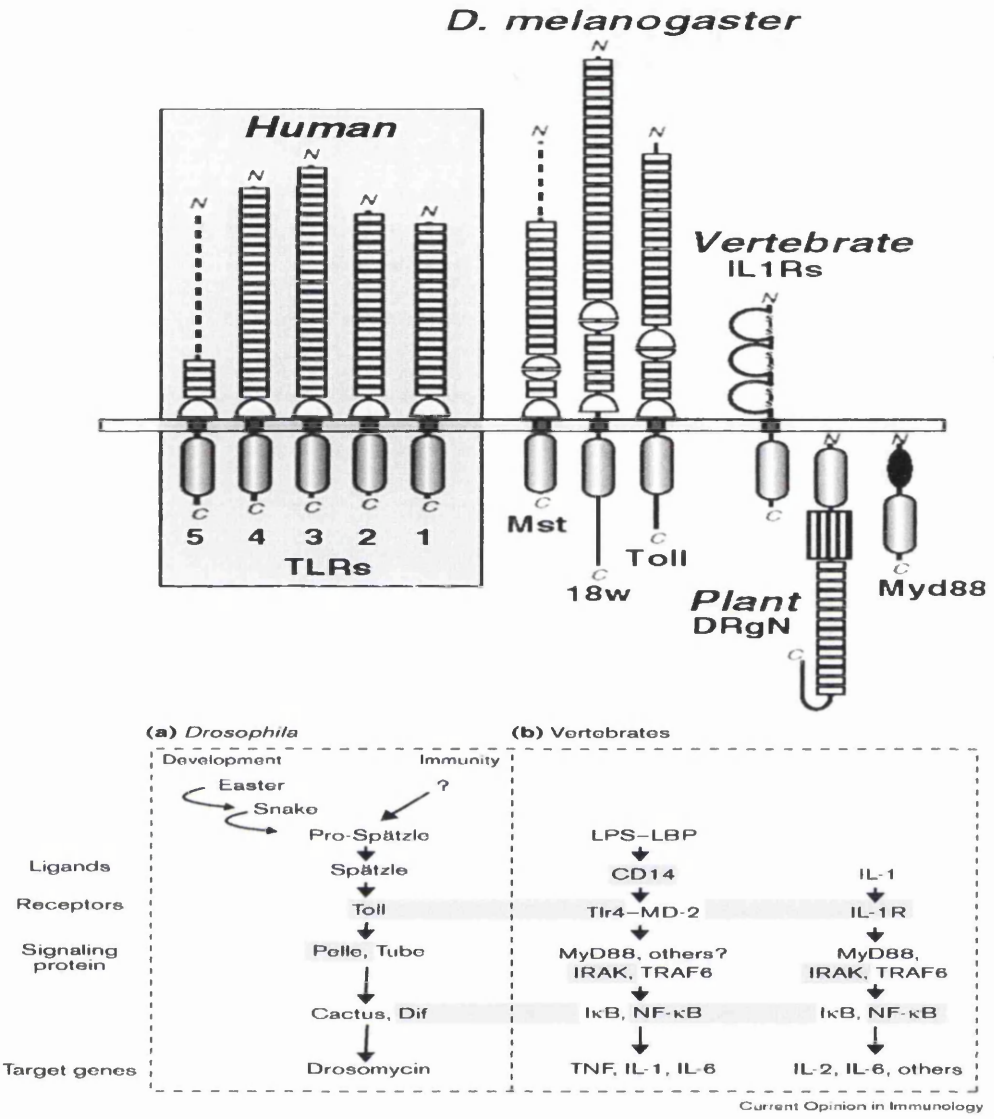

Figure 5.1Comparison of Toll family of receptors and signalling pathways in humans and *Drosphila.*

The toll pathway in *Drosophila* is involved in innate immune responses against fungal infections in addition to its role in embryogenesis. Human and Drosophila homologues are shaded in grey. There is currently no human homologue for drosophila protein, *Spatzle*, which is generated by proteolytic cleavage of pro-*Spatzle*, of serine proteases. Taken from (Kopp & Medzhitov 1999b).

Much of the initial work on the role of TLR's in LPS signalling concentrated on two toll-like receptors, TLR2 and TLR4. Yang and workers first identified TLR2 as a cellular signalling receptor for LPS, since over expression of TLR2 gene construct induced LPS responsiveness in LPS unresponsive cell lines (Yang et al. 1998). However, the story was complicated by the identification of the mutations in LPS hyporesponsive mice. Poltorak et al showed that one strain of mouse, C3H/HeJ, has a point mutation in TLR4 whilst the other (C57/10ScCr) is

null for TLR4 (Poltorak et al. 1998). Interestingly, both strains of mice will respond to LPS at high doses, and even respond normally to certain types of LPS (Tanamoto et al. 1997), indicating that other members of the TLR's might be able to "back-up" defective TLR4 (Wright 1999). In addition, TLR2 may play a more prominent role in signalling to non-myeloid cell such as endothelial cells, because it has been shown that response to LPS is enhanced by the presence of soluble CD14 with TLR2 (Kirschning et al. 1998). Contrary to what might have been predicted, they reported that overexpression of TLR4 in transfected cells did not confer increased LPS responsiveness (Kirschning et al. 1998). However, TLR4 requires the presence of an adapter molecule, MD-2, which has homology to TLR receptors, to induce LPS signals (Shimazu et al. 1999), and this molecule may not be expressed in some clones of A293 cell lines used in experiments reported by Kirschning et al (Kirschning et al. 1998). The balance of opinion now is that TLR4 is the dominant signalling receptor for LPS (Beutler 2000;Poltorak et al. 2000). Importantly, cells from mice that lack TLR2 have a normal response to LPS (Heine et al. 1999). However, the response to LPS may depend critically on type of cell, including clone of cell lines, the species from which it is derived and the chemical structure of LPS itself. Detailed study of the role of TLR4 in LPS signalling in humans is still lacking.

Recent work has shown that TLR2 in particular may be responsible for signalling in response to a number of different microbial products. These include gram positive bacterial components such as lipoteichoic acid and peptidoglycans (Schwandner et al. 1999), lipoproteins from mycobacteria (Brightbill et al. 1999a) and *Borrelia burgdorferi* (Hirschfeld et al. 1999) and cell wall components from *Staphlycoccus aureus* (Yoshimura et al. 1999) all through TLR2. This type of evidence has lead some researchers to postulate that TLR2 is mainly responsible

for recognising gram positive bacteria whereas TLR4 is mainly responsible for gram negative recognition. For example, Takeuchi et al, constructed mice deficient in TLR2 and TLR4. Fascinatingly, TLR2 deficient mice responded to an LPS challenge in the same way as wild type mice, whereas TLR4 deficient mice failed to respond. Moreover, TLR2 negative cells did not respond to a number of gram positive cell wall components, although, curiously, TLR4 deficient cells failed to respond to lipoteichoic acids (from gram positive bacteria) (Takeuchi et al. 1999b). In another study, point mutations in TLR2 resulted in failure to respond to yeast and gram positive bacteria, but had little effect on response to gram negative bacteria or LPS (Underhill et al. 1999).

5.1.6. The LPS/TLR activation pathway

Characterisation of the subsequent signal cascade triggered by LPS recognition by human TLR's links in with another highly evolutionary conserved mechanism that is core to the innate immune system. There is a striking similarity of the toll activation pathway to that of the IL-1 Receptor family (Dinarello 1996;Medzhitov & Janeway-CA 1998) as shown in (figure 5.2). Engagement of either pathway results in activation of two families of transcription factors, NF-κB and the c-jun/activator protein-1 family (Muzio et al. 1998). This pathway can be summarised as follows: the adapter protein MyD88 recruits IL-Receptor kinase (IRAK), which subsequently interacts with the TNF receptor-activated factor TRAF6. This allows engagement with Nf-κB inducing kinase or NIK. This activates another complex, I-κB that phosphorylates I-κBα, forming a complex with cytoplasmic Nf-κB (Muzio et al. 1998). The same study showed that, as well as activating the Nf-κB system, TLR/MyD88/IRAK can activate the stress

activated protein kinase, SAPK (also known as Jun activating kinase, JNK), which phoshorylates c-Jun/ATF2 transcription factors (Muzio et al. 1998) (see figure 5.2)

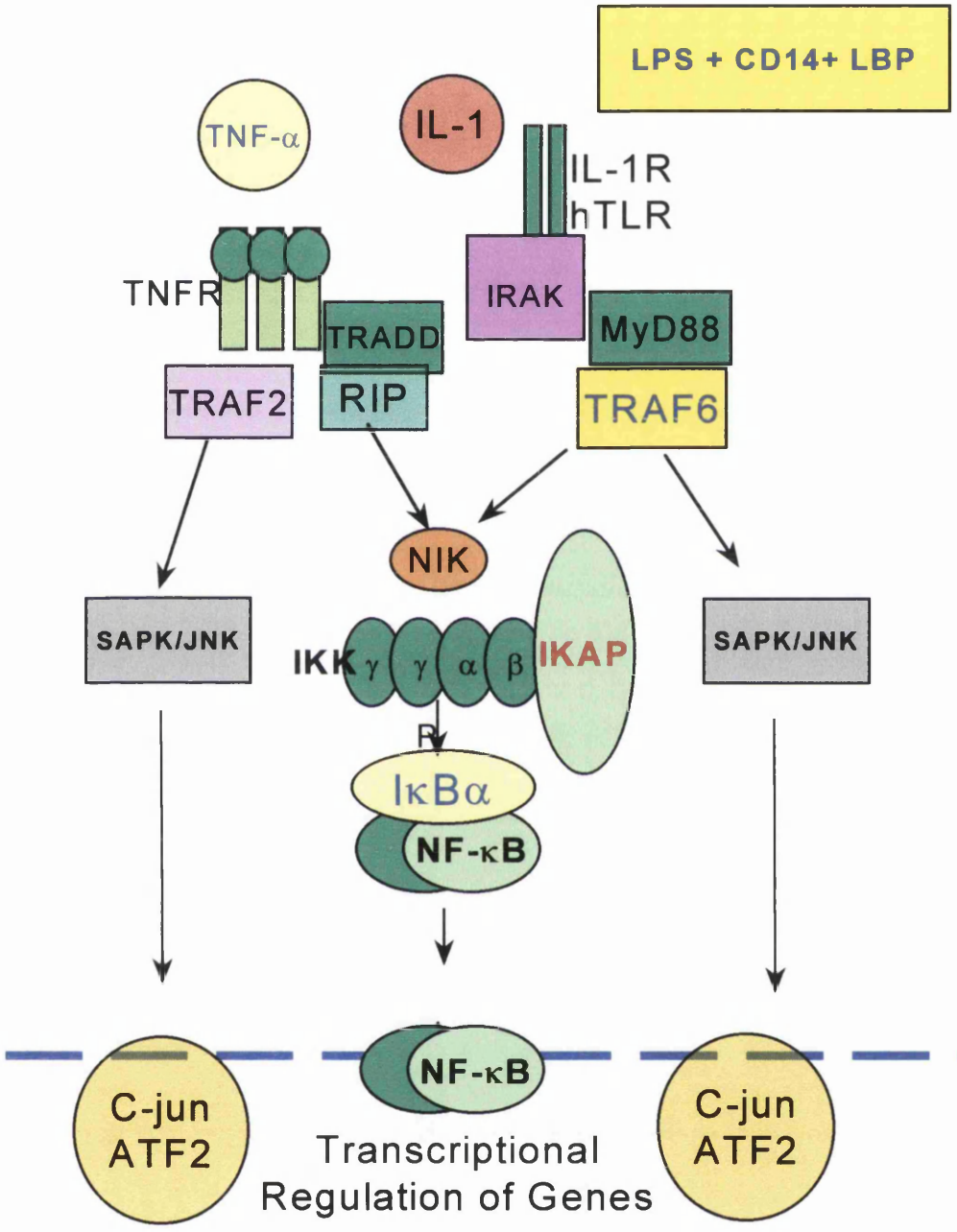

Figure 5.2. Signal transduction pathways of NF-κB and c-Jun/ATF2 of TNF-α, IL-1 and LPS.

Both IL-1 and Toll-like receptors signal through TRAF6 whereas TNF-α is through TRAF2. TRAF=TNF receptor activated factor; TRADD=TNF receptor associated death domain; RIP=Receptor interacting protein; NIK=NF-κB inducing kinase; SAPK=Stress activating protein kinase; MyD88; IKK= I kappa B kinase; IKAP= IKK associated protein; IκBα= Inhibitor of kappa B; JNK=Jun activating kinase; IRAK=IL-1 receptor activating kinase; NF-κB= Nuclear Factor kappa B; ATF2= Activator of transcription Factor 2. Adapted from (Ghosh, May, & Kopp 1998).

LPS signalling via TLR4 will induce multiple pathways that will have consequences for both up-stream and downstream events, as illustrated in figure 5.3. The situation with signalling induced by gram-negative bacteria will be yet more complex. Activation of not only other TLR's such as TLR2, but also a number of pathways that may be unique to a particular bacterium will be responsible for the overall pattern of signalling events, and hence the likely phenotype of the host inflammatory response

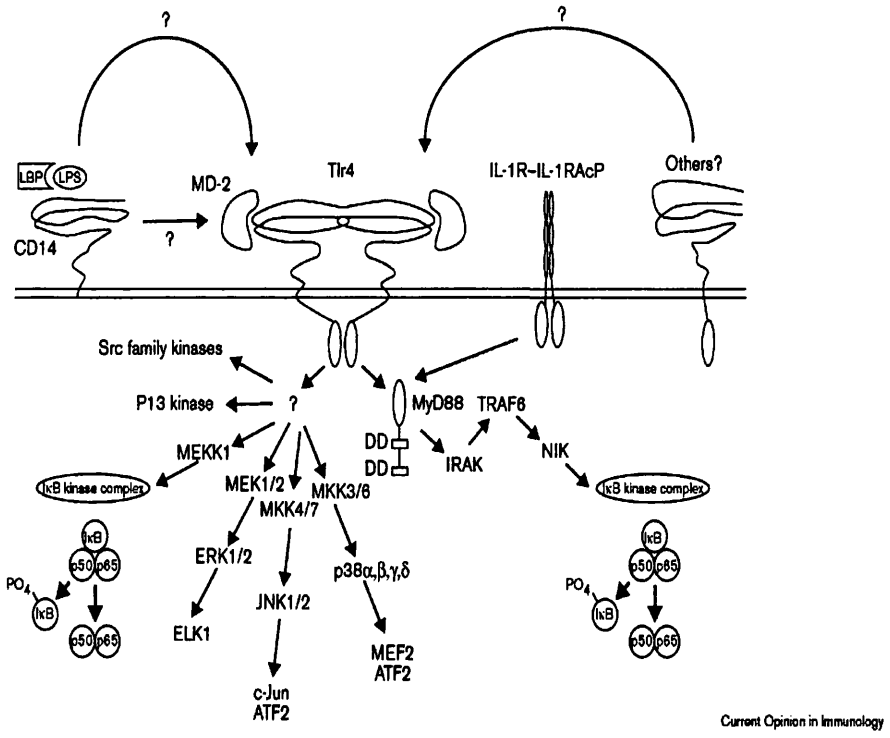

Figure 5.3. Main signal transduction pathways activated by TLR4/LPS

Engagement of TLR4 by LPS activates numerous intracellular pathways. In addition to activating IRAK (IL-1 activating kinase), and therefore ultimately release of activated NF-κB, TLR4 activates a number of other important pathways (such as MEKK1=mitogen activating kinase kinase -1) by an as yet undetermined mechanism. These ultimately can activate both NF-κB but also c-jun via JNK and ATF2 via p38. All these are critically involved in initiating gene transcription of a number of inflammatory and immune response genes. Taken from (Beutler 2000).

5.1.7. The Nf-κB family

The Nf-κB family of transcriptional proteins are evolutionarily conserved and mediate not only many aspects of the adult immune response but also embryogenesis and development of immune system (Ghosh, May, & Kopp 1998). Each has a conserved N-terminal domain or Rel-homology domain (RHD) in which lies the DNA-binding, dimerization, and nuclear location signals (May & Ghosh 1998). There are five, structurally related proteins, p65, c-Rel, Rel B, p50/p105 and p52/100. However, the latter two are produced as precursor molecules that are processed to active, smaller forms. All can exist as homo or heterodimers, which is of considerable importance in their activities as transcriptional regulators, as will be discussed later.

5.1.8. Nf-κB is sequestered in the cytoplasm in an inactive form bound to the inhibitory complex, IκB

Dimers of Nf-κB are bound to a family of related inhibitory proteins, IκB, of which the best studied are IκBα and IκBβ which are thought to be involved in p50p65 and p50c-Rel heterodimers (Thompson et al. 1995) . This non-covalent association is between an ankyrin repeat sequence of IκB, and the RHD domain of Nf-κB, effectively masking the nuclear location signal preventing its nuclear translocation (reviewed in (Ghosh, May, & Kopp 1998)). It seems that the crucial step in the cascade is the phosphorylation of IκBα, which leads to a series of steps: phosphorylated IκBα is ubiquinated (altered conformation) which triggers degradation within proteosome (reviewed in (May & Ghosh 1998)). Only once this has occurred, is free Nf-κB released. What then, is the mechanism of IκB phosphorylation? This crucial process is carried out by an unusually large and complex molecule called IκB kinase. This complex is formed from at least three

subunits, α,β and γ (Israel 1997;Scheidereit 1998). In addition, the scaffold protein, IKAP, is an integral part of this complex (Cohen, Henzel, & Baeuerle 1998). It seems at least that this may be a convergence pathway for the multiple extracellular stimuli that can activate Nf-κB. It has been shown that a number of activators of IκB kinase exist. These include TNF-α, IL-1 and LPS, via NIK, members of the protein kinase C family, and MEKK-1 (mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1), which is also, importantly, a strong activator of JNK and thus AP-1/c-jun (Karin & Delhase 1998). Additionally, small GTPases such as RhoA, Rac-1 and Cdc42 may activate IκB kinase complex (Perona et al. 1997a), probably through the activation the MEKK-1 as well.

5.1.9. Activated Nf-κB binds to the promoter elements of a wide variety of immune response genes

Once activated, Nf-κB is released from its inhibitory complex and rapidly enters the nucleus where it is able to bind to DNA containing a consensus sequence in the promoter region of various genes. There are a large number of Nf-κB inducible genes including vascular cell adhesion molecules ICAM-1, VCAM-1 and CD62E, cytokines such as IL-1, TNF, IL-6, IL-8, acute phase proteins, procoagulant molecules such as tissue factor (Mackman 1995) and regulatory proteins themselves (see extensive review (Ghosh, May, & Kopp 1998)). Many Nf-κB complexes are transcriptionally activating, such as p65: p50, p50/c-rel, p65: p65 and p65: c-rel but others are repressive or inhibitory, such as p50 and p52 homodimers (Plaksin, Baeuerle, & Eisenbach 1993). It has been proposed that this may be due to the lack of C terminal domain in these two isoforms (Ghosh, May, & Kopp 1998).

5.1.10. Transcriptional regulation of endothelial CD62E (E-selectin) involves co-ordinate activation of Nf-κB and JNK-c-Jun/AP-1/ATF2 pathways

Initial studies into the transcriptional regulation of CD62E demonstrated the complexity of multiple pathways in the activation of a single gene. In 1991, De Lamarter and workers identified an Nf-κB element that was "essential, but not sufficient" for CD62E transcription, and identified another *cis* regulatory element that was required for this process (Whelan et al. 1991). In the same year, Pohlman et al, characterised transcriptional elements involved in TNF-α, IL-1, LPS and PMA stimulated HUVEC (Montgomery et al. 1991). The result of this work has some important implications. They identified both an Nf-κB site but also an up stream, AP-1 site, to which activated c-Jun/Ap-1 protein product may bind. They showed that TNF-α, IL-1 and LPS were strong inducers of Nf-κB binding, CD62E mRNA and surface expression, but did not activate AP-1 proteins. Activation of protein kinase C by phorbol esters such as PMA only weakly induced Nf-κB binding and CD62E transcription, but did induce activation of AP-1/c-jun. Blockade of PKC activation in TNF-α, IL-1 and LPS stimulated HUVEC did not block Nf-κB binding, but did block CD62E gene transcription. All this indicated that another element, other than Nf-κB, was essential for CD62E transcription.

The full organisation of the cytokine responsive, human CD62E promoter element has now been characterised (Collins et al. 1995). As Figure 5.4 shows, there are four regulatory domains; PD1, 3 and 4 which bind Nf-κB, and PD2 site, which is a cAMP like response element (CRE-like), at –157 in the CD62E promoter region (Collins et al. 1995). It is this site which appears to be the

essential non-NF-κB site. A further, NF-ELAM-2 site at –100 has been identified, although it is not certain which proteins bind to this site (Hooft et al. 1992).

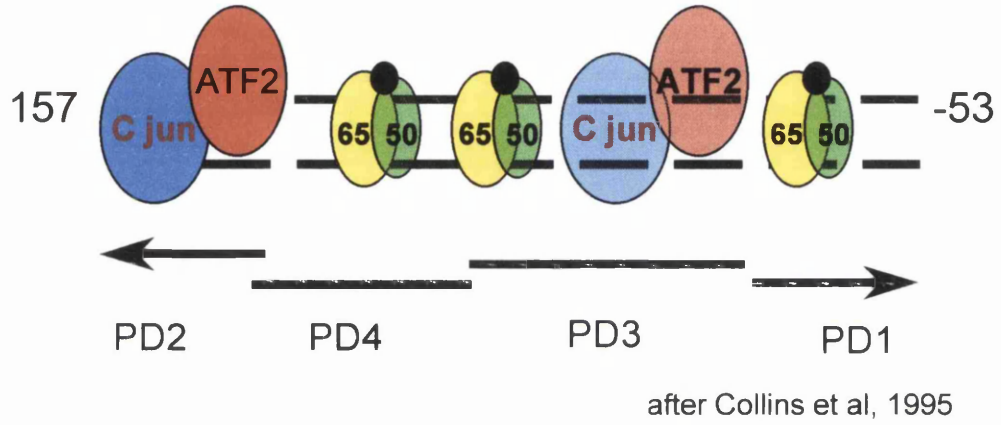

Figure 5.4 Cytokine response element within the promoter of the CD62E gene.

Occupancy of all sites is required for initiation of CD62E transcription. C-jun/ATF2 proteins, including ATF2 homodimers, C-jun/ATF2 heterodimers are constitutively bound to the promoter whereas activated NF-κB binds once activated and is translocated into the nucleus. Adapted from (Collins et al. 1995).

A number of different transcription factors from different families can bind this consensus site. These include ATF2 (Activating transcription factor-2) homodimers, ATF2/c-Jun heterodimers, and other members of jun/fos family and CREB protein (Kaszubska et al. 1993). Mice null for ATF2 genes have deficient CD62E expression (Reimold et al. 1996). It is the complex alterations of these transcription factors bound to this site that seem to be essential for the maximal up-regulation of CD62E. In particular, it has been shown that this CRE-like element is bound constitutively by three sets of protein complexes including ATF2 homodimers, ATF2/c-Jun and CREB. Upon TNF-α stimulation of endothelial cells, it was demonstrated that there was a decrease in complex with ATF2 alone but increase in the ATF2/c-Jun complex, but no change in CREB

protein (DeLuca 1994). This process has been further elucidated. Phoshorylation and activation of ATF2 and c-Jun/AP-1 by two nuclear located kinases, JNK and p38 MAP (mitogen activated protein kinase), which is essential for maximal transcription of CD62E (Read et al. 1997). Both of these are activated in response to TNF-α, IL-1 and bacterial endotoxin.

Convergence of these pathways, it seems, maybe the key process to the maximal induction CD62E expression (Read et al. 1997). A further and important part of the story has also been unravelled recently. It has been observed that activation of Protein kinase C (PKC) by PMA synergistically increases CD62E transcription by IL-1 but not TNF-α in HUVEC (Tamaru & Narumi 1999). This study demonstrated that it is the CRE-like/ATF2 site, which is responsible for this synergism. Therefore two separate signals, one cytokine mediated and the other via protein kinase C, can converge on this promoter site and not on Nf-κB sites. Given that LPS and IL-1 signal mechanisms are very similar in HUVEC (Zhang et al. 1999), it would be reasonable to postulate that such synergy could be associated with both LPS stimulus and activation of other pathways that activate AP-1/c-jun and ATF2 transcription factors

5.1.11. *Neisseria gonorrhoeae* induces cytokines in host epithelial cells by activating transcription factors Nf-κB and AP-1

In order to explain the rationale for investigating transcription factor activation by meningococci, the current state of knowledge about signalling mechanisms induce by the related bacteria *N. gonorrhoeae* needs to be outlined. Work conducted by T. Meyer and workers has elucidated some of the molecular mechanisms involved in the activation of epithelial cells by gonococci. They showed that pathogenic gonococci could induce Nf-κB rapidly (after 10 minutes)

in epithelial cells and AP-1 more slowly, with kinetics similar to that seen with PMA. This signalling seemed not to depend on invasion of epithelial cells (Naumann et al. 1997). Further studies demonstrated a novel way in which bacteria activate JNK and AP-1. This involves activation by small Rho GTPases, Rac1 and Cdc42, (by an as yet unknown mechanism), which in turn stimulates p21 activated kinase (PAK) to activate JNK (Naumann et al. 1998). Crucially, this is appears to be dependent on presence of either pili or Opa proteins, but not LPS. In addition, the pathway appears to be separate to classic PKC activation by PMA.

This demonstration of a separate pathway for signal transduction by pathogenic *N. gonorrhoeae* is important. As previously mentioned, the presence of additional pathways to those activated by LPS, may have significant effects on transcriptional control of CD62E gene. Although *N. meningitidis* and *N. gonorrhoeae* are different organisms, they are related and share a number of outer membrane proteins that have been implicated in inducing these pathways. It seems reasonable therefore, that signals induced by meningococci when they come into contact with endothelial cells may affect the pattern of transcription factor activation, which when bound to the CD62E promoter, results in gene transcription and thus the pattern surface expression shown in chapters 3 and 4. The following study investigates the activation of the transcription factors Nf-κB, Ap-1/c-jun, and ATF2 in HUVEC stimulated with parent organisms, unencapsulated mutants, LPS deficient mutant and purified LPS. In this way, the contribution of bacterial structure and both LPS and non-LPS components of meningococci in activating these transcription factors in endothelial cells was explored.

5.2 Methods

5.2.1. Introduction

The experimental work presented in this chapter involves the use of techniques specific to this chapter, and is thus described in detail. The aim of the experiments described in this section was to investigate the pattern of transcription factor activity in HUVEC stimulated with purified LPS and meningococcal strains. Transcription factors under study are proteins that are able to bind to particular consensus sequences in the promoter region of a gene of interest. On binding they either initiate, or in some cases inhibit gene transcription

The electrophoretic mobility shift assay (EMSA) employs this DNA binding property to identify, and quantify specific transcription factors of interest. Radioisotope labelled, double stranded DNA oligonucleotides with certain consensus sequences are used as probes to detect such proteins within nuclear extracts of stimulated cells. The resulting DNA/Protein complexes are resolved on non-denaturing polyacrylamide gels. If the probe has transcription factor bound, it will retard the movement of the complex causing a 'shift' that can be detected as a band by autoradiography. Further identification of a band can be achieved by preincubating protein extract with antibody raised against a transcription factor or component, which will retard the band yet further, and is thus called a 'supershift'.

5.2.2 Buffers.

The following nuclear extraction buffers were adapted from a number of sources but were based on the protocol used in the Molecular Rheumatology Laboratory, Windeyer Institute, University College London. All the buffers were made up in sterile, nuclease-free water and stored at -20^0 C. Nuclear extraction buffers were made up fresh prior to each extraction protocol.

5.2.3 Nuclear extraction buffers		
Buffer A		
	Hepes	10 mM
	$MgCl_2$	1.5 mM
	KCl	60 mM
	NaCl	10 mM
	EDTA	0.5 mM
	DTT	0.5 mM
	Protease Inhibitor Cocktail	1 tablet/25mls
Buffer C		
	Hepes	40 mM
	Glycerol	50%
	$MgCl_2$	3 mM
	EDTA	0.5 mM
	DTT	1.0 mM
	NaCl	400 mM
	AEBSF	1μl/ml
	Protease Inhibitor Cocktail	1tablet/25mls

5.2.4 General Buffers and Solutions		
T4 Polynucleotide Kinase 10 times Buffer		
	Tris-Hcl	700 mM
	$MgCl_2$	100 mM
	DTT	50 mM
Tris-Borate-EDTA 10 times Buffer		
	Tris Borate	0.89 M
	EDTA	0.02 M
Poly(dI.dC) poly (dI.dC) binding Buffer		
	EDTA	1 mM
	KCl	40 mM
	$MgCl_2$	5 mM
	DTT	2.5 mM

5.2.5 DNA/Protein binding Buffers(10 times)		
ATF2		
	Hepes	200 mM
	NaCl	800 mM
	EDTA	1 mM
	DTT	10 mM
	Glycerol	40%
NF-κB		
	Tris-HCl	100 mM
	NaCl	4 M
	DTT	50 mM
	Bovine serum albumin	100μg/ml
	EDTA	10 mM

5.2.6 Preparation of HUVECS for nuclear extracts

HUVEC were isolated from fresh human umbilical cords as described in chapter 2. The nuclear extraction method requires large numbers of cells therefore primary culture cells were grown in MCDB 131 medium with added growth factors (see section 2.1. Media) diluted 1:2 with normal medium with 20% fetal calf serum. Cells derived from cords from different donors were pooled into 175 cm^2 tissue culture, surface treated flasks. When 60 to 70 % confluent, cells were passaged into tissue culture treated flasks of various sizes depending on experimental design. Culture flasks were surface treated with ECAF prior to passaging. HUVEC were grown in MCDB 131 medium with 20% FCS until confluent. Medium was changed to RPMI 1640 with 20% FCS at least 24 hours prior to stimulation. Cells were checked for characteristic appearance by phase contrast light microscopy and for the absence of contaminating leukocytes.

5.2.7 Bacterial Culture

Suspensions of bacteria were prepared as described in chapter 2

5.2.8 Stimulation of HUVECS

HUVEC were stimulated with either purified LPS or organisms. In some experiments, live organisms were used. In most instances, paraformaldehyde-fixed organisms were used. The constraint of having to use a Class I safety cabinet for work with live meningococci meant that these experiments were technically difficult to perform. In addition, overall the results were compatible whether using live or paraformaldehyde fixed organisms.

5.2.9 Nuclear Extraction protocol.

The nuclear extraction method used is based on the protocol first published by Dignam et al (Dignam, Lebovitz, & Roeder 1983). Modifications, where made, were according to method used in department of Molecular Rheumatology (J.Cheshire, personal communication and (Dent 1993).). In addition, a number of other changes were made. These included alterations to centrifuge speeds and time, and the inclusion of the second wash step in point 7 below. All reagents were put on ice and centrifuges pre-cooled to 4^0 C. Nuclear extraction buffers composition is described above. All extract buffers contained protease inhibitor cocktail, due to high protease activity in lysed cell preparations, which would rapidly degrade transcription factors.

1. HUVEC washed twice in ice cold RPMI 1640 and scraped off with cell scraper into 15-ml conical tube.
2. Cells centrifuged at 200g for 5 minutes.
3. Cell pellets resuspended in 0.5ml Buffer A (see table 2.1) and incubated on ice for 10 minutes.
4. Lysate centrifuged at 200g for 5 minutes.
5. Pellet resuspended in 0.5ml Buffer A with 0.1% TRITON X-100, vortexed for 15 seconds and incubated for further 10 minutes.
6. Evidence for cell lysis was then checked by light microscopy
7. Extract centrifuged at 500g for 5 minutes, and washed once more in Buffer A and centrifuged 750g for 5 minutes to leave cytoplasmic fractions and crude nuclear pellet. In some experiments, cytoplasmic fraction was taken off and aliquotted.
8. Nuclear pellet resuspended in equal volume of lysis Buffer C as described in table 6.2.

9. Suspension rotated for 30 minutes at 4^0 C and centrifuged at 20,000g for 30 minutes.
10. Supernatant was then aliquotted into small volumes and snap frozen in liquid nitrogen and stored at -70^0 C until required.
11. Protein estimations of nuclear extracts were performed using Bradford method.

5.2.10 Oligonucleotide sequences

Single stranded oligonucleotide sequences corresponding to consensus sequences known to bind a various transcription factors were taken from published journals. CD62E-1-κB binding site, 5'-3' GCCATTGGGGATTTCCTCTTT contains 21 nucleotides from -100 to -71 of the proximal κB sequence of the CD62E promoter (Tamaru & Narumi 1999). The sequence, GAGACAGAGTTTCTGACATCATTGTAA contains 27 nucleotides taken from -166 to -129 part of the CD62E promoter region corresponding to NF CD62E ATF cis-element (Tamaru & Narumi 1999).

The above sequences and their corresponding anti-sense sequences were obtained commercially at an amount of 0.2μmol. Stocks were made up to concentration of 1μg/μl in sterile water and stored at -20^0 C. Single stranded oligonucleotides were then made up to 20-picomol/μl according to the following formula:

Concentration (μg/μl) = pM/μl×330×length of oligonucleotide/1×10^6.

5.2.11 Preparation of Radio-labelled Oligonucleotide Probes

Compound	Amount µl
Single stranded oligonucleotide (20pmol)	2
[γ-^{32}P] ATP (3000mCi/mmol)	3
dd$H_2$0	27
10 times binding buffer	4
T4 polynucleotide kinase (10 units/µl	4
Total (µll)	40

1. Single stranded oligonucleotides were end labelled with [γ-^{32}P] ATP using T4 polynucleotide kinase in a binding buffer (commercially prepared with T4 Kinase). Typical reaction mixtures are given above.
2. Reaction mixtures were incubated at 37^0 C for 30 to 45 minutes.
3. Mixtures from sense and anti-sense sequences were then added together at total volume of 100µl and, heated to over 90^0 C in a heating block and left to anneal slowly over 6 hours to room temperature.
4. Purification of annealed oligonucleotides and removal of unincorporated [γ-^{32}P] ATP on Chromaspin G10 gel columns. Purified end labelled, double-stranded oligonucleotide probes were

checked for activity using Geiga-Muller counter. An adequate incorporation of radioisotope was an activity of >500 counts/second of 1µl of probe at 1cm.

5. Probes were stored in appropriately shielded containers at -20^0 C until required.
6. Unlabelled, double stranded (cold competitor) probes were prepared by incubating sense and anti-sense sequences in equimolar amounts at 95^0 C, leaving to cool over 5 to 6 hours to anneal, purification on chromatography columns and stored at -20^{0}C until required.

5.2.12. Preparation of polyacrylamide gels.

4 or 6% polyacrylamide (19:1 polyacrylamide: bis-acrylamide) gels were prepared, using 0.25 or 0.5M Tris Borate-EDTA (TBE) buffer depending on experimental design and the transcription factor under investigation. Gels were poured one day prior to running experiments. Gels were 1.5 mm thick with 14 0.5cm wells and were 17.5cm by 15.0 cm.

5.2.13 Electrophoretic Mobility Shift Assay (EMSA)

The conditions used for resolving protein-oligonucleotide complexes were according to which transcription factor were under study. In particular, analysis of different transcription factors required different binding buffers as detailed in the previous section on buffer composition. All binding reactions were performed on ice and electrophoresis performed between 2 and 6^0 C.

5.2.13.1 Protocol

Gels were placed clamped into a vertical mini-sequencing gel tank. Gels were pre-run at 125mV (10mV/cm) at 4^0 C for several hours until mA was at least half of the initial value. The running buffer varied according to transcription factor under investigation. For Nf-κB, 0.5M TBE buffer was used, for ATF2 0.25M TBE

was used. In some experiments, loading buffer with bromophenol blue was included in one lane but was not used in sample because of possible interference with assay.

5.2.13.2 DNA binding reactions

Reaction mixtures were set up to include the following components. Nuclear protein extracts were equalised for crude protein concentrations as described. For individual experiments, equal amounts of protein were included in the reaction mixtures. Between experiments, this varied from 1 to 5μg of total protein extract. 3-6μg poly (dI-dC). poly(dI-dC) was included in each reaction mixture as a non-specific competitor DNA source. The composition of gel shift binding buffer used dependent on which transcription factor was being assayed. In some experiments, 100-fold molar excess of unlabeled double stranded probe was included to demonstrate specificity of binding. One reaction mixture did not include any nuclear protein extract.

5.2.13.3 Supershift analysis

Identification of DNA/Protein complexes was performed using polyclonal antibodies to transcription factor components. These were added 45 to 60 minutes prior to addition of radiolabelled oligonucleotide probe at 4^0 C, at a concentration of antibody used were 2μg/20μl

5.2.13.4. Polyacrylamide gel electrophoresis

Reaction mixtures were assembled and made up to total of 20μl with ddH_2O. Loading of gel was performed with appropriate radioisotope shielding. 20μl was loaded into each well. Gels were run at 125mV over 4 to 6 hours, until radioactivity could just be detected in the bottom tank by Geiger-Muller detector. The gel was then removed for the tank apparatus, and transferred to several layers

of Whatman 3M paper, wrapped in cling film and dried under vacuum at 80^0 C. This was then exposed to auto radiography film in a shielded cassette at -70^0 C for between 12 and 72 hours and developed in automated film developer.

5.2.14 Analysis of Gels

Exposed films were scanned on a video densitometer using Alpha Ease (Alpha Innotech Corporation) imaging software. Laser densitometry of bands on film was expressed as integrated density values, linked to background of unexposed film. Assuming equal total protein concentration loaded into each well (based on Bradford protein assay), direct comparison of Integrated Density values from different stimuli was achieved.

5.3 Results

5.3.1 Nuclear translocation of the transcription factor NF-κB in HUVEC stimulated by *N. meningitidis* and purified LPS.

Using EMSA, it was possible to detect protein complexes that bound to double stranded oligonucleotide containing a known binding site for NF-κB that had translocated to the nuclei of HUVEC by meningococci, or purified *E. coli* LPS. The pattern of protein complexes observed in HUVEC stimulated with either LPS or the unencapsulated *siaD-* mutant was similar. In unstimulated cells, there was little specific band detected, indicating that there was little or no activated NF-κB in the nuclei of resting cells. This is in agreement with previously published findings of NF-κB binding in endothelium stimulated with the pro-inflammatory cytokines TNF-α, IL-1 and CD40 ligand (Karmann et al. 1995). Figure 5.5 shows the binding pattern in HUVEC stimulated for 2 hours with either medium, purified *E. coli* LPS (100ng/ml), parent B1940, or the *siaD-* mutant (both 10^7 CFU/ml). Two distinct specific bands could be detected in nuclei from stimulated cells that were only faintly detectable in unstimulated cells. The pattern observed did not reveal any major differences in composition of complexes induced in HUVEC by the different stimuli at this time exposure. There was a subtle difference in the lower band intensity of the complex in cells stimulated with the parent strain, although this is unlikely to be of any significance.

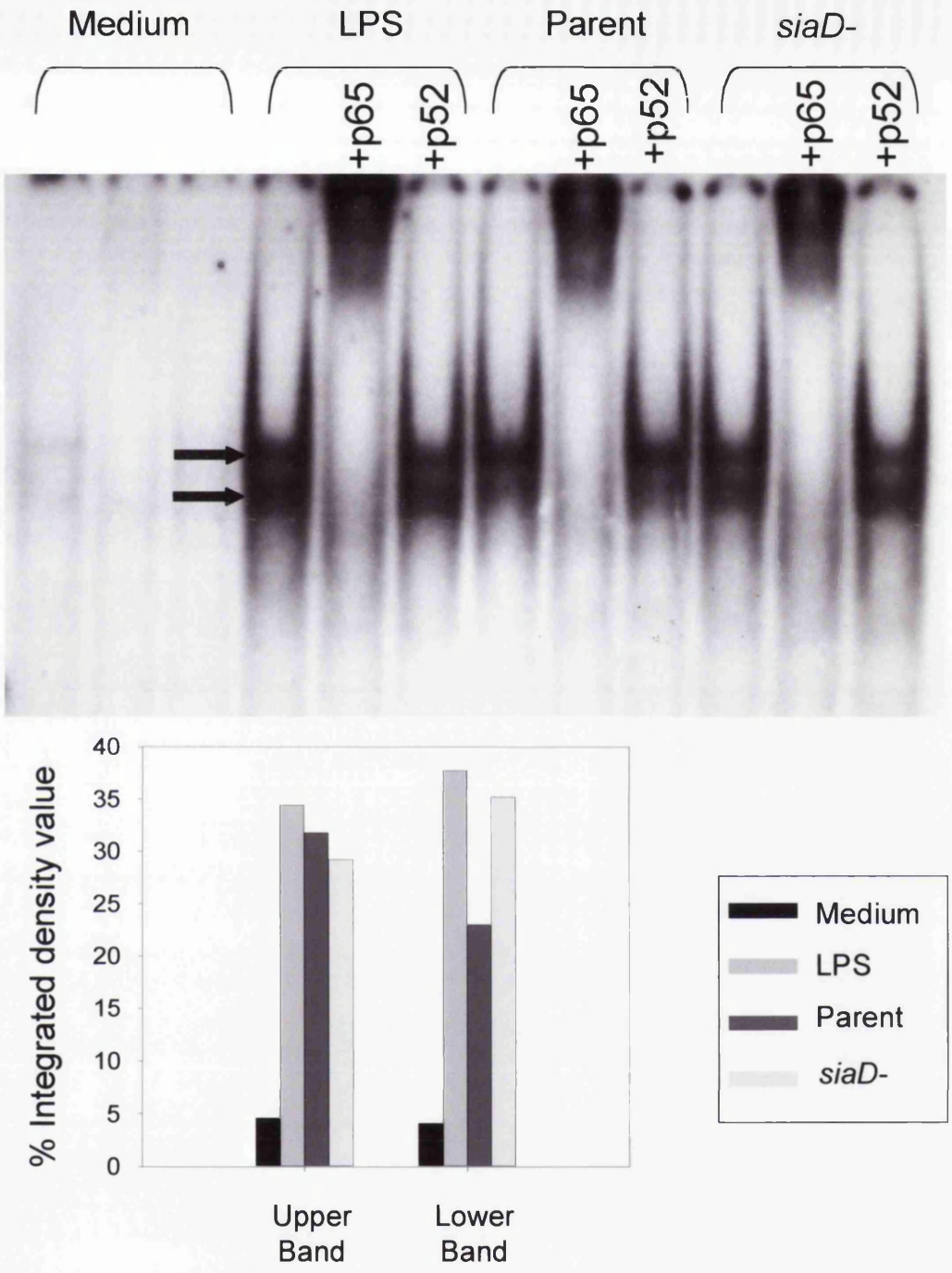

Figure 5.5. EMSA of protein complexes bound to NF-κB specific oligonucleotide probes in HUVEC stimulated with *N. meningitidis* B1940 parent and *siaD*- strains and LPS.

HUVEC were incubated with medium, 10^7 CFU/ml of *N. meningitidis* B1940 parent, *siaD*- strains, and 100ng/ml LPS for 2 hours. Nuclear extracts were prepared as described and incubated with ^{32}P-labelled oligonucleotide containing a NF-κB binding sequence within the CD62E promoter region. Supershift analysis using anti-p65, anti-p52 antibodies was used to determine the protein composition of induced complex.

5.3.2 Identification of protein complex bound to the NF-κB oligonucleotide.

In order to identify the composition of the proteins bound to the NF-κB oligonucleotide, supershift analysis was performed. In figure 5.5, the presence of a polyclonal antibody raised against the p65 sub-unit of NF-κB, shifts the strong specific band to the top of the gel, leaving a smaller, fainter lower band. This is consistent with previous studies that identify the strong upper band as p65/p50 heterodimers (Gilbert et al. 1996b). Antibody raised against p52 sub-unit induced no shift at all, indicating that this sub-unit is unlikely to participate in binding to this particular DNA sequence. In addition, this antibody also acts as a control to demonstrate specificity of binding of antibody to various sub-units of NF-κB. The identification of the lower band is likely to be a p50 homodimer, since it is shifted in the presence of antibody raised against p50, as seen in fig 5.9 (discussed later).

5.3.3 Effect of time exposure of on NF-κB translocation.

The results so far indicated that the nature of the complexes bound to the NF-κB site did not differ between purified LPS, or the meningococcal strains. However, there may be quantitative differences in NF-κB translocation at different time exposures. With this in mind, the HUVEC were stimulated with LPS, parent and *siaD*- strains at various time points and nuclear extracts prepared, as shown in Figure 5.6. NF-κB complexes could be detected at 15 minutes in HUVEC stimulated with meningococci and LPS. There was no consistent pattern in strength of signal between al the stimuli at this short time exposure.

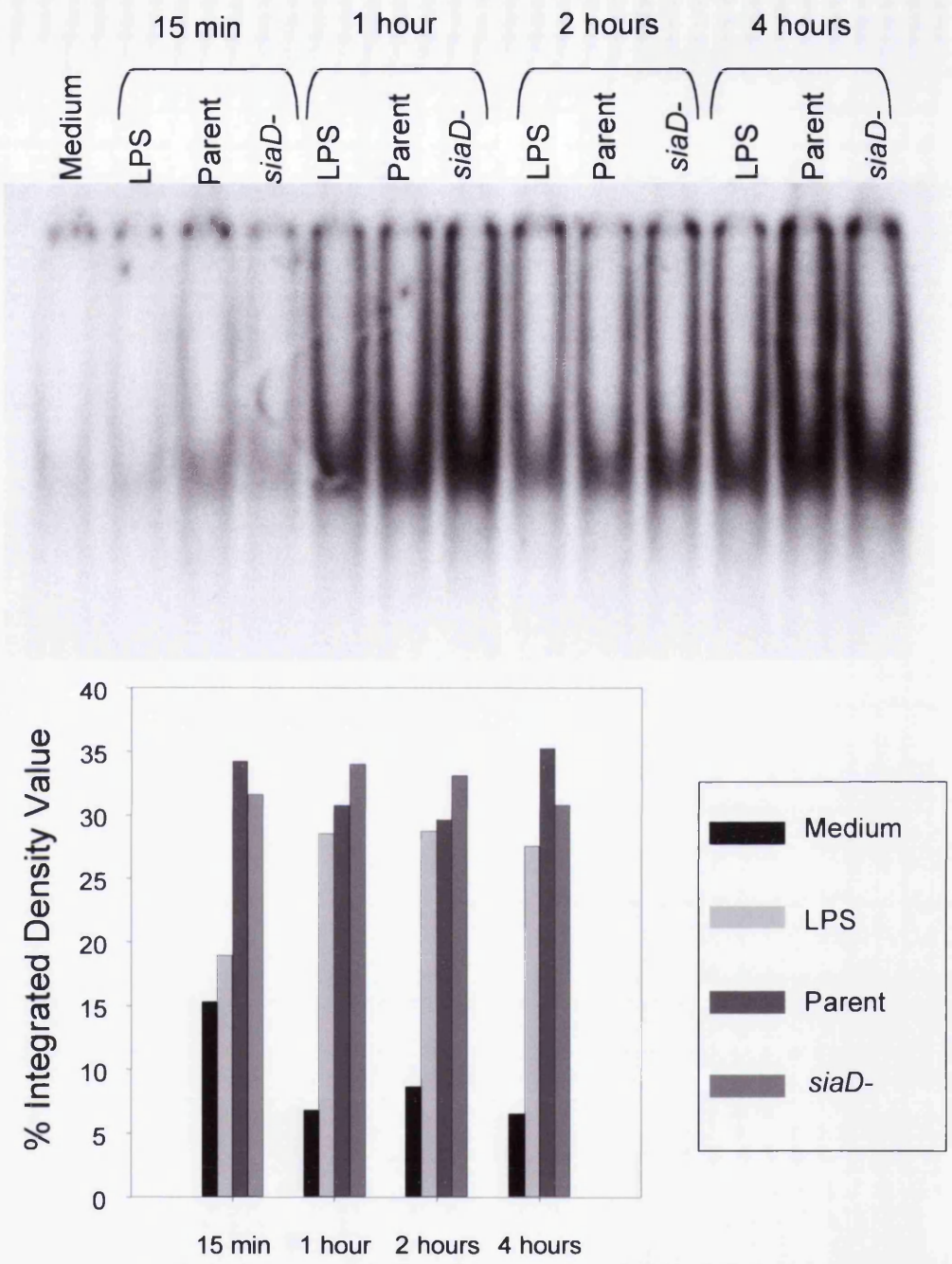

Figure 5.6 Time course of NF-κB protein binding complex in HUVEC stimulated with LPS and *N. meningitidis* B1940.

Nuclear extracts were prepared from HUVEC stimulated with 10ng/ml LPS, 10^6 CFU/ml B1940 parent and *siaD-* strains for the indicated time periods. These were incubated with ^{32}P-labelled oligonucleotide containing a NF-κB binding sequence within the CD62E promoter region.

After 1-hour exposure, strong bands could be detected in response to all the stimuli. However, in this case, the intensity of band extracts from cells stimulated with the *siaD-* mutant was greater than either the cells stimulated by parent

organism or purified LPS. This pattern was observed also at 2 hours exposure. After 4 hours exposure, both meningococcal strains induced similar signal strength, which was greater than that seen in response to LPS. Over a number of repeated experiments, the intensity of Nf–kB specific band was consistently greater in HUVEC stimulated with the *siaD-* strain than the parent strain or purified LPS after 1 hour stimulation.

5.3.4 Activation of transcription factors ATF2/c-jun/AP-1 in HUVEC stimulated with *N. meningitidis* and purified LPS.

As discussed in the introduction to this chapter, induction of CD62E transcription is dependent on the co-ordinate action of both NF-κB and members of the ATF2 and c-jun families of transcription factors. Therefore, EMSA was used to detect any changes in the pattern of binding of these transcription factors to the CRE-like ATF2 site. In contrast to NF-κB proteins, transcription factors that can bind to this consensus sequence are constitutively found in nuclei of unstimulated cells. Additionally, the appearance of the bands is critically dependent on a number of important experimental variables. This includes the composition of buffer within the DNA binding assay, the ionic concentration within the running buffer and the concentration of polyacrylamide gel used to resolve the reaction mixture. In order to identify the composition of the bands seen, it was necessary to include antibodies to both ATF2 and c-jun/AP-1 proteins to produce a supershift.

Figure 5.7 shows the pattern observed in nuclear extract preparations of HUVEC stimulated with LPS, parent B1940 and the *siaD-* mutant for 1 hour. There are 2 detectable band complexes in unstimulated cells. Both of these 2 bands shifts with both ATF2 and AP-1/c-jun antibodies, and indicates that these

represent complexes that include both ATF2 and AP-1/c-jun proteins, likely to be heterodimers. Video densitometry shows that these complexes are increased in HUVEC stimulated with LPS and both strains of meningococci.

In order to improve the resolution of these complexes, changes were made to both composition of DNA binding buffer, running buffer and polyacrylamide content of the gel, according to the method used by Tamara and Narumi (Tamaru & Narumi 1999). This resulted in much better resolution of the protein complexes (see fig 5.8). In addition to the two complexes shown in the previous figure, there is another feint but discrete upper band (C1 or complex1) that shifts with the ATF2 antibody. In agreement with figure 5.7, the second, lower complex (C2) shifts with both antibodies, indicating a complex containing both proteins, probably as heterodimers. The third, lowest specific complex (C3) is shifted partially by AP-1/c-jun antibody and not by the ATF2 antibody. It is possible that this complex contains proteins of the c-jun/c-fos family, which may cross react with the polyclonal antibody used in this study. However, no further identification of the proteins in this 3rd complex was attempted.

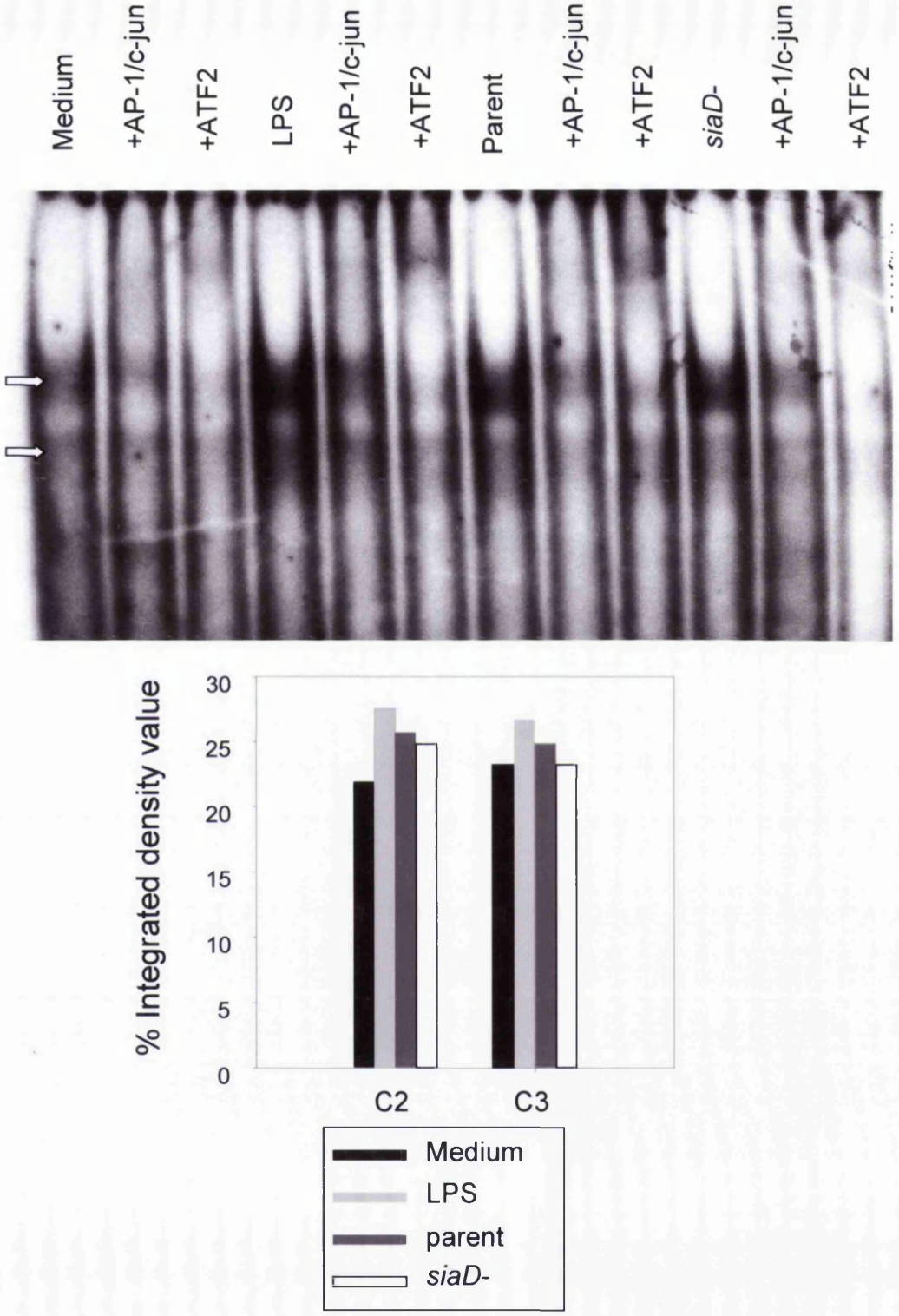

Figure 5.7. EMSA of protein complexes bound to ATF/CRE-like sequence containing probe in HUVEC stimulated with *N. meningitidis* B1940 parent and *siaD*- strains and LPS.

HUVEC were incubated with medium, 10^6 CFU/ml of *N. meningitidis* B1940 parent, *siaD*- strains, and 10ng/ml LPS for 1 hours

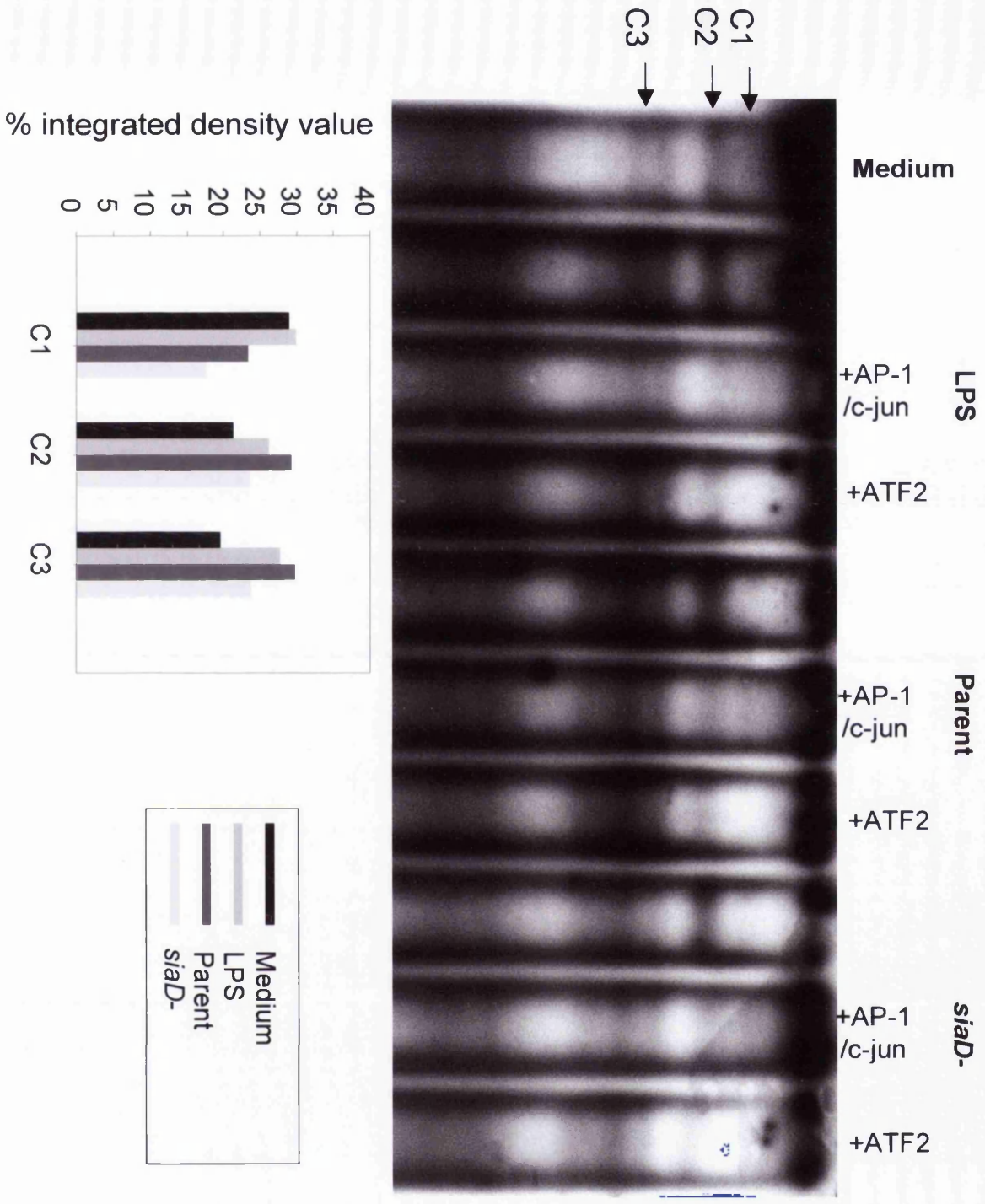

Figure 5.8. EMSA of protein complexes bound to ATF/CRE-like sequence containing probe in HUVEC stimulated with *N. meningitidis* B1940 parent and *siaD*- strains and LPS.

HUVEC were incubated with medium, 10^7 CFU/ml of *N. meningitidis* B1940 parent, *siaD*- strains, and 10ng/ml LPS for 4 hours. Nuclear extracts were prepared and binding to radiolabelled ATF2/CRE oligonucleotide sequence analysed by EMSA. Supershift analysis was performed using antibody against AP-1/c-jun and ATF2 proteins. Band densities were quantified using video densitometry.

When analysed by video densitometry, this feint band is denser in both unstimulated and LPS stimulated cells compared to extracts from cells stimulated with parent or *siaD-* strain. In addition, the complexes C2 and C3 are quite clearly increased in all the stimulated samples as compared to the control. Interestingly, and perhaps in contrast to what might have been expected, the results consistently showed that the *siaD-* mutant induced less of an increase in both of the two protein complexes C2 and C3 compared to LPS of the parent strain.

5.3.4 NF-κB translocation and changes in binding pattern of the ATF2/c-jun complexes induced by *N. meningitidis* parent, LPS deficient *lpxA-* strain and purified LPS

The results so far indicate that LPS and both the meningococcal strains are potent inducers of translocation of NF-κB into nuclei of HUVEC and changes in the binding pattern of the ATF2 and c-Jun protein complexes, both of which are required for initiation of CD62E transcription. Some differences in these binding patterns were observed in HUVEC stimulated with LPS compared to the organisms. To extend this study further, nuclear extracts in HUVEC stimulated with the group B meningococcal parent 44/76, and the LPS deficient mutant, *lpxA-* was analysed by EMSA. In this way, the contribution of non-LPS components of meningococci in signals which affect the activation of these transcription factors, and hence CD62E transcription, was assessed. The first question asked was does the LPS deficient strain induce same composition of transcription factors, and secondly can we quantitate the strength of the signal induced.

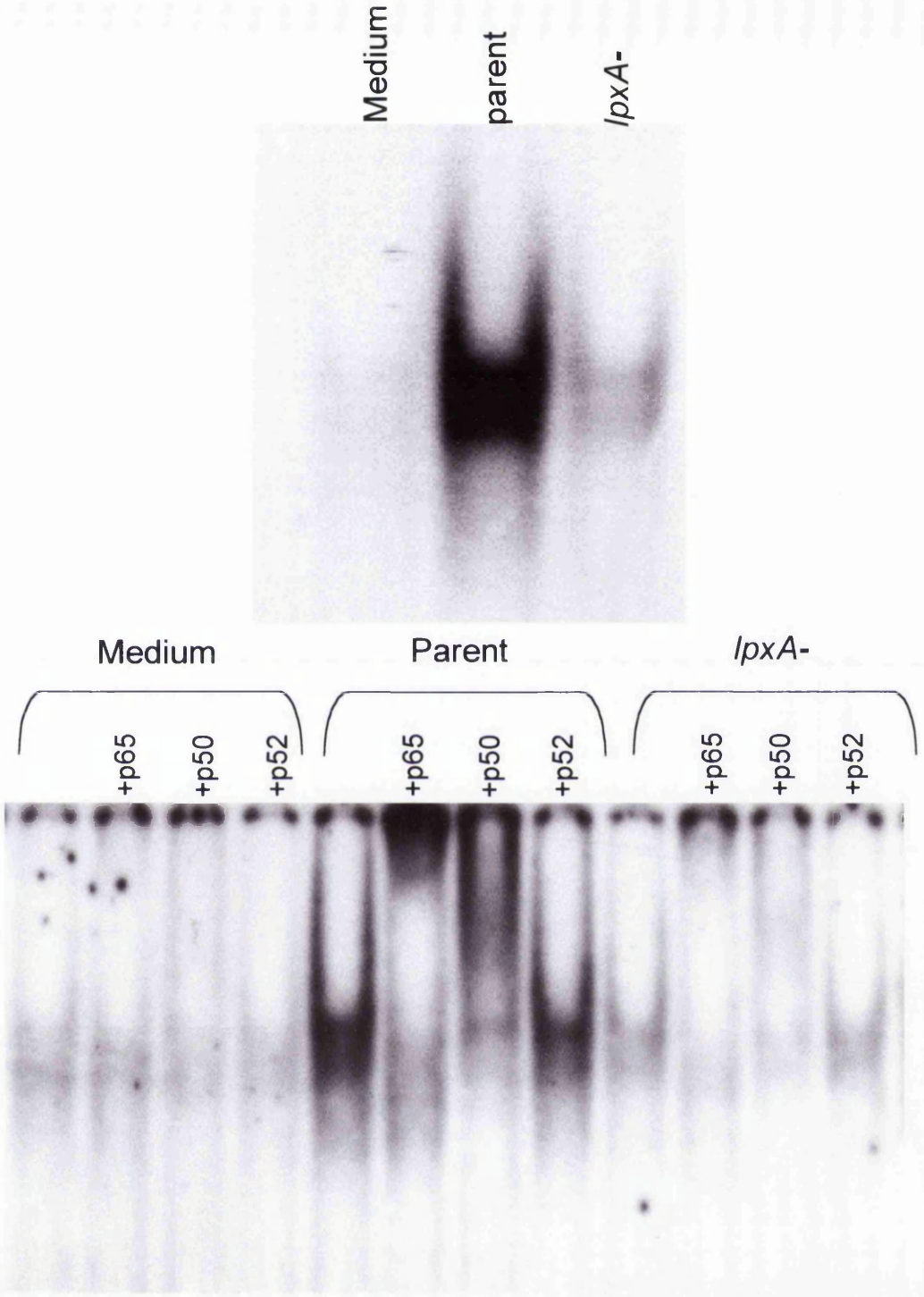

Figure 5.9 EMSA of protein complexes bound to NF-κB specific oligonucleotide probes in HUVEC stimulated with *N. meningitidis* H44/76 parent and *lpxA*- strains.

HUVEC were incubated with medium, 10^7 CFU/ml of *N. meningitidis* H44/76 parent, and *lpxA*- strains, for 2 hours. Nuclear extracts were prepared as described and incubated with ^{32}P-labelled oligonucleotide containing a NF-κB binding sequence within the CD62E promoter region. Supershift analysis using anti-p65, anti-p50, anti-p52 antibodies was used to determine the protein composition of induced complexes.

Figure 5.9 shows that, at stimulating dose of 10^7 organisms/ml, both the parent strain and *lpxA*- strain can induce NF-κB translocation into the nucleus. Supershift analysis confirms that the upper band corresponds to p65:p50 heterodimers whereas the lower band is probably p50 homodimers, since the lower band shifts completely with p50 antibody. There is a strong discrete band that is strongly shifted by p65 antibody but not by the p50. It may be that this band is p50 homodimer that have shifted up, or even another NF-κB complex (but not p52), possibly p65 homodimers. What is striking is that the *lpxA*- is far less potent in inducing NF-κB translocation than the parent strain. Even at 10^8 organisms/ml, the concentration at which the *lpxA*- can induce high levels of surface CD62E expression, and the level of NF-κB is less than either the parent or even high dose (100ng) of LPS (data not shown). Interestingly, almost no NF-κB specific band could be detected in response to 10^6 /organisms of *lpxA*- mutant (data not shown).

The *lpxA*- mutant also induces a small, but definite increase in complexes C2 and C3 bound to the ATF2/c-jun sequence, as shown in fig 5.10. Complex C1 observed in fig 5.8 was not seen in this assay. This may relate to the fact that the complex may be present in very small quantities and the protein concentration of the extract not high enough for this complex to be detected. At the higher stimulating dose of 10^8 CFU/ml, after 4 hours exposure, although very feint, it can be seen that the complex C1 is present in the unstimulated and LPS stimulated HUVEC (fig. 5.11). By contrast, this complex is reduced in the nuclear extracts from HUVEC stimulated with parent, *lpxA*- and *siaD*- organisms. This is shown in figure 5.11.

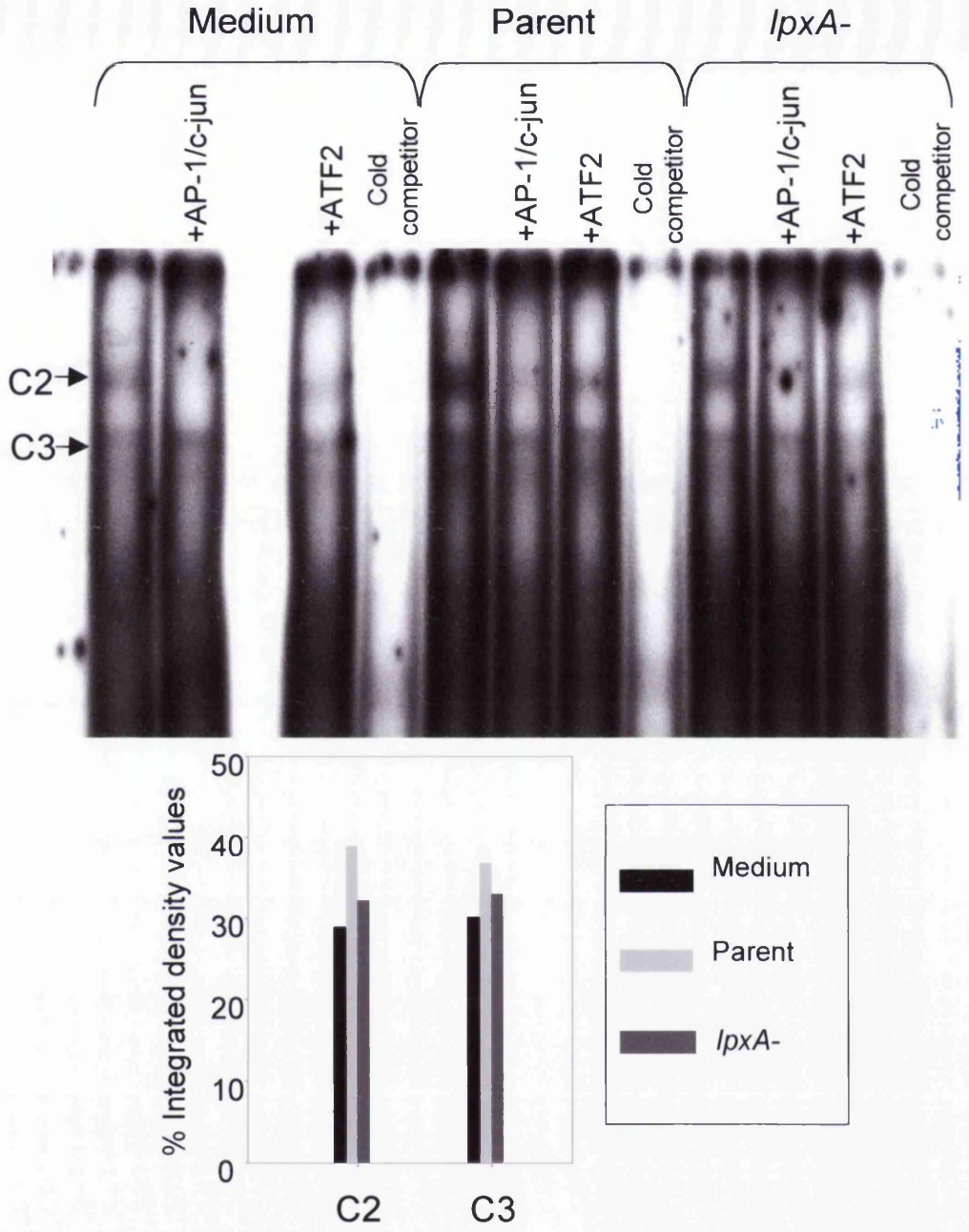

Figure 5.10 EMSA of protein complexes bound to ATF/CRE-like sequence containing oligonucleotide in HUVEC stimulated with *N. meningitidis* H44/76 parent and *lpxA*- strains.

HUVEC were incubated with medium, 10^7 CFU/ml of *N. meningitidis* H44/76 parent and *lpxA*- strains for 2 hours. Nuclear extracts were prepared and binding to radiolabelled ATF2/CRE oligonucleotide sequence analysed by EMSA. Supershift analysis was performed using antibody against AP-1/c-jun and ATF2 proteins. Band densities were quantified using video densitometry.

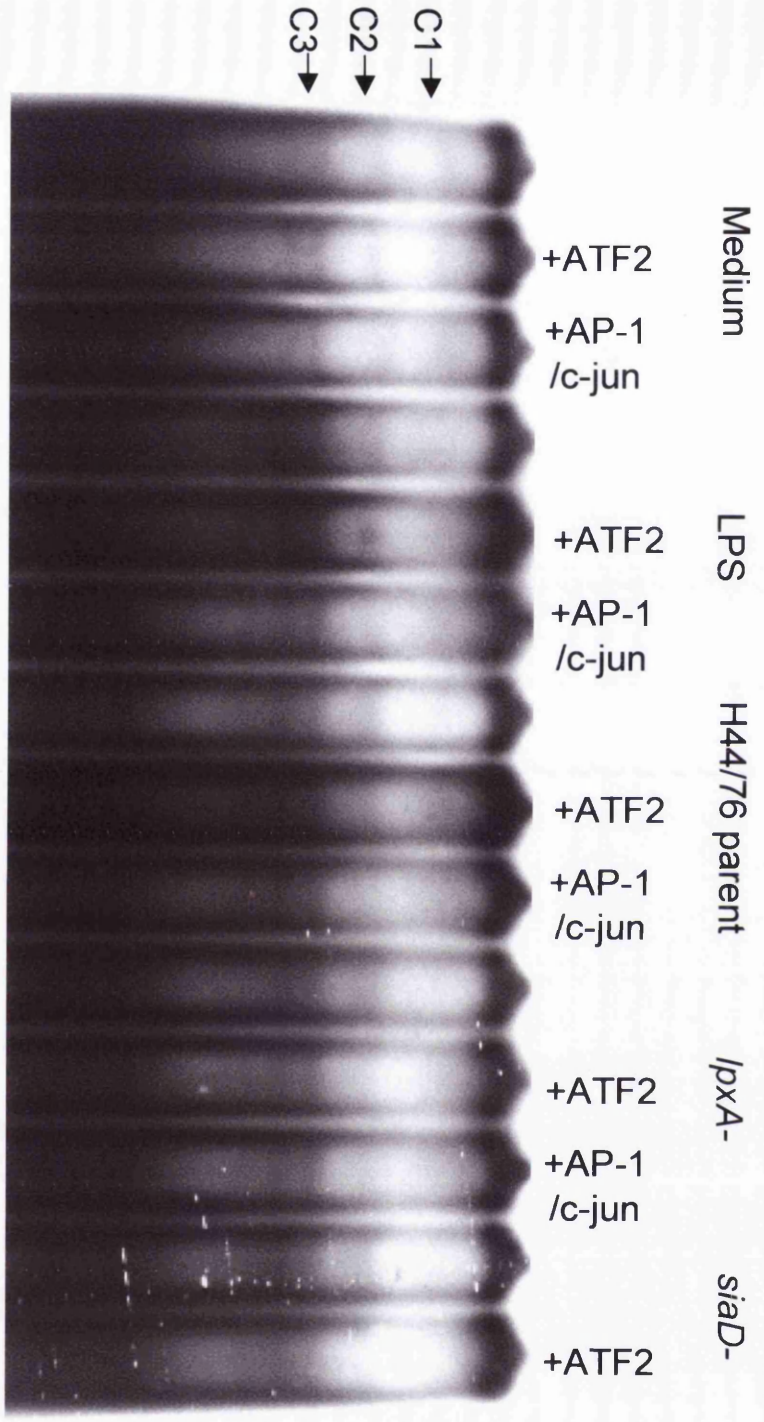

Figure 5.11. EMSA of protein complexes bound to ATF/CRE-like sequence containing probe in HUVEC stimulated with 10^8CFU/ml of *N. meningitidis* H44/76 parent, *lpxA*- and *siaD*- strains and 100ng/ml LPS.

HUVEC were incubated with medium, 10^8 CFU/ml of *N. meningitidis* H44/76 parent, *lpxA*-, *siaD*- strains, and 100ng/ml LPS for 4 hours. Nuclear extracts were prepared and binding to radiolabelled ATF2/CRE oligonucleotide sequence analysed by EMSA. Supershift analysis was performed using antibody against AP-1/c-jun and ATF2 proteins. Band densities were quantified using video densitometry.

5.4 Discussion

The present study used EMSA to investigate the activation of transcriptional regulators known to control the induction of expression of CD62E in HUVEC. Since such a marked difference in surface CD62E expression was observed in response to purified LPS and meningococci, particularly unencapsulated mutants it was postulated that meningococci were delivering additional signals to LPS alone to the transduction machinery involved in transcriptional control of this molecule. Additionally, it was clear that even in the absence of LPS, meningococci could induce high levels of surface CD62E expression, at least at high bacterial concentrations. It was thus important to establish whether non-LPS components in meningococci could activate these transcription factors, critical not only to CD62E expression but also a large number of different genes induced in the inflammatory response.

The data clearly shows that meningococci and LPS are potent inducers of nuclear translocation of the activated component of NF-κB in to nuclei of HUVEC. The composition of the NF-κB complex induced by all the stimuli was similar. This study utilised one NF-κB specific consensus sequence from the CD62E promoter. There are three such sequences in this promoter region, all of which have the capacity to bind p65: p50 heterodimers (Collins et al. 1995). It remains unknown whether the use of other consensus sequences would have revealed any differences in composition of NF-κB proteins induced. Different consensus sequences will have different affinities for NF-κB proteins, but will bind the same composition of protein (J. Cheshire, personal communication)

Analysis of the time course revealed some differences between LPS and meningococci. The NF-κB specific band detected in cells after 1 hour stimulation

with meningococci was consistently stronger than that seen with LPS stimulated cells. These results could be due to a number of reasons. It may simply be a dose effect, which is hard to control for unless LPS is specifically quantified in meningococcal samples at the time and compared to purified LPS. As shown in both chapters 3 and 4, dose of LPS does not explain the differences in relative potencies of the meningococci to induce CD62E expression as compared to LPS. Even if concentration of LPS were equivalent, differences in binding of LPS that is either in micelles of purified LPS and that within bacteria to CD14/LBP/TLR4 receptor complex may alter both pattern and kinetics of the downstream signal transduction mechanisms.

Despite the clear differences in surface expression the pattern of NF-κB activation in HUVEC stimulated with either purified LPS and the organisms did not differ greatly. This may not be that surprising. Activated NF-κB is involved in the transcriptional regulation of a large number of genes, including ICAM-1 and VCAM-1 (Collins et al. 1995), which are as potently up-regulated by LPS compared to meningococci. Binding of NF-κB to a promoter region is an absolute requirement for transcriptional up-regulation of CD62E, but although the amount of activated NF-κB is a measure of the potency of the signal, EMSA does not measure transcriptional activity of the promoter in question (Dent 1993). In addition, despite the increased potency of the unencapsulated *siaD-* strain to induce surface CD62E expression, it is interesting to note that the difference in capacity of the two strains to induce translocation of NF-κB were minor. As mentioned, the only consistent difference observed was the increase in signal after 1-hour stimulation by the *siaD-* strain. It is tempting to speculate that this is because the binding of LPS (or any component of meningococci involved in signal

transduction) to CD14/LBP/TLR4 may depend on bacterial endothelial contact, which may be altered significantly by the presence or absence of capsule, as is the case for meningococcal adhesion to endothelium.

Although NF-κB binding to the promoter is necessary for CD62E gene transcription, transcription factor binding to the CRE-like/ATF2 site is indispensable for this process. The data shown here demonstrate that both LPS, parent and *siaD*- strains induce alterations in protein complexes bound to this consensus sequence site, which is consistent with previous studies that have shown similar patterns in response to TNF-α or IL-1a stimulated endothelial cells (De et al. 1994;Kaszubska et al. 1993). Cytokine driven CD62E gene transcription involves the co-ordinate action of both NF-κB activation and kinases present in the nuclei, c-jun terminal kinase and p38, which phosphorylate and activate members of c-jun/c-fos family and members of the ATF family (Read et al. 1997). Analysis of the changes in transcriptional binding activity upon cytokine stimulation shows that there is a loss of ATF2 homodimers, and increase in ATF2/c-jun heterodimers (De Luca 1994). The results in this study show that when HUVEC are stimulated with LPS and meningococci, there is an increase in band that relates to increase amounts of ATF2/c-jun heterodimers in a similar fashion. In addition, all the stimuli increase signal strength in the third, lower band, which contains a protein that is either c-jun/AP-1 or a related molecule, or more likely both. What is perhaps more interesting is the effects that the meningococci have on the feint upper complex, which previous work has shown are composed of ATF2 homodimers (De Luca et al 1994)

The importance of the ATF2 site on the synergistic up-regulation of both CD62E expression and transcriptional activity of CD62E gene observed with a combination of stimuli was demonstrated recently by Tamaru and Narumi

(Tamaru & Narumi 1999). They were able to show that phorbol esters, which activate Protein kinase C, synergises with IL-1a but not TNF-α to induce maximal levels of CD62E expression, which was dependent on the integrity of the ATF2 site within the CD62E promoter. The binding pattern of transcription factors bound to the ATF2 site observed in the study by Tamaru and Narumi was very similar pattern to those presented in this chapter. That is, a fall in the complex 1 (thought to be ATF2 homodimers) and increases in complexes 2 and 3 (made up of ATF2, c-jun heterodimers and probably other combinations of proteins). The most marked fall in this complex was only observed in HUVEC stimulated by meningococci after 4 hours exposure. However, as Tamaru and Narumi also show, this binding pattern does not fully explain the synergy seen between IL-1a and PMA because the same changes in transcription binding to ATF2 site are seen with TNF-α and PMA, where there is no synergy (Tamaru & Narumi 1999). The authors postulates that protein-protein interactions between ATF2, c-jun and NF-κB are important determinants of transcription factor activity, which cannot necessarily be predicted by EMSA.

The demonstration that the *lpxA*- strain induces activation of NF-κB, and increases in activated ATF2 and c-jun protein complexes in HUVEC clearly shows that non-LPS components contribute to the overall signalling induced by parent meningococci. It is also clear that these components are far less potent inducer of these transcription factors than the LPS in the context of bacteria. Indeed, it was very hard to detect any NF-κB activation at 10^6 organisms of *lpxA*- strain, consistent with the surface expression shown in Chapter 4. Why this is so is not entirely clear. However, it is interesting to note that gram positive bacteria are generally poor inducers of CD62E expression (Noel-RF et al. 1995). One

explanation for why this is the case might be due to differences in expression or activity of TLR's on endothelial cells. Whereas TLR4 appears to be specific receptor for LPS, TLR2 seems to be able to recognise a number of microbial products, some of which, like peptidoglycan and lipoproteins, are present in gram-negative and gram-positive bacteria, whereas lipoteichoic acid is found only in gram-positive bacteria (Brightbill et al. 1999b;Schwandner et al. 1999;Takeuchi et al. 1999b).

It seems likely therefore, that a combination of both TLR4 (LPS) and TLR2 (for example from lipoproteins and peptidoglycan) dependent pathways are activated by meningococci on contact with endothelial cells. This in itself does not explain why expression of CD62E is so potently induced by meningococci compared to LPS. The surface expression data indicates that both LPS and LPS independent signals may be synergistic, at least in the case of CD62E expression. As mentioned above, Il-1a and PMA synergistically up-regulate CD62E gene expression at a transcriptional level. Demonstrating this synergy would be technically difficult for a number of reasons. The fetal calf serum in medium activates classic protein kinase C (Tamaru & Narumi 1999). Serum is required for both maintenance of endothelial cells in culture and to provide a source of soluble CD14 for LPS mediated signalling, making it hard to distinguish between all the possible signals that may be activated in this system. Theoretically one could provide an exogenous source of CD14 and stimulate cells in serum free conditions.

There are a number of TLR independent mechanisms that could theoretically provide the necessary signals. As discussed in the introduction to this chapter, the GTPases CDC42 or RAC are activated during interactions between

N. gonorrhoeae in epithelial cells (Naumann et al. 1998). This pathway activates c-jun/AP-1 kinase JNK, which can phosphorylate and activate c-jun/AP-1 (fig 5.12).

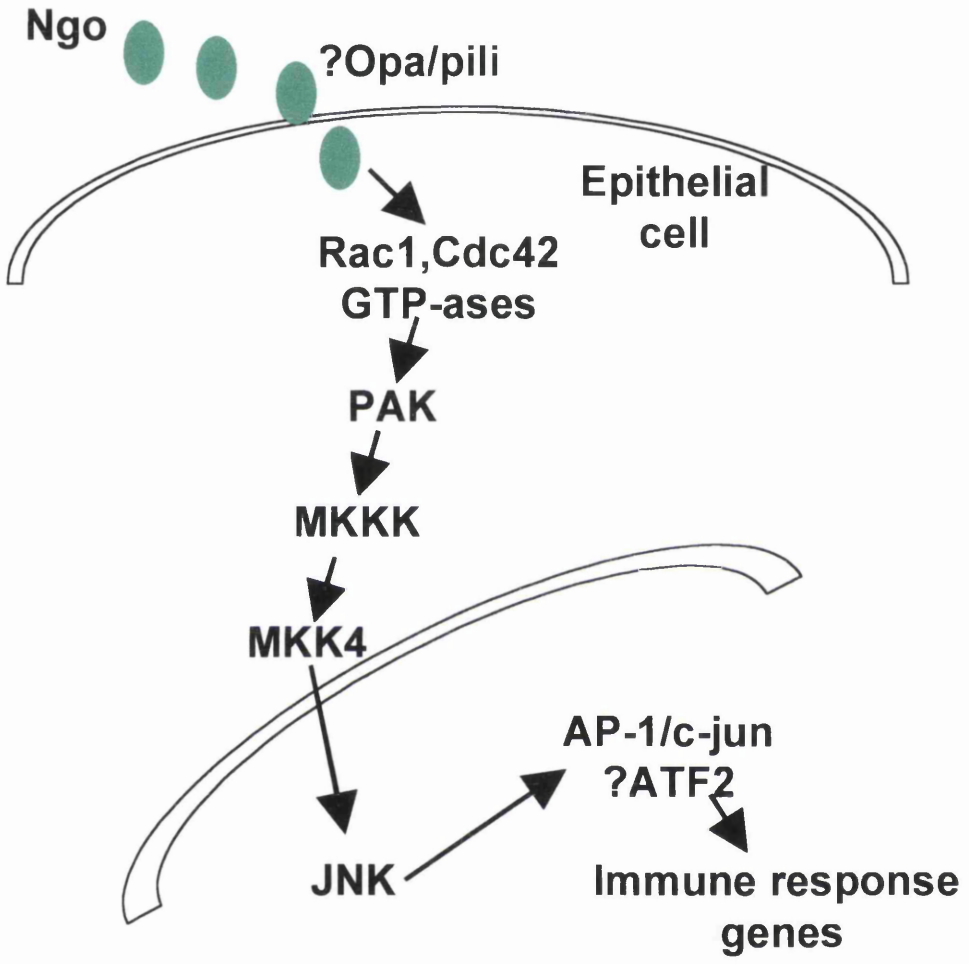

Figure 5.12. Pathogen specific activation of Jun kinase (JNK)by *Neisseria gonorrhoeae*.

Contact between virulent *Ngo* and epithelium results in activation of JNK via a cascade of stress response kinases to induce pro-inflammatory cytokines. Adapted from (Naumann et al. 1998).

Theoretically at least, activation of this pathway could phosphorylate ATF2 and c-jun/AP-1 proteins, and act in concert with TLR dependent pathways to induce the necessary signals to cause synergistic activation of CD62E expression as described in (Tamaru & Narumi 1999). This would depend upon demonstration of

a specific activation pathway in meningococci, and thus remains speculative. In addition, receptor mediated internalisation of bacteria involves activation of small GTPases (Caron & Hall 1998) which can activate both Nf-κB and JNK (Perona et al. 1997b). This would provide a link between mechanisms responsible for adherence and invasion of cells by meningococci and activation of a signal transduction pathway that could act in concert with TLR signalling. This would be a fascinating explanation of how unencapsulated mutants, which display greater adherence to endothelia and appear to be more efficiently internalised than capsulated strains, induce high levels of CD62E expression. Clearly further studies would be needed in order to dissect out these multiple pathways.

In addition, there remains a strong possibility that the increase in surface expression of CD62E in meningococci depends on post-transcriptional and post-translational mechanisms. This could include stabilisation of messenger RNA and increase in its half-life, post-translational modification of CD62E protein. In addition, since surface expressed CD62E is re-internalised via endocytic removal (Von Asmuth et al. 1992), it may be that meningococci somehow interfere with this process and contribute to high surface expression of surface CD62E expression. If this was specific to meningococci, it would be an interesting and novel reason for the observed differences in cell adhesion molecule profiles demonstrated in this thesis.

Chapter 6

6. Dendritic cell responses to *N. meningitidis:*

6.1. Introduction

6.1.1 Background

This study was undertaken for a number of reasons. The previous chapters demonstrated that bacterial structure is a critical determinant of endothelial inflammatory response to meningococci. From a conceptual point of view, how bacterial properties such as capsulation, LPS structure and non-LPS components, effect different cell types may provide valuable insight into host-bacterial interactions generally. In addition, how DC's respond to meningococci *in vitro* seeks to address important biological questions. DC's are highly adapted to interact with invading micro-organisms, and as such are likely to have a prominent role in the development of protective immune responses against pathogenic bacteria such as *Neisseria meningitidis,* both in the course of natural infections and also after exposure to vaccines.

Dendritic cells are highly specialised antigen presentation cells that form a gateway between the innate and adaptive immune system. Exposure of DC's to an invading pathogen triggers a series of activation events involving antigen uptake and processing and also migration to specialised lymphoid tissue for antigen presentation to T-lymphocytes(Banchereau & Steinman 1998a). In addition, activated DC's generate signals that alert the immune system to potentially dangerous foreign material that modulates subsequent lymphocyte activation and differentiation (Lanzavecchia 1998;Rescigno et al. 1999). Some of these signals are mediated by direct contact through co-stimulatory molecules CD40, CD80 (B7.1) and CD86 (B7.2) that are increased upon DC maturation and whilst others are mediated by cytokines and chemokines (Ridge, Di, & Matzinger 1998;Sallusto et al. 1998;Sallusto, Lanzavecchia, & Mackay 1998)

6.1.2 Origin of Dendritic cells.

Dendritic cells have been classically defined as being either myeloid or lymphoid depending on the nature of stem cell progenitors, cell surface phenotype and location in the body (Cella, Sallusto, & Lanzavecchia 1997). Lymphoid DC's develop from CD4 low precursors and become CD8α positive and Fas ligand positive, and are prominent in both thymus and other lymphoid tissue (Cella, Sallusto, & Lanzavecchia 1997;Wu, Li, & Shortman 1996;Zhang et al. 1999). Myeloid DC's can be generated *ex vivo* from either CD34 positive stem cells or from CD14 positive monocytes. Both these lineages require GM-CSF for differentiation (Cella, Sallusto, & Lanzavecchia 1997). However, peripheral blood monocytes require IL-4 in addition to GM-CSF to become immature DC's, a process that has been exploited in recent years to produce large quantities of DC's for experimental purposes. In the presence of M-CSF rather than GM-CSF, immature DC's assume macrophage behaviour and phenotype. In the case of CD34 positive precursors, those that are CD14 positive develop into DC's in the presence of GM-CSF, whereas those that are CD1a positive develop into Langerhan's cells (Cella, Sallusto, & Lanzavecchia 1997).

Recently, two different sub-populations of DC's, termed DC1 and DC2 have been described. DC1 cells are derived from peripheral blood monocytes, which in response to GM-CSF and IL-4, differentiate into immature myeloid DCs, whereas DC2 cells differentiate from plasmacytoid cells (CD4+, CD3-, CD11c-) in the presence of IL-3 and mature in the presence of CD40 ligand (Rissoan et al. 1999). The influence of these two types of cells will be discussed later.

6.1.3. Antigen capture by DC's

Dendritic cells are particularly prominent at sites close to the external environment, such as the skin, naso- pharyngeal and gastro-intestinal mucosa

(Austyn 1996). When DC's come into contact with potentially dangerous material, they undergo a remarkable maturation process. The behaviour of DC's is critically dependent on their stage of maturation, which defines their response to the micro-environmental triggers that they encounter. Immature Dendritic cells are characterised by their ability to sense, capture and internalise foreign material (Lanzavecchia 1996). They express a large number of receptors such as FcγR, mannose receptors, complement receptors such as CR3, and exhibit high levels of receptor-mediated phagocytosis and fluid phase uptake of antigen such as macro- and micropinocytosis (Cella, Sallusto, & Lanzavecchia 1997;Steinman & Swanson 1995). Production of chemokines and cytokines such as TNF-α and IL-1 attract and stimulates local inflammatory cells such as NK cells, macrophages and other DC's which are necessary for effective innate response (Austyn 1996). Once antigen capture has taken place, a number of these processes are effectively down regulated; including loss of macropinocytosis activity and mannose receptor expression (Sallusto et al. 1995).

6.1.4. DC's migrate to lymphoid tissue after antigen capture

After uptake of antigen, DC's undergo migration to lymphocyte areas of secondary lymph organs, where they change their behaviour from one of high antigen capture to one of efficient antigen processing (Lanzavecchia 1996;Sallusto et al. 1995). Immature DC's express low levels of MHC molecules but once maturation takes place, these are synthesised by DC's and are loaded with processed antigen. This is dependent on both the route of processing and the nature of antigenic material itself. Typically, intracellularly processed antigen is presented by class I MHC to CD8+ve, cytotoxic T lymphocytes. MHC Class II presents extracellular antigen to CD4+ve T helper lymphocytes (Banchereau & Steinman 1998a). However, cross presentation of exogenous antigen to MHC class

I molecules can also occur (Albert et al. 1998). Migration itself is a complex process that involves alteration in DC behaviour, including the 'co-ordinated switching' of chemokine and chemokine receptors (Sallusto et al. 1998;Sozzani et al. 1998;Yanagihara et al. 1998).

6.1.5 Maturation of DC's involves up-regulation of co-stimulatory molecules and soluble mediators required for optimal interaction with T-lymphocytes

In addition to their role as antigen presenting cells, DC's also provide critical signals that modulate subsequent lymphocyte activation and development (Ridge, Di, & Matzinger 1998). Some of these involve direct contact between dendritic cells and T lymphocytes through co-stimulatory molecules such as CD40, CD80 (B7.1) and CD86 (B7.2), which are up-regulated on DC maturation. Others are provided by soluble mediators produced by DC's, such as cytokines and chemokines (Sallusto, Lanzavecchia, & Mackay 1998). DC's are the only antigen presentation cells that have been shown to prime naive, $CD4^{+ve}$ T lymphocytes, which is dependent on expression of co-stimulatory molecules (Banchereau & Steinman 1998a). Helper T-lymphocytes recognise antigen presented in a MHC restricted manner but also require secondary signals for optimal functioning (Schoenberger et al. 1998).

6.1.6. Complex reciprocal signalling between DC's and T lymphocytes contributes to T cell polarity

The balance between a Th1 and Th2 response is thought to be controlled at the interaction between the antigen presenting cell and cognate lymphocyte. Hence, when DC's interact with CD4+positive T lymphocytes via antigen presented by MHC class II and T cell receptor, interactions between CD40 on DC's and CD40 ligand stimulates the DC to produce IL-12 (Ridge, Di, &

Matzinger 1998;Schoenberger et al. 1998). Production of IL-12 is pivotal to the development of Th_1 response, and is critical to protection against intracellular pathogens (Macatonia et al. 1995;Trinchieri 1993). IL-12 stimulates T cells to produce IFN-γ, , favouring a Th1 type response. Importantly, production of IFN-γ itself by memory T lymphocytes further induces DC's to produce IL-12 (Cella et al. 1996). Blocking of IL-12 completely suppresses Th1 type responses *in vitro* and *in vivo* (Trinchieri 1995). The presence of IL-12 during antigen presentation to naive T cells primes them for a Th1 response that remains when they are expanded clones (Manetti et al. 1994). Naïve cells that are expanded in the absence of IL-12 are poor producers of IFN-γ and do not display typical Th1 features upon subsequent stimulation. This suggests that the events that take place at the initial stages of naive T cell priming are critical to subsequent immune response.

The Th2 type response is considered important in immune response against extracellular pathogens such as helminths. The presence of cytokines IL-4, IL-10 and TGF-β, which are important mediators of a Th2 type responses, could be produced by antigen specific Th2 cells, committed memory T cells or mast cells (Trinchieri 1995). IL-4 is inhibitory to Th1 cells and directs the development of Th2 type profile. (Swain et al. 1990). Antigen specific, Th2 transgenic cells inhibit the production of IL-12 by dendritic cells and thus Th1 type responses. In one study, this was found to be due to production of IL-10, and not IL-4, by Th2 lymphocytes (Swain et al. 1990). The overall balance of the response will depend not only on the nature of antigen and associated danger signals, but by the presence of soluble mediators and the effector cells that produce these can have a propound influence on T cell polarity during the priming of naïve T cells (Trinchieri 1995). It is likely that, with the right conditions, secondary or recall responses can be skewed to produce either Th1 or Th2 type cytokine mediators,

either by rescuing the minority of T cells with a different phenotype or altering the response of cells to specific cytokines (Trinchieri 1995).

The identification that there are two different DC subsets that are involved in determining T cell polarity has provided a further insight into the processes that determine Th1 and Th2 differentiation. DC1 cells produce large quantities of IL-12, and direct a Th1 response, DC2 cells, on the other hand, produce very low levels of IL-12 and produce a "permissive" environment for the development of Th2 cells (Bottomly 1999). Intriguingly, the DC2 cells fail to produce IL-4, which indicates the existence of another mechanism, as yet unidentified, that regulates Th2 responses following contact between T cell and DC2 dendritic cells.

IL-12 production by DC's is critical for the development of Th_1 responses (Cella et al. 1996;Macatonia et al. 1995;Trinchieri 1993). The requirements for the production of IL-12 by DC's are somewhat controversial. It seems likely that more than one signal is required to generate IL-12 in DC's. For example, interactions between DC's and naive T lymphocytes fail to induce IL-12 in DC's probably because of the lack of IFN-γ production by naïve T lymphocytes (Snijders et al. 1998). Other workers have shown that antigen specific interaction between Th1 cells and DC's induce IL-12 production that is dependent on CD40 ligand, but not IFN-γ(Ria, Penna, & Adorini 1998). This could be dependent on both the nature of the antigen being presented by T lymphocytes, and also whether the lymphocytes are committed memory cells. Another important feature of DC's is their ability to respond to a wide variety of environmental stimuli. In this regard, either CD40 ligand or IFN-γ (but not both) can be replaced by the bacterial LPS to induce IL-12 (Snijders et al. 1998). This has important implications. The presence of specific microbial products in this regard is acting as

an 'adjuvant', determining the nature of the subsequent immune responses (Lanzavecchia 1998).

6.1.7 Influence on B lymphocyte development and differentiation by DC's

Although characterised by their ability to stimulate T cell development, DC's also have significant effects on B cell growth, development and antibody isotype switching. B-lymphocytes have been shown to interact with specific types of DC's, such as non-lymphoid, interstitial DC's that stimulate B cell proliferation, antibody production in naïve B cells that have been stimulated by CD40L on T cells (Dubois et al. 1997). Activated B cells interact with a unique, non-bone marrow derived phenotype of DC's, or follicular dendritic cells (Liu et al. 1996). These maintain the viability, growth and proliferation of B cells in germinal areas of lymphoid tissue (Banchereau & Steinman 1998a).

IL-12 release by DC's has specific effects on B-lymphocytes. It suppresses IgE production in IL-4 stimulated B cells in the presence of T cells (Kiniwa et al. 1992). Mice treated with IL-12 together with hapten-protein conjugate displayed inhibition of IL-4 secreting cells, reduce serum IgG1, IgG2b, and enhancement of IgG2a production (McKnight et al. 1994). In the same study, anti-IL12 antibodies blocked Th1 type response, as shown by the decrease in IFN-γ and IgG2a production.

6.1.8 Pattern recognition molecules and the innate immune response

Understanding of the role of innate immune system has shifted from regarding it as a "first line of defence" to one that is pivotal in directing acquired immune responses (Fearon 1997). Central to this process are the mechanisms by which immune system is able to recognise potentially dangerous, pathogenic

material. This depends on a highly evolutionary conserved set of pattern recognition molecules (PRM's) that recognise pathogen associated molecular patterns (PAMPS) (Medzhitov & Janeway-CA 1997). These PRM's are able to recognise a number of key pathogen derived molecules including bacterial lipopolysaccharide (LPS), lipoteichoic acid, lipoproteins from gram negative and gram-positive bacteria respectively, and unmethylated CpG motifs characteristic of bacterial DNA and double stranded viral RNA. Immature or precursor DC's are constantly sampling their microenvironment by means of such receptors (Medzhitov & Janeway-CA 1997), which play a critical role in phagocytosis of pathogens and triggering of the innate immune response (Austyn 1996), two processes that are intimately linked. In particular, type 1 DC's have a high capacity for receptor mediated phagocytosis, and are richly adorned with mannose receptors, complement receptors and Fcγ receptors.

6.1.9. Interactions between bacteria and DC's

Whole bacteria, protozoa as well as microbial products such as lipopolysaccharide (LPS) can induce DC maturation *in vitro* and *in vivo* resulting in increased expression of co-stimulatory molecules and production of pro-inflammatory cytokines (McWilliam et al. 1996;Rescigno et al. 1999;Roake et al. 1995;Verhasselt et al. 1997). Bacteria are potent stimulators of DC maturation and antigen specific T cell responses (Rescigno et al. 1998a). Knowledge of how this process occurs has increased dramatically by discovery of the host receptors that are able to recognise microbial components and activate both innate and adpative immunity (Bendelac & Fearon 2000). In particular, human Toll-like receptors have a central role in discriminating between different types of microbial components (Takeuchi et al. 1999b), and the signals that this generates has profound influence on both innate and adaptive immune responses (Medzhitov & Janeway-CA

1997;Medzhitov, Preston, & Janeway-CA 1997). It is now known that DC's express a characteristic pattern of TLR's (Muzio et al. 2000b;Muzio et al. 2000a). The nature of the response of DC's to gram-negative bacteria such as *N. meningitidis* will be profoundly influenced by the interactions between pattern recognition receptors like TLR's and the large number of microbial products present on meningococci.

6.1.10 Vaccine strategies against *N. meningitidis*

Development of effective vaccines against *N. meningitidis* has been hampered for a number of reasons. Polysaccharide capsule has been used as the base of vaccines against groups A and C meningococci, inducing broadly protective, anti-capsular bactericidal antibody (Frasch 1995). Pure polysaccharide vaccines have reduced efficacy in infants (Goldschneider et al. 1973). A more successful approach has been to conjugate group C polysaccharide to a protein carrier, which elicits B cell affinity maturation, class switching and induction of memory in infants (Richmond et al. 1999). The likely explanation for this is because this engages T cell help through antigen presentation cells and production of optimal co-stimulatory signals (Ada 1990). However, meningococcal group B polysaccharide capsule is poorly immunogenic even when conjugated, probably because of its molecular similarity to human neural cell adhesion molecule, N-CAM (Finne, Leinonen, & Makela 1983). Efforts to develop vaccines to serogroup B meningococci have concentrated on the use of outer membrane vesicles (OMV's), that contain outer membrane proteins such as porins, and opacity proteins (Andersen 1997). Although there has been some success in inducing protection in field trials (Bjune et al. 1991;Sierra et al. 1991), there remain significant questions in terms of cross strain protection and immunogenicity, particularly in infants. Also, there are doubts as to whether the current vaccines

could protect against heterologous strains (Tappero et al. 1999). Protection across strains maybe afforded by use of vaccines based on multiple antigens (Cartwright et al. 1999).

There have been several studies to date investigating specific T and B cell responses to group B meningococcus both in response to natural infection (Lehmann et al. 1999;Wiertz et al. 1996), and after administration of various OMV vaccines (Naess et al. 1998;van-der et al. 1997). There is evidence that this can result in the induction of antigen specific T-cell memory response and specific bactericidal antibody production. Interestingly, nasal administration of one OMV vaccine induced specific T-cell responses and production of bactericidal antibody in the absence of any adjuvant (Oftung et al. 1999). This raises two important points: first, whether route of administration effects responses because of engagement of antigen presenting cells like dendritic cells and second, whether other components of the vaccine may act as an adjuvant. The OMV vaccine used in the above study contains some native LPS (Fredriksen et al. 1991), which may be acting as an adjuvant (Alving 1993). Additionally, specific LPS antibodies to a number of epitopes are detectable in both normal and convalescent sera (Griffiss et al. 1984), which appear to be strain specific (Zollinger et al. 1997). Carbohydrate structure of LPS may significantly affect immune response to both LPS itself and associated OMP's (Andersen et al. 1997).

There are currently no studies that directly look at how DC's respond to *N. meningitidis*. It was hypothesised that meningococcal structure, especially presence of capsule and differences in LPS structure would effect the pattern of DC activation and maturation. This was achieved by determining changes in surface expressed molecules that have critical roles in DC biology, including co-stimulatory molecules and MHC molecules and production of cytokines. In

addition, since it was found that meningococci could activate endothelial cells independently of LPS, the study asked the question as to whether DC's could be activated by meningococci independently of LPS.

6.2. Methods

6.2.1 Media and reagents

RPMI 1640 medium with 2mM L-glutamine, penicillin/streptomycin supplemented with 10% Fetal Calf Serum was used throughout the experiments. Human recombinant IL-4 and GM-CSF were gifts of Schering-Plough. Meningococcal LPS from *N. meningitidis* serogroup B (strain 44/76) was prepared as previously described in Chapter 2.

6.2.2 Bacterial strains

This study used a number of different strains, which have been described, in section 2.6. One set of experiments compared the parent B1940 to its unencapsulated non-sialylated isogenic mutant *cps-*, and the second set compared parent H44/76 to its LPS deficient isogenic mutant, *lpxA-*. Other mutants were used as described.

6.2.3 Bacterial culture

Bacteria were grown and prepared for experimentation in RPMI medium as described in Chapter 2. In all experiments, organisms were fixed in 0.5% paraformaldehyde.

6.2.4 DC culture

The culture of DC's from human peripheral blood mononuclear cells has been described previously (Woodhead et al. 1998). Peripheral blood mononuclear cells (PBMC's) were prepared from blood samples obtained from healthy volunteers. The blood, anticoagulated with 4.2 mM EDTA, was diluted in an equal

volume of phosphate-buffered saline, layered over lymphoprep and centrifuged at 400g for 30 minutes at room temperature. The cells present at the interface were recovered and washed three times in HBSS. The PBMC's were diluted in RPMI-1640 medium supplemented with 10% fetal calf serum, 0.05 M 2-mercaptoethanol, 100 U/ml penicillin/streptomycin and 2.4 mM L-glutamine to a concentration of approximately 3 x 10^6 /ml and placed in six-well tissue culture plates (a volume of 3 ml per well). They were incubated for 3 hours at 37^{0}C in an atmosphere of 5% CO_2. The non-adherent cells were removed and discarded. The adherent cells were incubated for 7 days in fresh culture medium supplemented with 100ng/ml human recombinant GM-CSF and 50 ng/ml human recombinant IL-4.

6.2.5 Stimulation of DC's

Each experiment was performed in parallel to enable analysis of both intracellular cytokine levels and surface phenotyping. The average yield of monocyte derived human dendritic cells was approximately 1x10^6 / well. Two wells were used for each experimental condition. After stimulation, DC's were incubated for a further 24 – 26 hours at 37^{0}C in an atmosphere of 5% CO_2 prior to cell harvesting. The non-adherent cells were recovered and each well washed with 1.5 ml HBSS to enhance cell yield. The samples used for phenotypic analysis were centrifuged at 200g for 5 minutes at room temperature to enable supernatants to be collected and stored at –70^{0}C. Cell pellets were resuspended and the resulting solution layered over lymphoprep and centrifuged at 400g for 30 minutes at room temperature. A similar lymphoprep step was performed on the samples for intracellular cytokine analysis. The interface was recovered and the cells washed three times in HBSS.

6.2.6 Surface phenotyping

The cells were incubated at 4^0C in HBSS supplemented with 0.1% sodium azide and either 1% BSA or 10% NRS for 15 minutes. Directly conjugated antibodies were added to the cells at a final concentration of 1 in 10 (diluted with HBSS/0.1% sodium azide /1% BSA solution) and incubated on ice for 30 minutes. The cells were washed three times in HBSS supplemented with 0.1% sodium azide prior to being fixed in 2% formaldehyde/PBS solution with 0.1% sodium azide added. For the unconjugated antibodies, the cells were initially incubated on ice for 30 minutes at a final primary antibody concentration of 1 in 2, (diluted with HBSS/0.1% sodium azide/10% NRS). The cells were washed three times in HBSS/0.1% sodium azide/10% NRS prior to the addition of the secondary antibody (FITC-conjugated rabbit anti-mouse IgG (diluted 1 in 20 in HBSS/0.1% sodium azide/ 10% NRS). They were incubated for a further 30 minutes on ice and then washed three times in HBSS/0.1% sodium azide solution prior to fixation with 2% formaldehyde/PBS with 0.1% sodium azide.

6.2.7. Detection of intracellular cytokine production

To aid detection of intracellular cytokine production, 10μg/ml of brefeldin A was added to each well prior to addition of medium, LPS or suspensions of bacteria. DC's were cultured with various stimuli for a total of 24 hours. Cells were checked for characteristic features of dendritic cells by light microscopy. Cells were removed from wells, diluted 1: 2 with HBSS layered onto lymphoprep and centrifuged at 400g for 30 minutes. The interface was recovered and washed three times in HBSS, as described in the previous section.

Staining of cells for detection of intracellular cytokine production was performed as described but with several modifications (Sander, Andersson, & Andersson 1991). Cells were suspended in 4% paraformaldehyde in PBS at 4^0 C

for 15 minutes, with intermittent vortexing. Suspensions were washed once in PBS and 0.1% sodium azide and washed once in permeabilisation buffer, containing 0.1% saponin, 2mM Hepes buffer and 0.05% sodium azide in HBSS with calcium and magnesium. Cells were then resuspended in 200µl of saponin buffer and incubated with monoclonal antibodies to raised against human cytokines and isotype controls for 45 minutes at room temperature in the dark. Cells were washed twice in permeabilisation buffer and resuspended in PBS and 0.1% azide for immediate FACS analysis.

6.2.9 FACS analysis

Flow cytometry was performed on a FACSCALIBUR using Cell Quest software 5000 events within gate corresponding to Forward/Side characteristics of DC's were collected. Representative histograms and dot-plot graphs contained in figures were produced using WINMDI software.

6.2.10 Statistical analysis

Differences in both percent positive events in or median fluorescence intensity of antibody staining to cytokine induced by various strains of *N. meningitidis* were performed using Wilcoxon signed ranks test or Friedman 2-way ANOVA for 3 different stimuli using SPSS version 8.01.

6.3. Results

6.3.1 Phenotypic characteristics of immature, *in vitro* cultured human dendritic cells

Cells cultured with medium alone displayed cell surface phenotypic characteristic of immature DC's. Fig 6.1 shows typical forward and side scatter properties of unstimulated DC's as analysed by flow cytometry. These were

typically CD14 ^{low}, CD83^{-ve}, CD86^{low}, CD25^{-ve}. (figs 6.1 and 6.2). They expressed HLA DR, HLA DQ, and HLA Class I, CD40 and CD1a. CD3 and CD19 expression was absent in the gated window, as shown in Fig 6.1. The cells also displayed characteristic features of DC's on light microscopy, including dendrite projections.

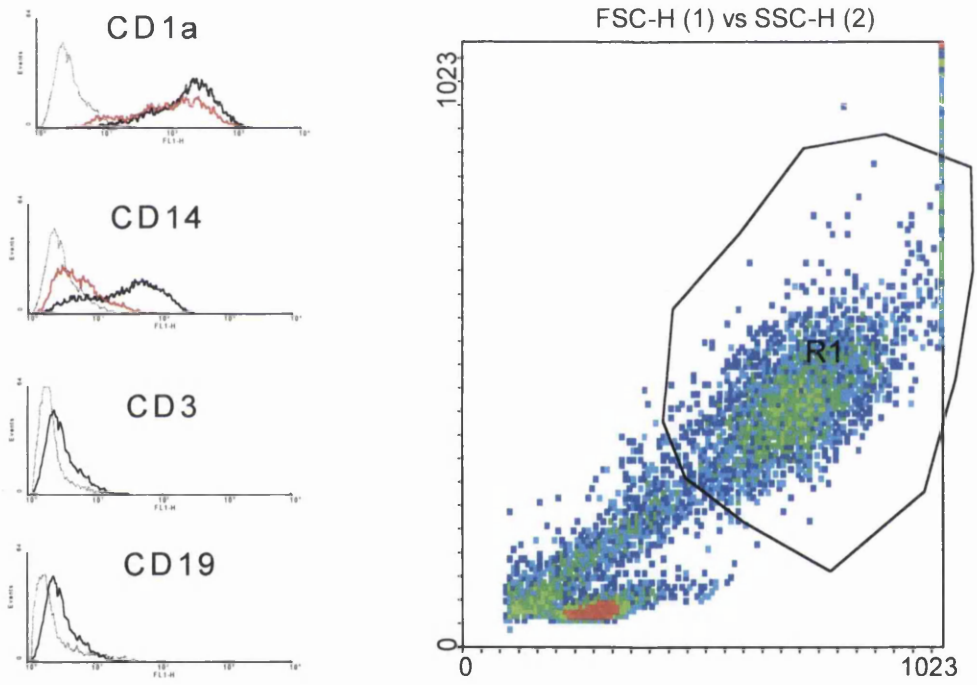

Figure 6.1 Characteristics of DC's on flow cytometry.

Dendritic cells were identified as a single population on forward and side scatter properties. As shown, there was minimal or no staining of CD3 or CD19 (continuous black histogram) in the gate. Cells were CD1a positive (continuous black line histogram), which decreased after stimulation with LPS (red line). Moderate staining of CD14 cells could be detected in immature day 8 DC's (represented by continuous black line) which was almost absent in mature DC's (represented by red line, in this case after stimulation with 100ng/ml LPS).

6.3.2 DC phenotype response to LPS and *N. meningitidis* B1940

After stimulation with either LPS or bacteria, DC's became CD14 $^{-ve}$ (fig 6.1). Stimulation with LPS or *N. meningitidis* B1940 increased expression of MHC molecules HLA-DR and HLA-DQ, co-stimulatory molecules CD40, CD86 (B7.2)

and CD25 (IL-2R) and CD83, all consistent with an activated, mature phenotype (fig 6.2). There were some differences between purified LPS and bacteria. In general, bacteria were more potent than LPS at inducing markers of DC activation and maturation. However, over multiple experiments, there were no significant differences in CD40, CD86 or HLA DQ expression induced by LPS or bacteria (fig 6.3). Over a range of experiments, induction of CD83 and CD25 was greater in response to bacteria than purified LPS. The degree of increase in CD83 expression is a particularly useful marker of DC maturation (Zhou & Tedder 1996). Changes in CD1a expression were variable but there was no consistent pattern observed.

6.3.3 Influence of structure of *N. meningitidis* B1940 on pattern of DC phenotype

Both parent and *cps*- (unencapsulated, with a truncated, non-sialylated LPS) strains were potent inducers of DC's maturation as determined by changes in surface markers. A number of patterns emerged. The unencapsulated, non-sialylated *cps*- mutant was at least as effective in inducing phenotypic changes as both the parent organism and high dose (100ng/ml) LPS. Expression of CD86 (B7.2), HLA-DQ and CD40 was greater with the *cps*- strain than the parent strain in a number of experiments (fig 6.3), although this did not reach statistical significance. The pattern seen with CD25 and CD83 up-regulation did not show any clear differences between the mutants, although both strains were effective in inducing expression of these molecules (fig 6.2).

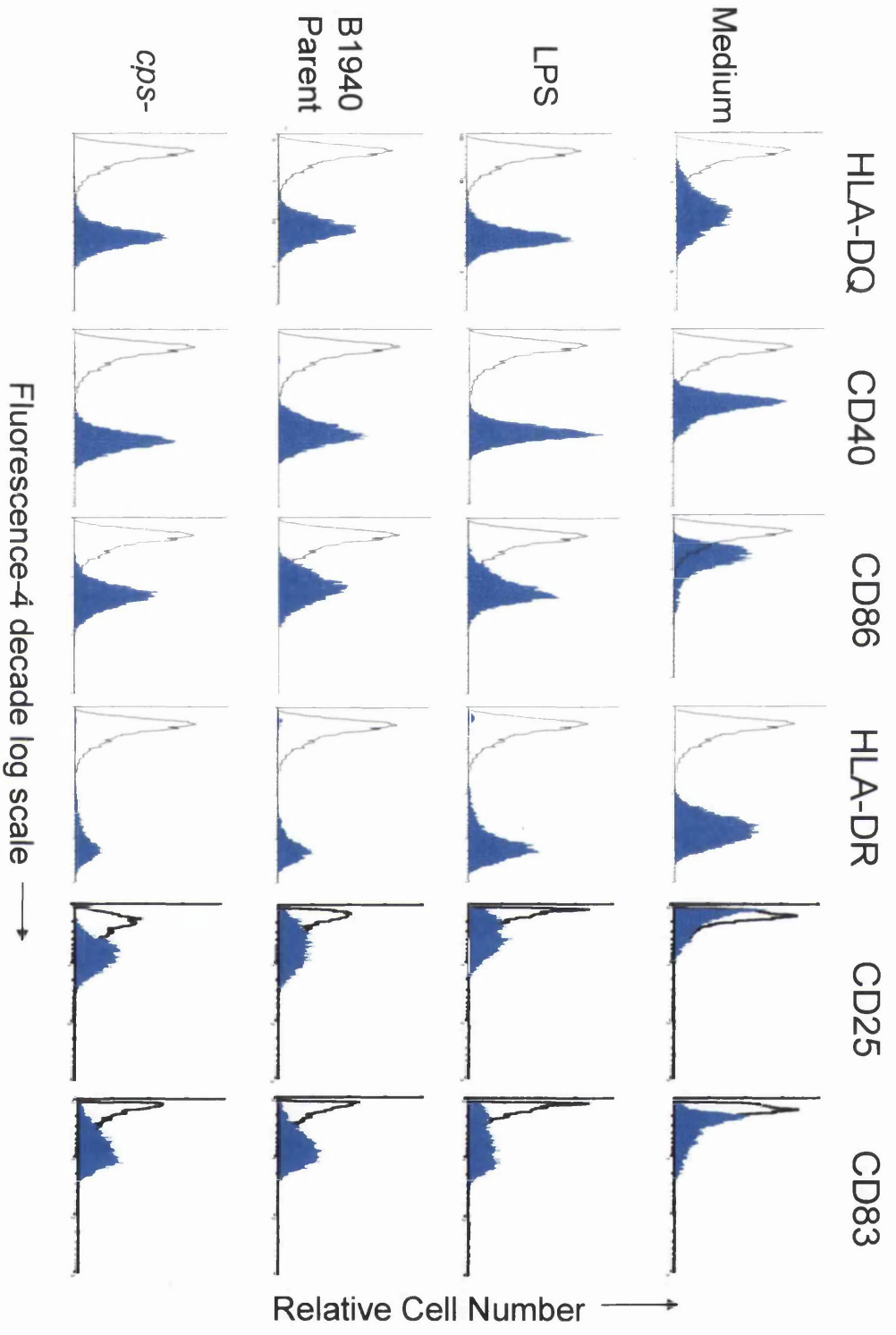

Figure 6.2. Representative flow cytometric profiles of surface phenotypic markers on day 8 DC's stimulated with Medium, LPS, *N. meningitidis* B1940 parent and *cps*- strains.

Day 7 DC cultures were incubated for 24 hours with medium, 100ng of meningococcal LPS, 10^7 cfu/ml of parent or *cps*- organisms. Thin black line histograms show appropriate isotype matched control staining. Filled histograms shows staining of the antibody raised against the indicated surface marker. This is representative of at least 5 separate experiments.

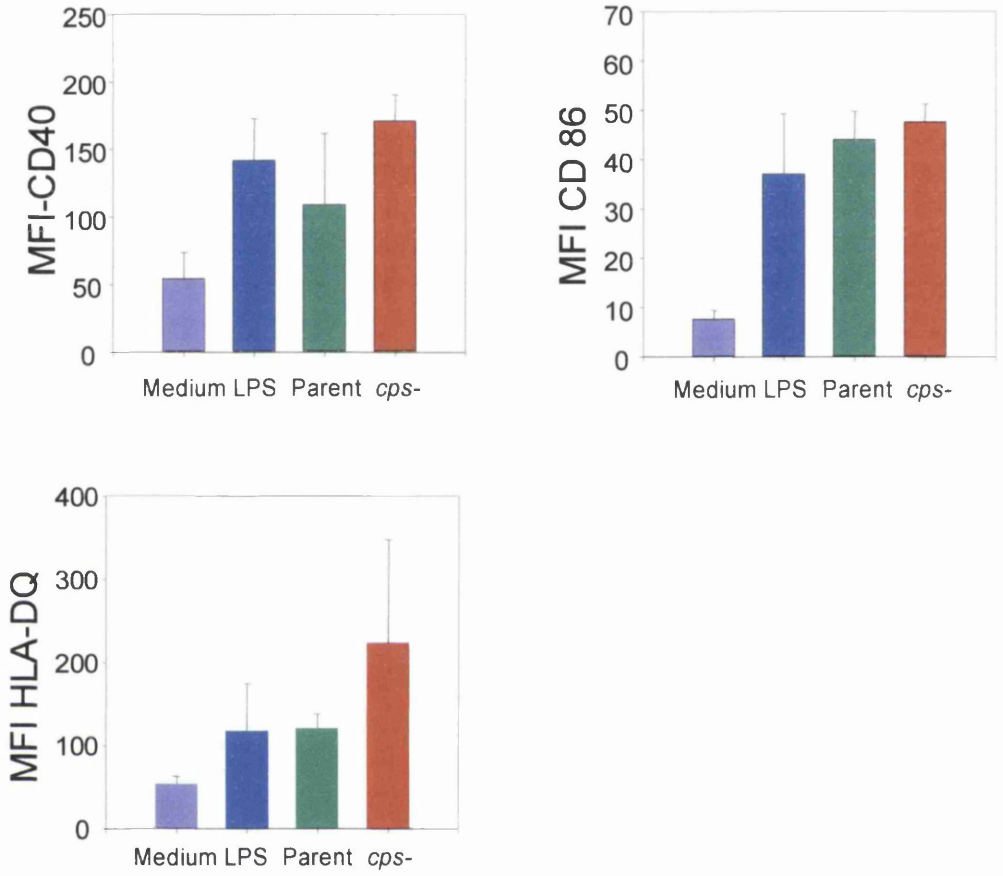

Figure 6.3. Relative capacity of purified LPS, and B1940 parent and *cps*- strain to induce expression of co-stimulatory molecules and HLA-DQ on DC's.

Day 7 DC cultures were incubated for 24 hours with medium, 100ng of meningococcal LPS, 10^7 CFU/ml of parent or *cps*- organisms. Results are expressed as mean MFI antibody staining of indicated surface markers ± SEM for three experiments.

Determination of expression of HLA-DR was problematic throughout all this set of experiments. Stimulated DC's stained for HLA-DR were noticeably clumped and often only a few events could be recorded by flow cytometry, which made analysis of this data difficult. One postulate for this problem was that expression on stimulated cells is so high that cross-linking took place resulting in large clumps of cells. In a number of experiments, HLA-DR expression appeared to decrease after stimulation, especially with the organisms. There are a number of

possible reasons for this. Clumping of cells with high expression were not recorded by the flow cytometer whereas those cells which had low expression remained in the DC gate and therefore recorded. It is also possible that this apparent down-regulation was a real phenomenon. This could be due to shedding of molecules or even internalisation, although none of these were formerly examined. It is interesting to note that, in the experiments where this phenomena occurred, other markers of maturation, particularly CD83 and CD86, were increased.

6.3.4. Intracellular cytokine production by DC's in response to *N. meningitidis* B1940 and LPS

The effectiveness of the bacteria or LPS in DC maturation was further investigated by measurement of intracellular cytokines by flow cytometry. This method determines cytokine production in individual cells, which is advantageous to ELISA methods that measure released cytokine into the supernatant, and can be confounded by different cell numbers in the wells and by production by other cells such as non-differentiated monocytes or lymphocytes. The surface markers suggested that both the unencapsulated, non-sialylated *cps-* mutant and 100ng LPS was at least as effective as the parent organism in inducing many markers of maturation. The cytokine studies revealed a number of striking differences.

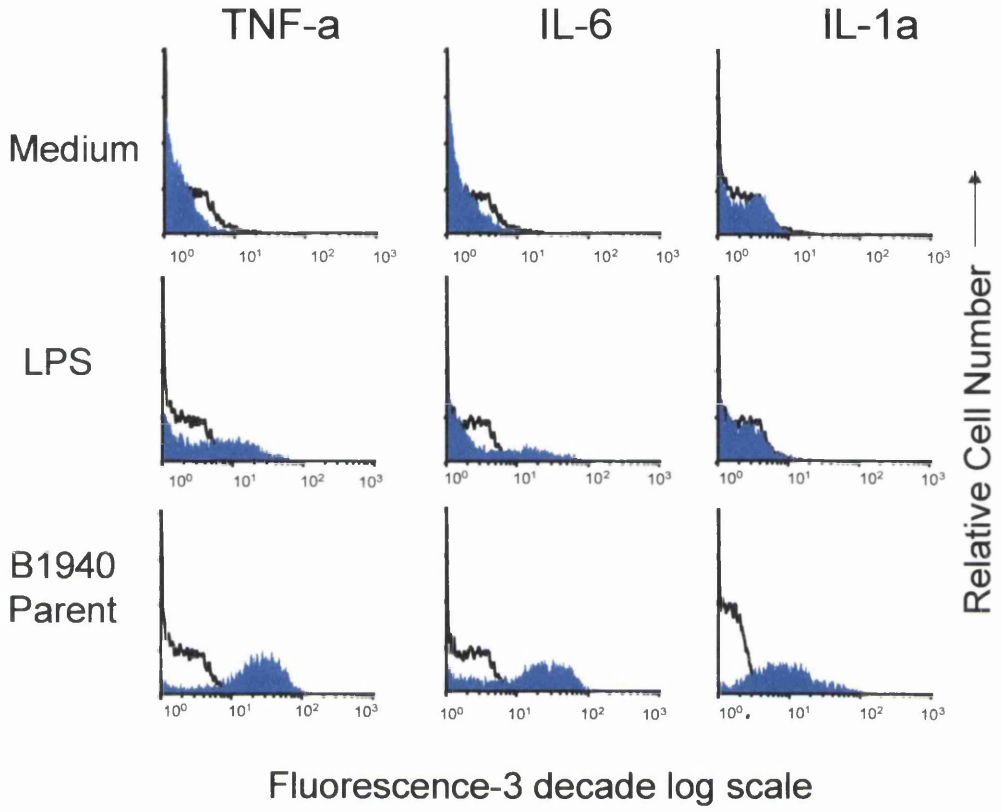

Figure 6.4. Representative flow cytometric profiles of cytokine production in unstimulated and stimulated DC's on day 8.

DC's were stimulated with either medium, or 100ng LPS or 10^7 cfu/ml B1940 parent strain in presence of 10μg/ml brefeldin A. Cytokine production was assessed by detection of intracellular staining with PE conjugated isotype control (thick line) and anti-cytokine antibody (filled histograms) as indicated.

Firstly, LPS, whether derived from *E. coli* or meningococci, was a poor inducer of cytokines in all experiments. As Figure 6.4 demonstrates, only IL-6 and TNF-α was detected to any degree in DC's stimulated by LPS (100ng/ml). Low levels of both IL-6 and TNF-α could be observed by analysing the percentage positive events, whereas the intensity of staining, as measured by MFI, was almost always low. In the case of IL-1a production, there was often no difference between medium and LPS stimulated DC's. In some experiments, small amount of IL-1a could be detected in LPS stimulated wells, as determined by percent positive events. This contrasts with the phenotype data, in which 100ng LPS induced both co-stimulatory markers such as CD40, CD86 and MHC molecules.

Importantly, no IL-12 could be detected in any experiments at any dose of LPS. At doses of 1 or 10ng/ml LPS, cytokine induction was almost absent (data not shown).

6.3.5 Cytokine production in DC's stimulated with *N. meningitidis* B1940 and *cps*- strain

In marked contrast, *N. meningitidis* B1940 were potent inducers of the cytokines tested. At doses of 10^5 bacteria/ml, expression of IL-1a and TNF-α was greater than the maximal dose of LPS used (100g/ml). This was not true for IL-6. However, concentrations of 10^6 organisms and above always induced higher levels of IL-6 than 100ng/ml of purified LPS. In contrast to what might have been predicted from the surface marker data, the parent organism appeared to be the more potent inducer of cytokine production than the *cps*- mutant (fig 6.5). Over a number of experiments, a complex pattern emerged. The parent organism induced higher levels of IL-6 than the *cps*- strain over a range of concentrations. This was statistically significant at both 10^7 and 10^6 cfu/ml, as shown in Fig 6.5. The difference could not achieve statistical significance at 10^5 cfu/ml, due to small sample size. In the case of IL-1a production, significantly greater levels induced by the parent organism were seen only at 10^6 cfu/ml. However, in some experiments, production of IL-1a was markedly increased in DC's stimulated by the parent organism at 10^7 bacteria per/ml as compared to the *cps*- strain.

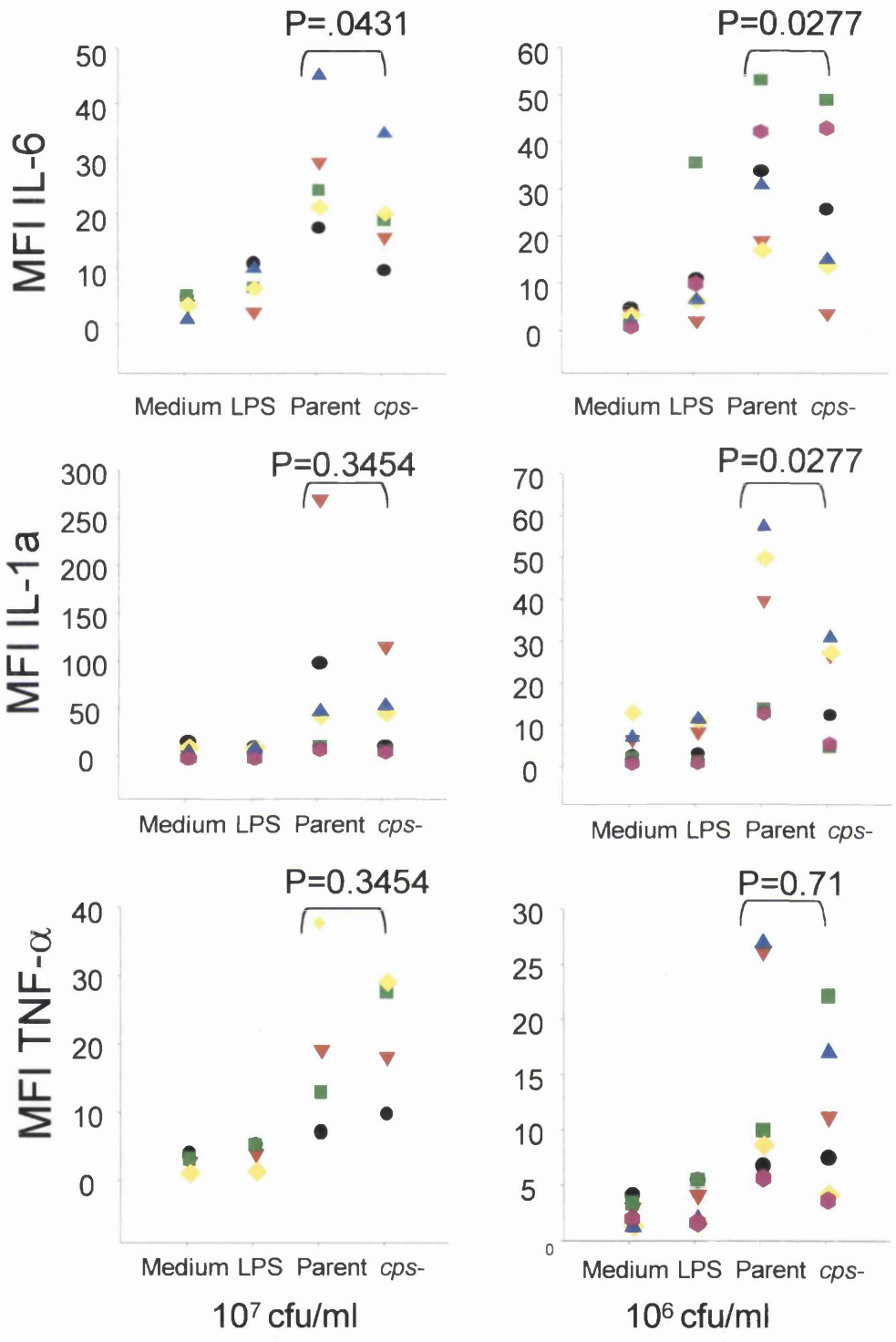

Figure 6.5 Relative capacity of purified LPS, and B1940 parent and *cps*- organisms to induce cytokine production in DC's.

DC's were stimulated for 24 hours with 100ng/ml LPS and indicated concentrations of B1940 and *cps*- organisms, and cytokine production assessed by intracellular staining protocol. Each data point represents MFI of PE conjugated anti-cytokine antibody staining, with lines linking individual experiments. Differences in cytokine produced in response to either the parent or *cps*- strain was analysed by Wilcoxon means ranks test.

Interestingly, there was no significant difference between the strains in terms of TNF-α expression, although at 10^6 cfu/ml, the TNF-α production by the parent was greater than the *cps-* mutant in the majority of experiments (fig 6.5). Both the parent and the *cps-* strain induced IL-12 production by DC's, whereas LPS did not (fig 6.6). Although the differences were small, the parent organism induced higher levels of IL-12 than the *cps-* strain, although the number of observations was not large enough to allow meaningful statistical analysis.

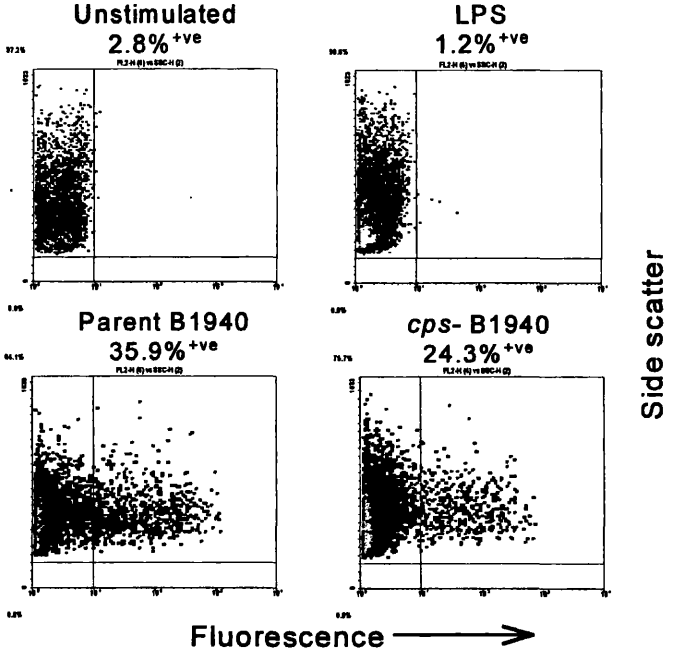

Figure 6.6 IL-12 production in DC's in response to *N. meningitidis* B1940 parent and *cps-* strains and 100ng/ml LPS.

Density plots of IL-12 staining (FL-2) plotted against side scatter of gated DC population. Percent positive staining is shown on each graph.

6.3.6 Response of DC's to *N. meningitidis* B1940, parent, *cpsD-*, *siaD-* and *cps-* strains.

To investigate further the role of bacterial structure on DC maturation, DC's were incubated with the parent strain, *cpsD-* (capsulated, truncated, non-sialylated LPS), *siaD-* (unencapsulated, sialylated LPS) and *cps-* (unencapsulated, truncated, non-sialylated LPS) mutants at 10^6 cfu/ml, intracellular cytokine production measured. Interestingly, the most potent inducer of cytokine

production was the parent organism, with the exception of Il-1a production where the unencapsulated *siaD-* mutant, which has normal LPS structure, was more potent. The least potent was the encapsulated, *cpsD-* mutant, which possesses a truncated LPS as shown in fig 6.7

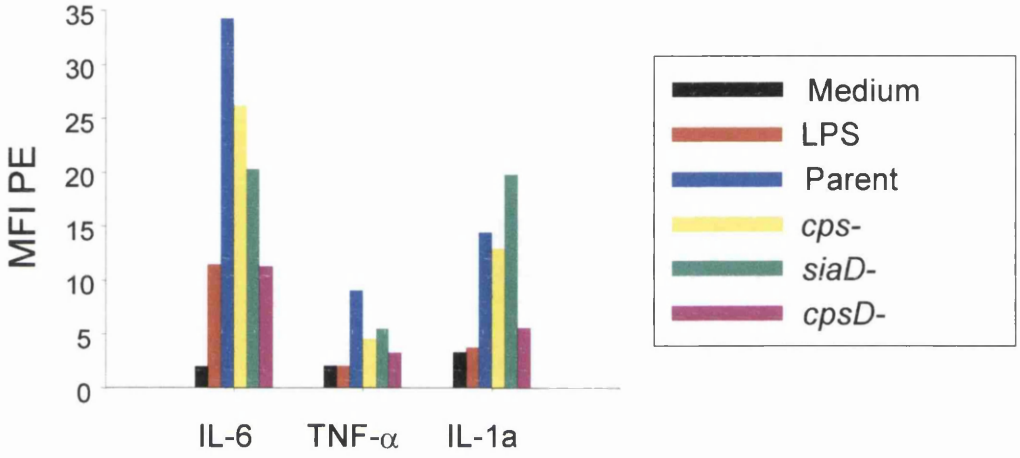

Figure 6.7. Cytokine induction in DC's in response to *N. meningitidis* B1940 parent, *cpsD-*, *siaD-* and *cps-* strains.

DC's were stimulated with 10^6 CFU/ml organisms and 100ng/ml LPS and cytokine production assessed using intracellular flow cytometry protocol. This is representative of three separate experiments.

6.3.7 Response of DC's to meningococcal LPS, *N. meningitidis* H44/76, and an LPS deficient mutant, *lpxA-*

The *lpxA-* strain was used to determine whether non-LPS components in meningococci could activate DC's. Both the parent and *lpxA-* strains increased expression of the co-stimulatory molecules CD86 and CD40, both HLA-ABC and HLA-DQ in DC's, and markers of DC maturation CD25 and CD83 (fig 6.8). Although the levels were comparable at doses of 10^7 CFU/ml of either bacteria, the parent was generally more potent stimulus. The pattern seen was similar to that seen with 100ng/ml of meningococcal LPS (fig 6.8)., although some differences were observed. MHC class I expression was poorly induced by LPS, but was up regulated by the organisms. Expression of most of the surface markers

was greater in response to the parent organism than with the *lpxA*- strain. CD83 expression was consistently higher in response to the parent strain as compared to either the *lpxA*- mutant, or 100ng/ml LPS. The response of DC's to the *lpxA*- mutant was markedly reduced below 10^7 CFU/ml (data not shown). In agreement with the previous set of experiments utilising the B1940 strain, the parent H44/76 organism induced high levels of all the cytokines tested (fig 6.9). The LPS deficient, *lpxA*- mutant also induced cytokines in DC's. However, the pattern of cytokine production induced by the *lpxA*- mutant differed from that of the parent in a number of ways. Firstly, intensity of staining was generally lower than that seen in response to the parent organism at 10^7 CFU/ml. but significantly higher than that seen in response to 100ng/ml of LPS (table 6.1). The response was also sensitive to dose of organisms. Whilst the parent strain cold induce relatively high-level cytokine production at 10^5 CFU/ml, it was almost absent when 10^6 CFU/ml or below of the *lpxA*- strain was used (fig 6.9). Interestingly, this is very similar to the concentration dependent pattern observed with HUVEC stimulated with the *lpxA*-, as shown in Chapter 4.

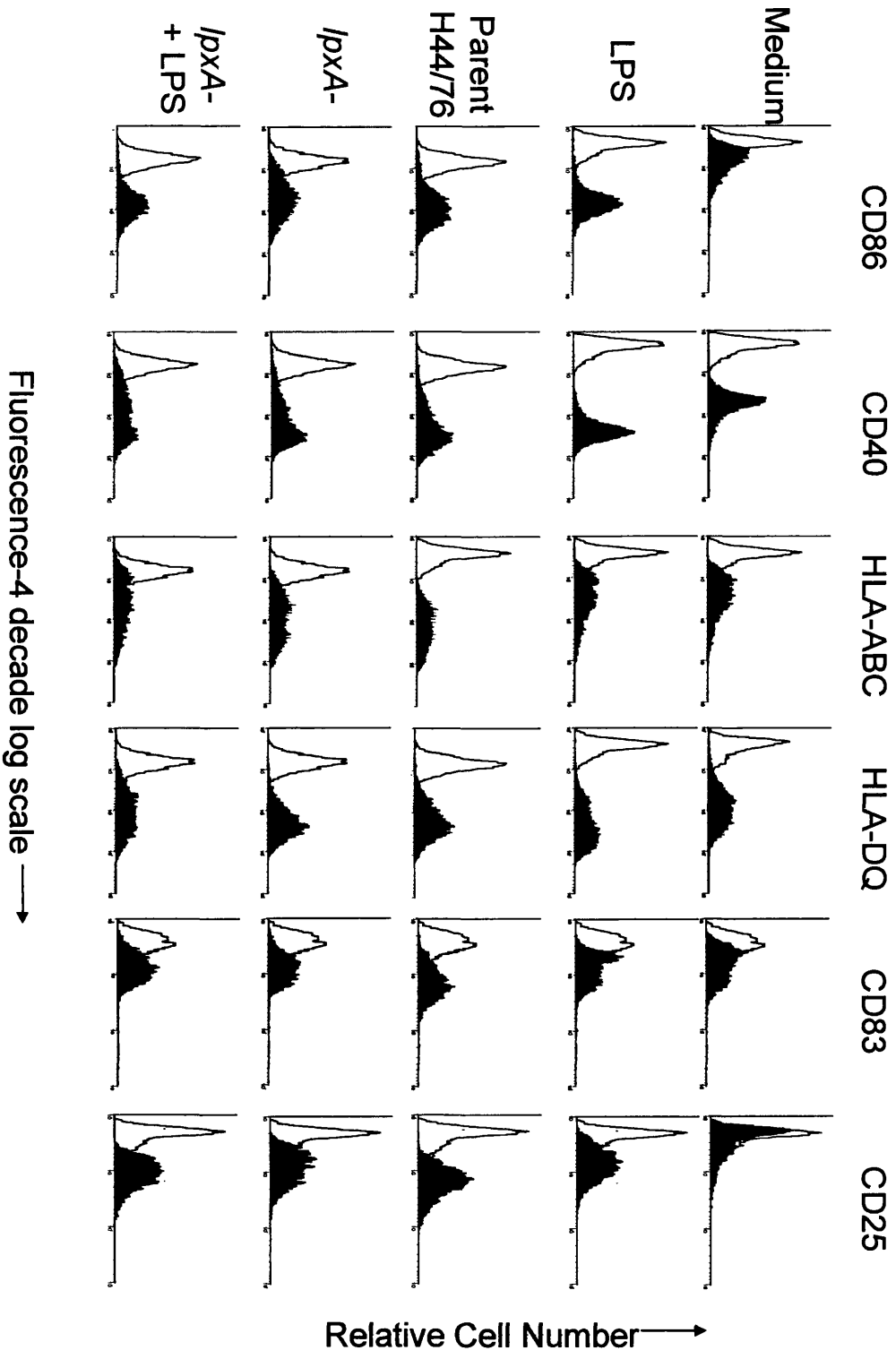

Figure 6.8. Surface phenotypic markers on day 8 DC's stimulated with Medium, LPS, *N. meningitidis* H44/76 parent and *lpxA*- strains.

DC cultures were incubated for 24 hours with medium, 100ng of meningococcal LPS, 10^7 cfu/ml of parent or *lpxA*- organisms. Thin black line histograms show staining of appropriate isotype matched controls. Filled histograms shows staining of the antibody raised against the indicated surface marker. This is representative of 3 separate experiments.

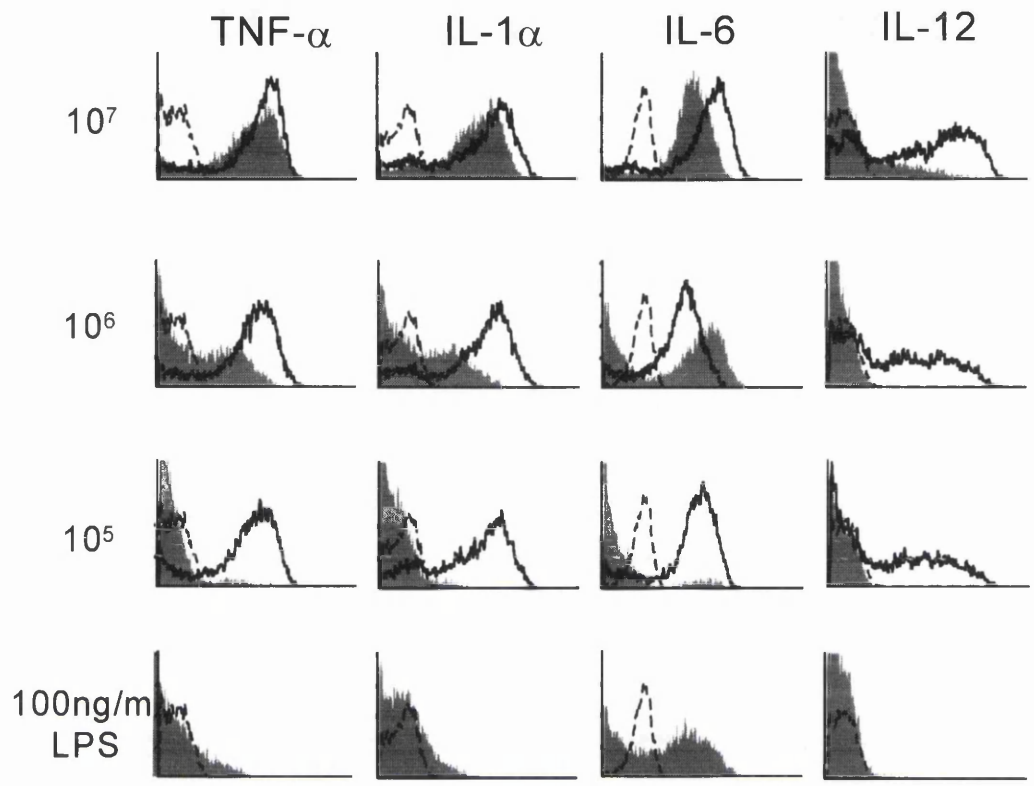

Figure 6.9. Dose dependent cytokine production in DC's in response to parent H44/76, *lpxA*- strains and meningococcal LPS.

DC's were stimulated with either medium, or 100ng LPS or 10^5- 10^7 CFU/ml H44/76 parent and *lpxA*- strains in presence of 10μg/ml brefeldin A. Cytokine production was assessed by detection of intracellular flow cytometry protocol. Thick line histogram represents staining in response to parent strain, filled histogram in response to *lpxA*- mutant or LPS as indicated, and dashed lines staining in unstimulated DC's. This is representative of 6 experiments yielding comparable results.

The parent strain induced far higher levels of IL-12 production than the *lpxA*- strain, even at 10^7 CFU/ml, at which dose the *lpxA*- induced comparable levels of IL-6, TNF-α and IL-1a to that seen with the parent strain. (Fig 6.9). In agreement with experiments using *E. coli* LPS, meningococcal LPS induced very low levels of cytokines compared to the organisms. Only IL-6 was produced in any quantity, as shown in fig 6.9. Importantly, very little IL-1a or TNF-α and no IL-12 could be detected in response to meningococcal LPS. Comparison of cytokine induction in DC's in response to the parent and *lpxA*- strains and meningococcal LPS are summarised in Table 6.1.

Table 6.1 Differences in cytokine production by DC's in response to *N. meningitidis* H44/76 parent, LPS deficient *lpxA*- strains, and purified meningococcal LPS.

Stimulus	*Median-MFI/ % positive events*	Interquartile range	P
IL1-a			
Medium	3.7	3.5-4.2	
	3	*2-3*	
LPS	3.5	3.2-3.5	
	6	*5-16*	P= 0.0067
H44/76 parent	15.1	12.9-33.1	
	59	*56.-64*	
lpxA-	6.4	4.9-8.5	
	27	*23-33*	
TNF-α			
Medium	2.1	2.0-2.6	
	1	*1-2*	
LPS	3.2	2.9-9.7	
	18	*15-32*	P=0.015
H44/76 parent	62.1	26.7-66.7	
	75	*62-86*	
lpxA-	7.9	2.8-21.8	
	48	*16-51*	
IL-6			
Medium	3.7	3.5-5.2	
	3	*2-4*	
LPS	5	3.2-14.2	
	36	*32-62*	P=0.015
H44/76 parent	33.1	15.1-66.1	
	66	*62-85*	
lpxA-	6.4	5.0-8.5	
	66	*47-77*	

Dendritic cells were stimulated with 10^7 /ml organisms, 100ng/ml purified meningococcal LPS, and medium in the presence of brefeldin A for 24 hours. Cytokine production was assessed by the intracellular fluorescence flow cytometry protocol as described in Materials and Methods. Results are expression as both MFI and percent positive events (Italics). Ranking order of cytokine production as measure by MFI, was compared using a Friedman two-way ANOVA, H44/76 parent>H44/76 *lpxA*->purified LPS.

6.3.8 Response of DC's co-stimulated with purified LPS and the *lpxA*- mutant

The results showed that neither the *lpxA*- mutant nor purified LPS could induce cytokine production in DC's to anywhere near the level seen in response to the parent strain. In order to test whether the *lpxA*- strain could be reconstituted by addition of purified LPS, DC's were co-incubated with 100ng of meningococcal LPS and the LPS deficient *lpxA*- mutant. Addition of LPS to the *lpxA*- strain resulted in small increase in cytokine production compared to incubation of the organism alone. However, this did not result in levels anywhere near to that seen with the parent organism, as shown in fig 6.10. There was a very small increase in percent positive cells producing IL-12 when exogenous LPS was added to 10^7 CFU/ml of the *lpxA*- mutant (fig 6.10). Co-stimulation resulted in small increases in surface expression of phenotypic makers, as shown in fig 6.8.

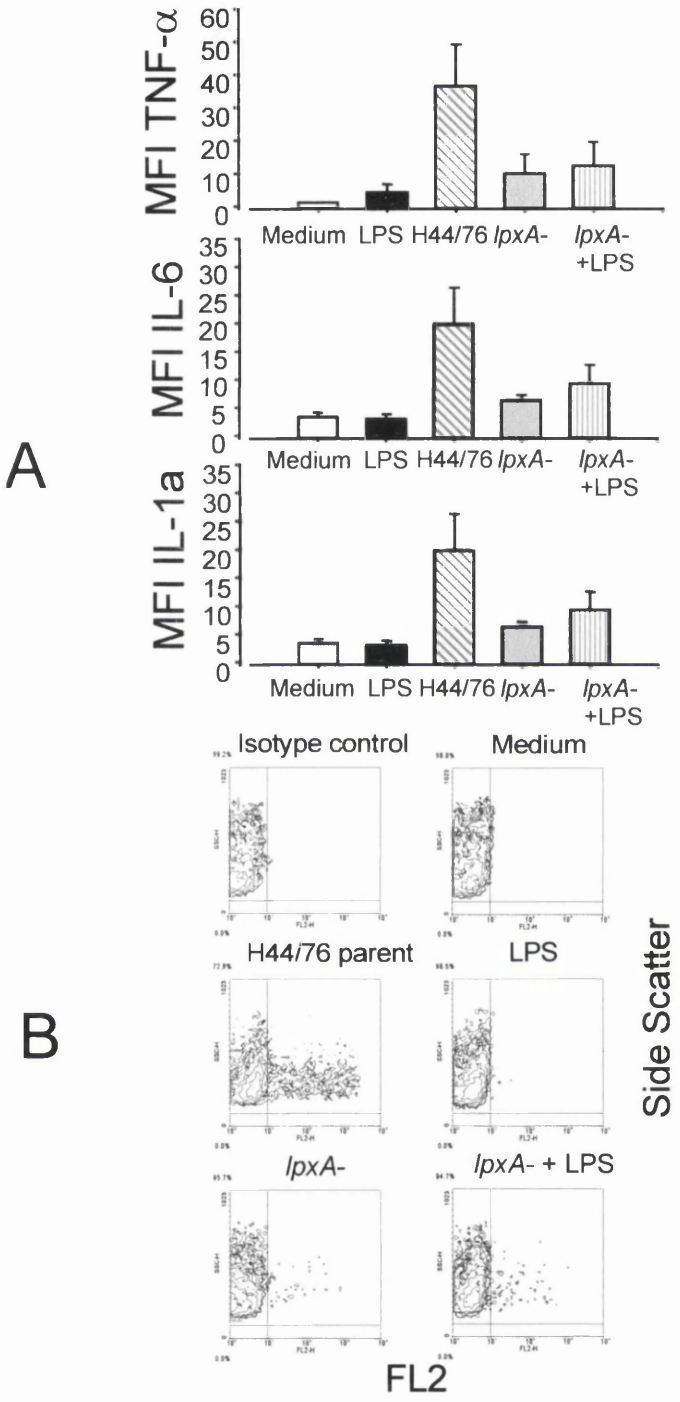

Figure 6.10. Addition of exogenous LPS only partially restores cytokine production by DC's in response to the *lpxA*- strain

DC's were stimulated with 10^7 CFU/ml of parent, *lpxA*- strain and 100ng/ml LPS and 10^7 CFU/ml of *lpxA*- strain together with 100ng/ml LPS in the presence of 10μg/ml brefeldin A. Fig 6.10 A, IL-6, TNF-α and IL-1a production, presented as mean MFI ± SEM from 3 separate experiments. Fig. 6.10 B, representative contour plots of IL-12 production from one experiment, representative of three experiments yielding similar results.

6.4. Discussion

Activation and maturation of DC's is critically affected by the nature of the antigen they encounter and microenvironmental milieu in which this takes place and this has important implications for the development of subsequent immune responses (Lane & Brocker 1999). One of the primary roles of DC's is the recognition and response to invading microorganisms, and due to their location at the interface with the external environment, interactions between DC's and intact bacteria are of great interest (Rescigno et al. 1999). Bacteria are potent stimulators of the innate immune and inflammatory responses and antigen specific adaptive immunity. There is to date no published work on how *Neisseria meningitidis*, gram-negative, exclusively human pathogen, interacts with human DC's. This study demonstrates a number of interesting and potentially important findings. Firstly, that variations in capsule expression and LPS structure effects nature and degree of DC maturation and activation. Secondly, that DC activation and maturation can occur independently of LPS, but that presence of LPS within bacteria is required for optimal DC response to meningococci, at least in terms of cytokine, particularly IL-12 production.

It has been shown previously that neutrophil and endothelial activation is more pronounced in organisms lacking capsule or sialylated LPS or both (Heyderman et al. 1997;Klein et al. 1996) and work presented in this thesis). Additionally, whole blood monocyte production of cytokines, and IL-8 production in endothelial cells is at least as potent in response to unencapsulated, non-sialylated *N. meningitidis*, including the *cps-* strain used in our study (G. Dixon and D. Jack, unpublished observations). The data from the surface expressed molecules suggested that the *cps-* mutant was at least as effective as the parent strain at activating DC's. It was somewhat surprising that that the parent organism

of B1940 was the more effective inducer of cytokines IL-6, IL-1a and IL-12 than the *cps-* strain. It is interesting that this difference was not observed for all cytokines tested, as shown by the TNF-α data, and not at all concentrations tested, as shown by the IL-1a results.

What is the reason for this apparent difference in cytokine production by DC's in response to these two strains? Group B meningococci possess α2-8 polysialic acid capsule and as the *cps-* strain is unencapsulated, this might account for the difference observed. However, meningococcal group B polysaccharide capsule is poorly immunogenic, probably because of its molecular similarity to human neural cell adhesion molecule, N-CAM (Finne, Leinonen, & Makela 1983). Additionally, DC's did not respond at all to purified meningococcal capsule from H44/76 in this study (data not shown). However, presence or absence of capsule might be important in interactions between DC's and bacteria in other ways, as discussed below.

The *cps-* mutant also differs from the parent strain in that is possesses a truncated, non-sialylated LPS. It is possible that this is the determining factor and not capsule expression *per se.* When the effects of B1940 parent, *cps-*, *siaD-* and *cpsD-* strains on DC maturation were compared, it was the unencapsulated *siaD-* mutant with normal LPS that was most similar in potency to the parent strain in terms of cytokine production. This suggests that it is the possession of the truncated, non-sialylated LPS that is the major factor in determining the production of cytokines in DC's in response to these strains of meningococci.

Phagocytic internalisation of micro-organisms is a critical function of immature, or precursor DC's (Rescigno et al. 1999). Cellular receptors such as the mannose receptor are highly expressed on immature DC's and are involved in phagocytosis, antigen processing and thus maturation of DC's (Austyn

1996;Sallusto et al. 1995;Stahl & Ezekowitz 1998). Particle adsorbed antigen, which is phagocytosed, induces up-regulation of IL-12, IL-1a and MHC Class II whereas soluble antigen does not (Scheicher et al. 1995). It is therefore possible that differences in the mechanisms of phagocytosis might contribute to the greater effectiveness of the parent compared to the *cps-* strain in induction of IL-1a, IL-6 and IL-12. However, this was not specifically explored in this study. The *cps-* strain were at least as effective as the parent at inducing surface markers of DC maturation such as CD40, CD86, CD83 and HLA-DQ. This indicates that certain aspects of DC maturation, such as the induction of co-stimulatory molecules or production of cytokines are differentially affected when interacting to microorganisms. The explanation may lie in the fact that the processes of bacterial internalisation and signals required for DC maturation are intricately linked. This would be a fascinating area for further investigation.

The relatively poor effectiveness of purified LPS to induce cytokine production in DC's in this study was surprising. LPS is a potent inflammatory mediator and is able to induce DC maturation (McWilliam et al. 1996;Verhasselt et al. 1997). In agreement with published literature, these results also show that LPS can induce many surface molecules implicated in DC differentiation and function. The results from experiments comparing the LPS deficient mutant, purified LPS and parent strain does give some clues as to an explanation for this finding. Firstly, the *lpxA-* mutant induces production of all cytokines tested, and to a greater degree than soluble LPS, albeit lower than the parent strain. This indicates several points. It suggests that DC's interact with intact bacteria in a different fashion to that of fluid phase stimuli. When exogenous LPS was added to the *lpxA-* mutant, although it augmented the cytokine response, it was nowhere near as effective as the parent was. Since LPS is a structural component of the outer membrane of the

bacteria, it is likely that the context in which LPS is presented to DC's is a key factor. It is possible that, since LPS can induce surface marker expression, that part of the explanation may be methodological. In these experiments, detection of intracellular cytokines employed the use of Brefeldin A, which blocks transport of secreted cytokine (and other proteins) from the Golgi apparatus. However, purified LPS is a potent inducer of cytokines in whole blood monocytes and cultured endothelial cells using this same method, arguing against this being a significant explanation for the results observed. In addition, similar results were obtained using either purified *E. coli* LPS or various batches of meningococcal LPS, making it very unlikely that the results were as a result of a poor batch of purified LPS with low biological activity. Dose of LPS is also unlikely to be the explanation for the findings. 100ng of meningococcal LPS is equivalent to or greater than LPS content of 10^7 bacteria. Measurement of the LPS content of *Neisseria meningitidis* based on spectrophotometric analysis of the LPS specific sugar 2-keto-3-deoxyoctonic acid (KDO) has demonstrated that there are approximately 1.5×10^5 molecules of LPS per bacterium. This makes 100ng/ml of purified LPS equivalent to about 10^8 bacteria/ml (M-van Deuren, personal communication).

What then is responsible for activation of DC's by the *lpxA*- strain? . Outer membrane proteins and pili from Neisseria, which are involved in adhesion and invasion of host cells and can induce signal transduction pathways known to control expression of cytokine genes (Naumann et al. 1997;Naumann et al. 1998). The *lpxA*- strain expresses the same outer membrane proteins as the parent strain (Steeghs et al. 1998), and thus provides a mechanism by which this strain is able to activate DC's. In addition, the *lpxA*- strain will contain other inflammatory components, such as lipoproteins, and pedptidoglycans.

As mentioned in the introduction, characterisation of the cellular pattern recognition receptors such as the human toll-like receptors (TLR's), has greatly aided enquiry into the molecular mechanisms by which the immune system detects and responds to microbial products (Medzhitov, Preston, & Janeway-CA 1997;Rescigno et al. 1998b). It now seems that TLR4, via CD14 is the major pattern recognition receptor for bacterial LPS (Beutler 2000), whereas TLR2 has been shown to recognise and signal in response to a number of components such as bacterial lipoproteins, peptidoglycans, gram-positive teichoic acid (Brightbill et al. 1999a;Takeuchi et al. 1999b). All of this has lead some workers to speculate that TLR's provide a mechanisms for discriminating between gram-positive and gram-negative pathogens (Takeuchi et al. 1999b). However, it is also becoming clear that a number of different components of the same organism, for example *Mycobacterium tuberculosis,* are recognised by different TLR's (Means et al. 1999). It seems more likely that the interaction between DC's and meningococci would involve both TLR4 and TLR2.

Of particular pertinence to interactions with microorganisms and phagocytic cells, were the findings of Underhill et al. They found that TLR2 was enriched within phagosomes of macrophages that had ingested yeast cell wall zymosan. Transfection with dominant negative mutant of TLR2 into macrophages abrogated TNF-α release in response to zymosan, even though still taken up into the phagosome, probably via mannose receptor internalisation (Underhill et al. 1999). This intriguing finding suggests that TLR's act in concert with particle internalisation receptors to initiate inflammatory responses like TNF-α production in phagocytic cells. The implications of this of DC's maturation in response to meningococci may be significant. In the parent organisms, LPS may engage with TLR's within the phagosome. In addition, other bacterial components, as

suggested by the data from the *lpxA*- strain, may interact with other TLR's and together with signals generated by the process of phagocytosis itself, provide effective stimulus for DC maturation.

Despite the ability of the *lpxA*- strain to activate DC's, it is noteworthy that the ratio of bacteria to DC required for this is at least a log order higher for the *lpxA*- strain than the parent is. This clearly indicates that presence of LPS within organisms is of great importance in generating the particular response in DC's, namely high IL-12 production. Production of IL-12 by DC's is considered to be a critical step in the generation of Th_1 responses (Trinchieri 1995), such as is required for protection against intracellular pathogens like *Listeria monocytogenes, Leishmania major* and *Mycobacteria* (Hsieh et al. 1993;Sher & Coffman 1992). Previous work suggests that DC's produce IL-12 only after interaction with T helper cells in the lymph node via CD40 ligation (Cella et al. 1996). However, the published literature contains some conflicting data. In one study, CD40 ligand transfected Jurkat cells induced high levels of IL-12 whereas LPS, TNF-α, a gram-positive (*Staphylococcus aureus*) and a gram-negative bacteria (*Pseudomonas aeruginosa*) induced very little or no IL-12 (Cella et al. 1996). Another study failed to demonstrate IL12 production in LPS stimulated DC's when using immunocytohistochemistry (Lore et al. 1998) Other studies have found that LPS is able to stimulate IL-12 production in human DC's (Buelens et al. 1997;Karp et al. 1998;Verhasselt et al. 1997). It is notable is that in these studies ELISA's of supernatants was used to assay IL-12. The method employed in this study used an inhibitor of protein transport from the Golgi apparatus, which would prevent release of cytokines into the supernatant. This could also affect autocrine and paracrine stimulation of DC's in culture.

One study found that high-level IL-12 production by human DC's required two signals, CD40 ligand and Interferon-γ(Snijders et al. 1998). In addition, it was found that LPS could replace either of these stimuli, but that individually the production was much reduced in response to these stimuli. This is important for a number of reasons. Firstly, the requirement of two signals would mean that most effective IL-12 production occurs when DC's interact with activated lymphocytes bearing CD40 ligand or producing IFN-γ, which would limit its action to stimulate antigen specific T cells (Lanzavecchia 1998). Secondly, this requirement would be a 'safety mechanism', controlling over exaggerated and thus potentially harmful Th1 responses (Snijders et al. 1998). IL-12 production in DC's seems to be also dependent on their state of maturity and presence of inhibitory cytokines especially IL-10. For example after 48 hours in culture, fully mature CD83+ve, DC's fail to respond to either LPS, or CD40 ligand bearing T cells to produce IL-12, in part due to IFN-γ hyporesponsiveness (Kalinski et al. 1999). IL-10 inhibits IL-12 production in LPS stimulated DC's but only partially inhibits its production in CD40 ligand stimulated DC's (Buelens et al. 1997). IL-10 has potent counter-regulatory effects on T cell responses, and is Th2 type cytokine (Moore et al. 1993). These differential effects may be important because quite different responses may occur depending on the ratio of pro- and anti-inflammatory stimuli in the environmental milieu when DC's are exposed to pathogenic material. However, DC's, in common with monocytes, become unresponsive to LPS induced production of IL-12, which cannot be explained by action of IL-10 or TGF-β, and may be a mechanism for immunological anergy seen after exposure to LPS (Karp et al. 1998).

The reasons for the failure to detect IL-12 in DC's stimulated by LPS in this study are unclear. It is possible, that given the generally low level of cytokine

production in LPS stimulated DC's, that the intracellular cytokine method is not sensitive enough to detect low levels of cytokine. In this case, the fact that cytokines are so clearly detectable in DC's stimulated with organisms means that they are induced at relatively large quantities. Since cytokine production was assayed at a per cell basis by flow cytometry, it at least allows us to conclude that the potency of the organisms, especially IL-12 and IL-1a production was markedly greater than LPS alone. There is a possibility that the presence of other cells within the culture wells would have an impact on the results. This applies particularly to CD40 ligand, or IFN-γ producing cells, which could act as co-stimulators for IL-12 production. However, this would apply equally to LPS stimulated cells, and the results indicate that this is unlikely to be a major mechanism. The finding that meningococci are able to induce high levels of IL-12 in the apparent absence of other stimuli is interesting and may be important. Certainly, Salmonella are able to enter DC's and induce IL-1, IL-6 and IL-12 production (Marriott et al. 1999). This provides a link between cytokine production, particulate entry and activation by bacterial components (Scheicher et al. 1995;Shibata, Metzger, & Myrvik 1997).

IL-12 is composed of two sub-units, p40 and p35, which combine to produce the bioactive heterodimer, but in addition p40 forms homodimers which are considered to act as IL-12 receptor antagonists (Trinchieri 1995). It is interesting to note that p40 is produced in larger quantities than p70 (Trinchieri 1995) and is also the target of many standard IL-12 ELISA assays. However, it is possible to assay the bioactive p70 heterodimer, or quantitate biologically active IL12 by means of measuring the production of IFN-γ in peripheral blood mononuclear cells stimulated with supernatants from DC cultures (D'Andrea et al. 1993). A number of studies could detect p70 IL-12 in LPS stimulated DC's, by

ELISA (Kalinski et al. 1999;Karp et al. 1998;Montgomery et al. 1991). The monoclonal antibody used in the results presented in this study recognises the p70 heterodimer, but also the p40 homodimer. If the IL-12 detected in this study contains significant amount of IL12 p40, then it noteworthy that LPS still fails to induce this component using the intracellular method.

Chapter 7

7. General discussion and future developments

7.1. Introduction

Meningococcal disease is still a cause of significant mortality and morbidity both in the United Kingdom and in many other counties worldwide. The mortality from this condition has dropped significantly in the last 20 years for a number of reasons. Early recognition of symptoms and signs, particularly by parents, relatives and primary care workers has lead to more rapid referral to expert medical care. Prompt effective resuscitation, early use antibiotics and access to modern intensive care facilities are all factors that are implicated. This is despite an increase in the incidence of invasive meningococcal disease that has occurred in many developed countries in the last ten years.

Most deaths from MD in the developed world are due to overwhelming sepsis. In pathophysiological terms, vascular damage and dysfunction can explain many of these features. In particular, activation and adherence of host inflammatory cells, particularly neutrophils, platelets and monocytes, in addition to the pro-coagulant and inflammatory consequences of widespread activation of clotting cascades are critical determinants of the severity, and outcome in severe invasive MD. Meningococci can be cultured from the vasculitic lesions from patients and can be seen to adhere and invade vascular endothelium *in vivo* often associated with invading neutrophils, platelets and microthrombi (Sotto et al. 1976). *In vitro* studies have shown that meningococci adhere to and invade to endothelium in a ligand specific manner that is dependent on bacterial structure (Virji et al. 1991;Virji et al. 1992a), can mediate endothelial damage in the presence of neutrophil (Klein et al. 1996) and induce procoagulant phenotype (Heyderman et al. 1997). At the beginning of this thesis, there was no information on how meningococci themselves induced expression of vascular adhesion molecules,

which are critical in the regulation of adhesion and activation of inflammatory cells to vascular endothelium.

This thesis has attempted to demonstrate that meningococci are not only potent inducers of important cell adhesion molecules on endothelial cells, but that the nature of this response is dependent on the structure of the bacteria. In particular, those factors, such as capsulation and LPS structure already shown to influence capacity of meningococci to adhere to and invade both endothelial and epithelial cells were also shown to be determinants of degree of endothelial activation, as assessed by expression of cell adhesion molecules on HUVEC.

It soon became clear from the results that the pattern of this activation induced by meningococci was different to that observed in response to purified LPS. Moreover, this difference could not be adequately explained by differences in dose or structure of LPS used, as has been discussed in Chapter 3. This raised a number of important questions. Firstly, can components other than LPS in meningococci induce vascular adhesion molecules, and secondly, can the differences in the expression of cell adhesion molecules be explained by differences in signal transduction mechanisms known to regulate their expression? The fortuitous construction of a completely LPS deficient meningococcal mutant helped answer the first question, as discussed in Chapter 4. The second question addressed in Chapter 5, proved far more difficult to answer, for a number of reasons, which will be discussed later. Many specific questions have been posed in the discussion sections of the various chapters. There are a number of important general considerations that should be addressed.

7.2 Validation of data and future studies

7.2.1 Is the use of isogenic mutants valid to what happens in vivo?

Isogenic mutants are very powerful tools for studying host bacteria interactions. Since they are all derived from the same strain, they are likely to be identical to the parent in every way apart from the specific phenotypic characteristic that the gene deletion(s) impart. In this way, comparison of a single strain and its derived mutants may be more useful than studying action of different strains of bacteria, which can vary in too many ways that conclusions drawn may be limited (Manger & Relman 2000). There is theoretical and experimental support for the notion that the phenotypic characteristics of particular isogenic mutants used have pathological relevance. Surface expressed components of meningococci, especially capsule and sialylated LPS may be advantageous for survival but may not allow for adhesion and transmigration across host epithelial and endothelial barriers. The parent B1940 does not appear to adhere to or transmigrate through epithelial cells (Hammerschmidt et al. 1994) or adhere well to endothelial cells (Klein et al. 1996). Modulation of expression of both these phenotypic characteristics can occur both *in vivo* and *in vitro* (Hammerschmidt et al. 1996b;Hammerschmidt et al. 1996a).

There are of course a number of problems that use of isogenic mutants entail. They are unlikely to be encountered in nature and since most of them will not have pathogenic potential, they may not give a true picture of the complex molecular events that occur during invasive disease. In addition, specific gene deletions may have unpredictable effects in terms of phenotypic characteristics and behaviour. This could be for a number of reasons. Either the construct has effects on other genes, or the targeted gene controls unknown phenotypic

characteristics. As the expression of many outer membrane components of meningococci are phase variable, it is possible that some colonies of mutant strains may not express some outer membrane components.

A number of other approaches could be used to investigate interactions between meningococci and host cells. Either expression of single genes can be tracked *in vitro* and *in vivo* by including a gene construct linked to a fluorescent tag, such as green fluorescent protein (GFP). This could be very useful especially in tracking what genes are expressed or switched off when meningococci invade nasopharygeal epithelial cells, enter the bloodstream or attach to and invade vascular endothelium. It is possible, for example, that only a very few bacteria are required to enter and escape the mucosal barrier. Once in the circulation, genes required for survival may be switched back on, and hence favouring bacterial growth. Another approach would be to use gene expression arrays that would allow simultaneous analysis of expression of a large number of genes in either host or bacterium (Manger & Relman 2000). Analysis of such data is likely to be formidable. It is likely that hundreds of genes will be activated during these interactions and deciding which of these is of primary pathological relevance will be difficult. Again, isogenic mutants could be very useful in this situation,

7.2.2.Is the use of HUVEC valid?

HUVEC are commonly used in models that study endothelial function. They are readily available from renewable sources, relatively easy to culture and are responsive, in terms of alteration in phenotypic characteristics, to commonly used stimuli, such as LPS and pro-inflammatory cytokines. However, a drawback that their use might entail is that they are derived from only one anatomical site. Since endothelial cells will vary in both phenotype and reactogenicity according to their

site of derivation, HUVEC might not be the best model for studying interactions with meningococci and EC's. The alternative would be to either culture cells from different anatomical sites or use established cell lines. However, there are also significant problems with this approach as well. Some cell lines are transformed by use for example SV40 transfection, whilst others have been serially passaged and require use of additional growth factors, all of which influence endothelial phenotype and reactogenicity to stimuli.

A number of approaches may help address these questions. For example, biopsy samples from vasculitic lesions from patients could be studied. This approach is already being used to investigate phenotypic and genotypic characteristics of meningococci invading vascular endothelium (R. Heyderman, personal communication). Cell adhesion molecule expression on vascular endothelial cells could be analysed by fluorescent confocal microscopy. This approach could then be used to address a number of questions. For example, what is the pattern of cell adhesion molecule expression in those endothelial cells to which meningococci have already adhered or invaded? Are there foci of meningococcal invasion and are these associated with activated and adherent neutrophils? Another intriguing question is whether activation of the endothelium itself may support further meningococcal adhesion and invasion. It may be that expression of selectins themselves may interact with meningococci in a ligand specific manner. Moreover, this may allow bacteria that may otherwise be poorly adherent to bind to endothelium. Damage to endothelium, and exposure of sub-endothelial matrix may also act as a focus for further meningococcal attachment

7.2.4. What is the importance of bacterial numbers, LPS levels and degree of host response?

The importance and role of the relationship between the host inflammatory response, and numbers of bacteria and levels of endotoxin has been discussed in previous chapters. Recent evidence has forced a revaluation of the relative role of the nature of the host inflammatory response in determining the outcome from bacterial sepsis in general (Bone 1996b;Bone 1996a). One proposed model states that the host responds by producing a pro-inflammatory response at the site of invasion, in order to clear organisms, but also produces a counter-regulatory, systemic anti-inflammatory response, in order to contain the action of potent pro-inflammatory molecules that might damage the host. When local invasive infection is not contained and becomes systemic, a number of problems may occur.

In the systemic inflammatory response, the relative balance of pro-inflammatory and anti-inflammatory mechanisms and their pathophysiological consequences of these may depend to a great degree on what stage of SIRS a patient in. A state of 'immunological dissonance' describes a state where these two processs are out of balance (Heckels 1989). The anti-inflammatory response induces anergy, which can result in failure of further clearance of micro-organisms. Any further failure to clear proliferating organisms may result in further damage being inflicted by the actions of organisms themselves, in addition to the host inflammatory responses already set in motion. Hence, organism dependent activation of host inflammatory processes, such as endothelial and neutrophil activation, activation of pro-thrombotic cascades may be particularly relevant. This is because degree of damage would depend on the presence of large numbers of organisms, and their associated toxic molecules rather than levels of pro-

inflammatory cytokines *per se.* In the light of this, the studies by Westendorp et al, which indicate that those patients who have the poorest outcome from MD are those who have a predisposition to mount predominantly anti-inflammatory profile, would seem all the more important (Westendorp et al. 1997).

All of this becomes particularly relevant if one considers what occurs to endotoxin production and elimination during the course of severe MD. Plasma endotoxin levels are highest at time of presentation of disease and then decline progressively after the administration of antibiotics (Brandtzaeg et al. 1989a). This decline occurs despite continued deterioration in the clinical status of the patient (van Deuren et al. 1992). This highlights several central points. Antibiotic treatment does not seem to greatly increase endotoxin release as it can in certain situations (such as Jarisch-Herxheimer reaction) and prompt use of antibiotics is of great importance. It also demonstrates that rise in endotoxin levels probably reflects presence of proliferating meningococci. Any process that favours the proliferation of organism over clearance is likely to have a profound effect on eventual outcome from MD.

Development and validation of new techniques that could quantify numbers of meningococci in the circulation in cases of invasive MD would seem particularly appropriate. This then could be used in conjunction with measurement of endotoxin activity, LPS levels, cytokine levels (both pro- and anti-inflammatory) and genetic data to identify genetic polymorphisms (such as tendency to produce high levels of IL-10) that may contribute to increasing risk of mortality from this condition.

7.2.5. Does the data from use of $rBPI_{21}$ help in assessing the use of anti-LPS adjuncts in MD?

Recent years have witnessed a great deal of effort and money directed towards development of novel strategies to counteract the inflammatory damage seen in bacterial sepsis. In particular, strategies that aimed to abrogate the effect of the powerful pro-inflammatory cytokines TNF-α and IL-1 have been developed but, have largely been unsuccessful (Bone 1996c). This could be due to a number of reasons. Firstly, the complexity of the regulatory mechanisms of cytokines *in vivo,* such as soluble TNF-receptors which may both inhibit action of TNF-α initially but prolong its activity (Aderka et al. 1992) may mean that pharmacological targeting of this molecule may have unforeseen effects. Secondly, for the reasons given in the previous section, blocking action of pro-inflammatory cytokines such as TNF-α may not always be beneficial, as the effect this may have will depend critically on the phase of the SIRS an individual is in (Bone 1996b;Bone 1996c).

Strategies that are primarily directed against bacterial components and toxins that induce inflammatory response in humans would seem appropriate. The best studied of these components is gram-negative endotoxin, or LPS. Gram-negative septicaemia is a significant cause of mortality (Glauser et al. 1991b), and effects of this potent molecule have been extensively studied. The reasons why early trials using monoclonal antibodies to LPS failed to improve outcome have been already been discussed. Development of the protein rBPI is important because it is host derived and has both anti-LPS activities and anti-biotic activities. A phase III placebo control trial that evaluated its safety and usefulness as

adjunctive therapy in paediatric meningococcal disease has been completed and its results eagerly awaited.

The results presented in this thesis indicate that this molecule is highly effective at abrogating endothelial cell adhesion molecule induction by purified LPS. They also showed that $rBPI_{21}$ can effectively abrogate this response induced by meningococci. However, it was also clear was that it only effectively inhibited endothelial response at bacterial concentrations of 10^5CFU/ml. In addition, its effects were partially determined by the LPS structure of meningococci. The lack of inhibition seen when higher concentrations of bacteria were used could be due to a number of factors. Firstly, stoichiometric ratios of LPS in organisms and rBPI molecules could dictate that rBPI simply cannot bind to lipid A, especially if sialylation of LPS confers resistance to this binding to lipid A buried in the bacterial membrane. In addition, the data from the effects of rBPI on the LPS deficient mutant *lpxA*- show that rBPI cannot inhibit cell adhesion molecule expression induced by non-LPS components of meningococci. This effect would have increasing relevance at large concentrations of bacteria, which, as has been stated, might be the cases in the very worst cases of MD. If rBPI is to be effective in MD, it may be that it needs to be given early in the course of the disease. As is the case of antibiotic therapy, this would serve to halt the logarithmic increase in bacterial numbers to levels that no amount of anti-bacterial therapy can combat. It would also interrupt pathogen specific, rather than host specific activators of the inflammatory response.

7.2.6. Is the transcription factor data useful?

When this thesis was started, a number of fundamental questions regarding the mechanisms by which the host recognises and responds to bacterial pathogens

remained unanswered. One of the most enduring puzzles had been how bacterial LPS is recognised (Ulevitch & Tobias 1995). In the last three years, a major discovery has been the discovery of mammalian toll-like receptors. Of great interest is the fact that TLR's of different kinds may help discriminate between certain classes of bacterial components (Takeuchi et al. 1999b;Underhill et al. 1999). It seems that TLR4 may be responsible for the recognising bacterial LPS (Beutler 2000) and transducing signals to a highly evolutionary conserved pathway that involves, amongst other processes, regulation many genes involved in inflammatory and immune responses.

The family of nuclear transcription factors NF-κB appear to be central to the toll signal transduction pathway (Hatada, Krappmann, & Scheidereit 2000). From the results presented in chapter 3, it was clear that there were differences in the pattern of endothelial adhesion molecule expression (particularly CD62E) induced by meningococci and that seen with purified LPS. For this reason, the binding of nuclear transcription factors to promoter region of CD62E (E-selectin) gene, critical to its transcriptional regulation, was assessed by EMSA. The results presented in chapter 5 contain a number of interesting observations. Firstly, that non-LPS containing meningococci induce very weak NF-κB activity. Also, that the binding pattern to the ATF2 consensus sequence shows that there might be some differences between the signal induced by the bacteria as compared to purified LPS. Identification of the specific mechanisms responsible for this difference would require further investigation.

Transcription factor binding does not measure transcriptional activity. However, this could be achieved by transfecting the promoter region controlling CD62E that is linked to a luciferase expression vector into HUVEC (Tamaru &

Narumi 1999). Transcriptional activity in response to bacteria and LPS could then be quantified. In addition, measurement of mRNA, either by quantitative PCR techniques or northern blot experiments may indicate whether this increase in CD62E expression is due to increase in transcription and translation, or due to post translational processes.

The reasons why NF-κB activity is quite so low when stimulated by the meningococci without LPS is unclear. Differential TLR expression on endothelial cells might be part of the answer. . In a recent study, it was found that both HUVEC and HMEC-1 microvascular cells expressed very low levels of TLR2, and did not respond to lipoproteins, known to signal through this receptor (Brightbill et al. 1999b). Moreover, response could be restored in HMEC-1 by transfecting TLR2 constructs (Faure et al. 2000). It may be that the LPS deficient mutant signals to a significant extent through TLR2, which may explain in part the relatively poor response to this organism seen in HUVEC. As yet, there are no details about how these non-LPS components are recognised, and which TLR's are used. In addition, it still needs to be explained how it is that at the high doses of bacteria, the LPS deficient organism can induce very high levels of particularly CD62E. Is there synergy between TLR receptor mediated signals and signals generated via other pathways, such as Rho GTPases? Does bacterial adhesion and invasion via the activation of novel pathways as described by (Naumann et al. 1998) influence this?

It is certain that the next few years will see an explosion of information on the how bacterial components and TLR's interact. Not the least of these could be the identification of genetic polymorphisms of the TLR's that may have a fundamental influence on how people react to microorganisms of different types.

Also, there is an intriguing idea that activation of TLR's by LPS may generate ligands by action of serine proteases, as it does in Drosophila (Bowie & O'Neill 2000). This would be analogous to a number of systems involving proteolytic cascades such as the coagulation pathway. How bacteria such as meningococci on contact with endothelial cells may modulate this would be fascinating

7.2.7.Can DC responses to *N. meningitidis in vitro* be useful in evaluating immune responses *in vivo*?

Following the recent well-publicised vaccination campaign using the meningococcal, C conjugate vaccine an encouraging fall in predicted cases due to this serogroup has been noted (CDR report, April 2000). This very welcome news must be tempered with a note of caution when considering vaccination strategies against *Neisseria meningitidis* as a whole. There is still no effective vaccine against serogroup B, which is still responsible for most meningococcal disease in the UK and a number of other countries. Unfortunately, it is unlikely that a similar strategy used in the development of the C conjugate vaccine would work in group B disease. This strategy requires generation specific antibody response to capsule, which, in the case of group B meningococci would either be ineffective or potentially harmful, due to its mimicry of host N-CAM (Finne, Leinonen, & Makela 1983).

There is increasing interest in the role of DC's in the course of both natural infections and in response to vaccines. This is not only due to there capacity to process and present antigen, but also due to their ability to provide co-stimulatory signals to lymphocytes via surface expressed ligands like CD40 and CD86 but also soluble mediators such as IL-12 (Banchereau & Steinman 1998a;Steinman 1991). DC's could be useful tools in predicting the nature of the

immune response to a particular bacterium, or its components. The results from this study point to the fact that the DC sees bacterial components, particularly LPS, in context of bacteria themselves, probably because of the correct molecular orientation within the outer membrane. The results also suggest that when LPS is presented to DC's in intact bacteria, it is the dominant inflammatory species in meningococci. In this regard, the powerful adjuvant property of LPS could be harnessed without its deleterious effects, either by chemical modification of reducing LPS content in the preparations (but not completely) (Alving 1993). Data derived from future studies could be used to augment screening of potential vaccines, especially as there are some doubts as to the suitability of animal models to test immune response to meningococcal antigen.

What could be the consequence of such findings? Understanding how DC's interact with either whole bacteria or modified components may help in the designing of novel vaccine strategy (Banchereau & Steinman 1998b). DC's engineered to produce IL-12 pulsed with antigen have been found to be potent at protecting mice form leishmaniasis (Ahuja et al. 1999). Modulation of antigen delivery may be beneficial. This may involve either conjugation of polysaccharide antigen to protein carrier, which has been used successfully against vaccination against *Haemophilus influenza* type B and now *Neisseria meningitidis* Group A and C (Richmond et al. 1999). Route of delivery of antigen may significantly alter the way is processed by the immune system. Delivery to mucosal routes such as intranasal route may be beneficial because such areas are so rich in antigen presenting cells like DC's.

Interestingly, heat-inactivated *lpxA*- bacteria or outer membrane complexes from this mutant elicit poor immune responses in mice (Steeghs et al. 1999). However, protective responses and bactericidal antibody production,

including class switching to IgG2a and IgG2b, were restored by addition of native LPS, as well as chemically altered LPS and less toxic, related compounds. Fascinatingly, this restoration of non-LPS components ability to elicit protective immunity only occurred when outer membrane preparations of *lpxA*-, and not with whole organisms, were given together with LPS. This suggests that LPS may complex with outer membrane preparations and not to whole organisms. This complements data presented in this thesis that optimal maturation of DC's and in particular IL-12 production only occurs with intact organisms with LPS in the outer membrane.

Another interesting feature of the results is the comparison of the two strains of meningococcus with different LPS structures. It is considered that it may be beneficial to include meningococcal LPS with short oligosaccharide structures, since these may contain epitopes that are preserved across strains (Tappero et al. 1999). It is interesting, therefore, that the *cps*- mutant, which has a truncated LPS, induces a pattern of DC maturation and cytokine production that is different from that seen with the parent strain. Why this should be so is not clear. However, part of the answer may lie in the relationship between internalisation of bacteria and molecular recognition of and response to pathogen associated motifs, particularly LPS by DC's. Hence, any bacterial property that effects the process of internalisation may determine the pattern of activation and maturation of DC's. It would be interesting therefore, to investigate whether phagocytic uptake of parent and isogenic mutants differed in any way, either by degree or rate.

A major recent development has been the complete characterisation of the genome of a single strain of a group B meningococcus, MC58 (Tettelin et al. 2000). There is hope that use of this enormous amount of information can be

used to predict and test which components of meningococci could be used a potential vaccine candidates. One approach has been to identify surface exposed structures that are conserved across strains and elicit bactericidal antibodies in mice (Gregory 2000). However, such an enquiry may be complemented by investigating how DC's present such epitopes, especially in context of danger signals generated by presence of LPS or its less toxic derivatives that still possess adjuvant activity.

All this should be stated with a note of caution. Since DC's play such a fundamental role in so many aspects of immunity, including tolerance, generation of autoimmunity and chronic inflammation, not all the activities of DC's may be beneficial (Sallusto & Lanzavecchia 1999). The evaluation the response of DC's to bacteria that have defined structural differences or the components that are derived from them may allow us to gain insight into potentially beneficial, or indeed deleterious, effects that such factors may engender.

7.3. Concluding remarks

Despite the huge amount of epidemiological and experimental data so far accumulated about the relationship between humans and *Neisseria meningitidis*, there is a great deal that remains a complete mystery. To understand a disease one must first ask the question of it; what is it in particular about this condition that defines it? This thesis started with a simple premise that this bacterium, once it has escaped from the normal commensal environment, can induce a pattern of vascular damage that is characteristic in both severity and rapidity. We are now in possession of the tools to attempt to answer two questions; what is it about this particular organism and what is it about this person that had lead to this? There is no more graphic illustration of the nature of this disease than in witnessing the first appearance of rash in a person through to established and fatal disease. The

answer, however complicated, however chaotic and however unpredictable lies there.

Appendix I:

Flow cytometry settings

HUVEC		
Detector	**Voltage**	**Amp gain**
Forward scatter	E-1	1.00
Side scatter	347	1.00
Fluorescence-1 (FITC)	396	1.00
Fluorescence-2 (PE)	417	1.00
Threshold	200	
Dendritic cells		
Forward scatter	E-1	4.07-5.27
Side scatter	380	1.00
Fluorescence-1 (FITC)	295	1.00
Fluorescence-2 (PE)	441	1.00
Threshold	72	

Appendix II:

Addresses of Suppliers

Amersham International,
Amersham Place,
Little Chalfont,
Bucks HP7 9NA

BDH Merck,
Merck House, Poole,
Dorset, BH15 1TD

Becton Dickinson,
Between Towns Road,
Cowley, Oxford, OX4
3LY

Bio-Rad Laboratories,
Maylands Avenue,
Hemel Hemptead
Herts. HP2 7TD

Boehringer Mannheim,
Bell lane,
Lewes,
E. Sussex, BN7 1LG

Clontech Laboratories,
Unit 2, Intec 2,
Wade Road,
Basingstoke,
Hants RG24 8NE

Dako Ltd.,
Angel Drove,
Ely,
Cambs. CB7 4ET

GIBCO BRL,
3, Fountain Drive,
Inchinnan Business
Park,
Paisley PA4 9RF

Globepharm Ltd.,
PO Box 89C.
Esher,
Surrey, KT10 9ND

Hayman Ltd.
111-3, Waldegrave
Road,
Teddington, Middx.

PAA Laboratories,
Suite 4,
2, Cambridge Road,
Kingston-upon-Thames,
KT 1

R & D systems,
4-10, The Quadrant,
Barton Lane,
Abingdon,
Oxon, OX14 3YS

Santa Cruz
Biotechnology, Inc.,
2161, Delaware
Avenue,
Santa Cruz,
California 95060
USA

Serotec,
22, Bankside,
Station Approach,
Kidlington,
Oxford, OX5 1JL

Sigma-Aldrich
(including Sigma-
Genosys),
Fancy Road,
Poole,
Dorset, BH17 7NH

Clonetics,
TCS biologicals,
Botolph Claydon,
Bucks MK18 2LR

National Diagnostics,
Unit 4,
Fleet Business Park,
Itlings Lane,
Hessle,
Hull, HU13 9LX

Whatman Ltd,
Springfield Mill,
Maidstone,
Kent

Appendix III:

Abbreviations

ADP	Adenosine diphosphate
AEBSF	4-(2Aminoethyl)benzenesulfonylfluoride
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CD	Cluster of differentiation
CFU	Colony forming units
DC's	Dendritic cells
DIC	Disseminated intravascular coagulopathy
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
ECAF	Endothelial attachment factor
EDTA	Ethenediamine tetraacetic acid
ELISA	Enzyme linked immune sorbant assay
EMSA	Electrophoretic mobility shift assay
FACS	Fluorescent antibody labelled cell sorter
FCS	Fetal calf serum
FITC	Fluoroscein isothiocyanate
GAGs	Glycosaminoglycans
GM-CSF	Granulocyte monocyte colony stimulating factor
GPI	Glycosylphosphatidyl inositol
HBSS	Hank's buffered saline solution
HLA	Human leukocyte antigen
HUVEC	Human umbilical vein endothelial cells
ICAM-1	Intercellular adhesion molecule 1
IFN-γ	Interferon gamma

kDA	Kilo daltons
LAD	Leukocyte adhesion deficiency
LBP	Lipolysaccharide binding protein
LFA	Leukocyte function antigen
LPS	Lipopolysaccharide
M	molar
MAC	Membrane attack complex
MBL	Mannose binding lectin
MCDB	Molecular and cellular and developmental biology
mCi	Milli curies
MCP-1	Macrophage chemoattractant protein 1
MD	Meningococcal disease
MFI	Median fluorescence intensity
MHC	Major Histocompatibility Antigen
mRNA	Messenger RNA
NCAM-1	Neural cell adhesion molecule
NO	Nitric oxide
NRS	Normal rabbit serum
OD	Optical Density
OMP	Outre membrane protein
OMV	Outre membrane vesicle
P	Probability
PAF	Platelet activating factor
PAGE	Polyacrylamide gel electrophoresis
PBMC's	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PE	Phycoerythrin
PECAM-1	Platelet endothelial cell adhesion molecule
PKC	Protein kinase C
$rBPI_{21}$	Recombinant bactericidal permeability

	increasing protein
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
SEM	Standard error of the mean
SIRS	Systemic inflammatory response syndrome
SMD	Systemic meningococcal disease
TBE	Tris borate-EDTA
TGF-β	Transforming growth factor beta
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor alpha
VCAM-1	Vascular cell adhesion molecule 1
VLA-4	Very late leukocyte antigen 4

References

Achtman, M. 1995, "Global Epidemiology of Meningococcal Disease," in *Meningococcal Disease*, K. Cartwright, ed., John Wiley and sons, Chichester, pp. 161-175.

Ada, G. L. 1990, "The immunological principles of vaccination [see comments]", *Lancet*, vol. 335,no. 8688, pp. 523-526.

Aderka, D., Engelmann, H., Maor, Y., Brakebusch, C., & Wallach, D. 1992, "Stabilization of the bioactivity of tumor necrosis factor by its soluble receptors", *J.Exp.Med.*, vol. 175,no. 2, pp. 323-329.

Ahuja, S. S., Reddick, R. L., Sato, N., Montalbo, E., Kostecki, V., Zhao, W., Dolan, M. J., Melby, P. C., & Ahuja, S. K. 1999, "Dendritic cell (DC)-based anti-infective strategies: DCs engineered to secrete IL-12 are a potent vaccine in a murine model of an intracellular infection", *J.Immunol.*, vol. 163,no. 7, pp. 3890-3897.

Albert, M. L., Pearce, S. F., Francisco, L. M., Sauter, B., Roy, P., Silverstein, R. L., & Bhardwaj, N. 1998, "Immature dendritic cells phagocytose apoptotic cells via alphavbeta5 and CD36, and cross-present antigens to cytotoxic T lymphocytes", *J.Exp.Med.*, vol. 188,no. 7, pp. 1359-1368.

Alving, C. R. 1993, "Lipopolysaccharide, lipid A, and liposomes containing lipid A as immunologic adjuvants", *Immunobiology*, vol. 187,no. 3-5, pp. 430-446.

Andersen, S. R. 1997, Lipopolysaccharide as component in an outre membrane vesicle vaccine against meningococcal disease caused by Neisseria meningitidis of serogroup B, Department of Vaccinology, NIPH. PhD.

Andersen, S. R., Bry, K., Thorseng, K., & Jantzen, E. 1996, "Heterogeneity of lipoploysaccharides of Neisseria meningitidis revealed by thin-layer chromotography combined with monoclonal antibodies", *J.Microbiol.Methods*, vol. 25, pp. 187-194.

Andersen, S. R., Bjune, G., Hoiby, E. A., Michaelsen, T. E., Aase, A., Rye, U., & Jantzen, E. 1997, "Outer membrane vesicle vaccines made from short-chain lipopolysaccharide mutants of serogroup B Neisseria meningitidis: effect of the carbohydrate chain length on the immune response", *Vaccine*, vol. 15,no. 11, pp. 1225-1234.

Andersen, S. R., Bjune, G., Lyngby, J., Bryn, K., & Jantzen, E. 1995, "Short-chain lipopolysaccharide mutants of serogroup B Neisseria meningitidis of potential value for production of outer membrane vesicle vaccines", *Microb.Pathog.*, vol. 19,no. 3, pp. 159-168.

Anderson, K. V. 2000, "Toll signaling pathways in the innate immune response", *Curr.Opin.Immunol.*, vol. 12,no. 1, pp. 13-19.

Anderson, M. S. & Raetz, C. R. 1987, "Biosynthesis of lipid A precursors in Escherichia coli. A cytoplasmic acyltransferase that converts UDP-N-acetylglucosamine to UDP-3-O-(R-3-hydroxymyristoyl)-N-acetylglucosamine", *J.Biol.Chem.*, vol. 262,no. 11, pp. 5159-5169.

Anon 1994, "The French National Registry of HA-1A (Centoxin) in septic shock. A cohort study of 600 patients. The National Committee for the Evaluation of Centoxin [see comments]", *Arch.Intern.Med.*, vol. 154,no. 21, pp. 2484-2491.

Anrather, D., Millan, M. T., Palmetshofer, A., Robson, S. C., Geczy, C., Ritchie, A. J., Bach, F. H., & Ewenstein, B. M. 1997, "Thrombin activates nuclear factor-kappaB and potentiates endothelial cell activation by TNF", *J.Immunol.*, vol. 159,no. 11, pp. 5620-5628.

Arditi, M., Zhou, J., Dorio, R., Rong, G. W., Goyert, S. M., & Kim, K. S. 1993, "Endotoxin-mediated endothelial cell injury and activation: role of soluble CD14", *Infect.Immun.*, vol. 61,no. 8, pp. 3149-3156.

Arditi, M., Zhou, J., Huang, S. H., Luckett, P. M., Marra, M. N., & Kim, K. S. 1994, "Bactericidal/permeability-increasing protein protects vascular endothelial cells from lipopolysaccharide-induced activation and injury.", *Infect.Immun.*, vol. 62,no. 9, pp. 3930-3936.

Arfors, K. E., Lundberg, C., Lindbom, L., Lundberg, K., Beatty, P. G., & Harlan, J. M. 1987, "A monoclonal antibody to the membrane glycoprotein complex CD18 inhibits polymorphonuclear leukocyte accumulation and plasma leakage in vivo", *Blood*, vol. 69,no. 1, pp. 338-340.

Astiz, M. E. a. R. E. C. 1998, "Septic Shock", *Lancet*, vol. 351, pp. 1501-1505.

Austyn, J. M. 1996, "New insights into the mobilization and phagocytic activity of dendritic cells [comment]", *J.Exp.Med.*, vol. 183,no. 4, pp. 1287-1292.

Banchereau, J. & Steinman, R. M. 1998a, "Dendritic cells and the control of immunity.", *Nature*, vol. 392,no. 6673, pp. 245-252.

Banchereau, J. & Steinman, R. M. 1998b, "Dendritic cells and the control of immunity.", *Nature*, vol. 392,no. 6673, pp. 245-252.

Baraff, L. J., Lee, S. I., & Schriger, D. L. 1993, "Outcomes of bacterial meningitis in children: a meta-analysis", *Pediatr.Infect.Dis.J.*, vol. 12,no. 5, pp. 389-394.

Beekhuizen, H., van-de, G. J., Olsson, B., van, B. I., & van, F. R. 1997, "Infection of human vascular endothelial cells with Staphylococcus aureus induces hyperadhesiveness for human monocytes and granulocytes", *J.Immunol.*, vol. 158,no. 2, pp. 774-782.

Belvin, M. P. & Anderson, K. V. 1996, "A conserved signaling pathway: the Drosophila toll-dorsal pathway", *Annu.Rev.Cell Dev.Biol.*, vol. 12, pp. 393-416.

Bendelac, A. & Fearon, D. T. 2000, "Innate immunity; Receptors and effectors of innate immunity", *Curr.Opin.Immunol.*, vol. 12, pp. 11-12.

Bennett, B. L., Cruz, R., Lacson, R. G., & Manning, A. M. 1997, "Interleukin-4 suppression of tumor necrosis factor alpha-stimulated E-selectin gene transcription is mediated by STAT6 antagonism of NF-kappaB", *J.Biol.Chem.*, vol. 272,no. 15, pp. 10212-10219.

Berry, L. J. 1985, "Introduction," in *Handbook of Endotoxin, Volume 3: Cellular Biology of Endotoxin*, L. J. Berry, ed., Elsevier Science Publishers, Amsterdam, pp. 17-21.

Beutler, B. 2000, "Tlr4: central component of the sole mammalian LPS sensor", *Curr.Opin.Immunol.*, vol. 12,no. 1, pp. 20-26.

Bevilacqua, M. P., Pober, J. S., Mendrick, D. L., Cotran, R. S., & Gimbrone-MA, J. 1987, "Identification of an inducible endothelial-leukocyte adhesion molecule", *Proc.Natl.Acad.Sci.U.S.A.*, vol. 84,no. 24, pp. 9238-9242.

Bierhaus, A., Zhang, Y., Deng, Y., Mackman, N., Quehenberger, P., Haase, M., Luther, T., Muller, M., Bohrer, H., Greten, J., & et, a. 1995, "Mechanism of the tumor necrosis factor alpha-mediated induction of endothelial tissue factor", *J.Biol.Chem.*, vol. 270,no. 44, pp. 26419-26432.

Bird, M. I., Foster, M. R., Priest, R., & Malhotra, R. 1997, "Selectins: physiological and pathophysiological roles", *Biochem.Soc.Trans.*, vol. 25,no. 4, pp. 1199-1206.

Bjerknes, R., Guttormsen, H. K., Solberg, C. O., & Wetzler, L. M. 1995, "Neisserial porins inhibit human neutrophil actin polymerization, degranulation, opsonin receptor expression, and phagocytosis but prime the neutrophils to increase their oxidative burst.", *Infect.Immun.*, vol. 63,no. 1, pp. 160-167.

Bjune, G., Hoiby, E. A., Gronnesby, J. K., Arnesen, O., Fredriksen, J. H., Halstensen, A., Holten, E., Lindbak, A. K., Nokleby, H., Rosenqvist, E., & et, a. 1991, "Effect of outer membrane vesicle vaccine against group B meningococcal disease in Norway [see comments]", *Lancet*, vol. 338,no. 8775, pp. 1093-1096.

Bone, R. C. 1991, "The pathogenesis of sepsis", *Ann.Intern.Med.*, vol. 115,no. 6, pp. 457-469.

Bone, R. C. 1996a, "Immunologic dissonance: a continuing evolution in our understanding of the systemic inflammatory response syndrome (SIRS) and the multiple organ dysfunction syndrome (MODS) [see comments]", *Ann.Intern.Med.*, vol. 125,no. 8, pp. 680-687.

Bone, R. C. 1996b, "Toward a theory regarding the pathogenesis of the systemic inflammatory response syndrome: what we do and do not know about cytokine regulation", *Crit Care Med.*, vol. 24,no. 1, pp. 163-172.

Bone, R. C. 1996c, "Why sepsis trials fail [see comments]", *JAMA*, vol. 276,no. 7, pp. 565-566.

Bonfanti, R., Furie, B. C., Furie, B., & Wagner, D. D. 1989, "PADGEM (GMP140) is a component of Weibel-Palade bodies of human endothelial cells", *Blood*, vol. 73,no. 5, pp. 1109-1112.

Bottomly, K. 1999, "T cells and dendritic cells get intimate [comment]", *Science*, vol. 283,no. 5405, pp. 1124-1125.

Bowie, A. & O'Neill, L. A. 2000, "The interleukin-1 receptor/Toll-like receptor superfamily: signal generators for pro-inflammatory interleukins and microbial products [In Process Citation]", *J.Leukoc.Biol.*, vol. 67,no. 4, pp. 508-514.

Braconier, J. H., Sjoholm, A. G., & Soderstrom, C. 1983, "Fulminant meningococcal infections in a family with inherited deficiency of properdin", *Scand.J.Infect.Dis.*, vol. 15,no. 4, pp. 339-345.

Brandtzaeg, P. 1995, "Pathogenesis of Menigococcal Infections," in *Meningococcal Disease*, K. Cartwright, ed., John Willey and Sons, Chichester, pp. 71-114.

Brandtzaeg, P., Hogasen, K., Kierulf, P., & Mollnes, T. E. 1996a, "The excessive complement activation in fulminant meningococcal septicemia is predominantly caused by alternative pathway activation", *J.Infect.Dis.*, vol. 173,no. 3, pp. 647-655.

Brandtzaeg, P., Joo, G. B., Brusletto, B., & Kierulf, P. 1990, "Plasminogen activator inhibitor 1 and 2, alpha-2-antiplasmin, plasminogen, and endotoxin levels in systemic meningococcal disease", *Thromb.Res.*, vol. 57,no. 2, pp. 271-278.

Brandtzaeg, P., Kierulf, P., Gaustad, P., Skulberg, A., Bruun, J. N., Halvorsen, S., & Sorensen, E. 1989a, "Plasma endotoxin as a predictor of multiple organ failure and death in systemic meningococcal disease.", *J.Infect.Dis.*, vol. 159,no. 2, pp. 195-204.

Brandtzaeg, P., Mollnes, T. E., & Kierulf, P. 1989, "Complement activation and endotoxin levels in systemic meningococcal disease", *J.Infect.Dis.*, vol. 160,no. 1, pp. 58-65.

Brandtzaeg, P., Osnes, L., Ovstebo, R., Joo, G. B., Westvik, A. B., & Kierulf, P. 1996b, "Net inflammatory capacity of human septic shock plasma evaluated by a monocyte-based target cell assay: identification of interleukin-10 as a major functional deactivator of human monocytes", *J.Exp.Med.*, vol. 184,no. 1, pp. 51-60.

Brandtzaeg, P., Ovsteboo, R., & Kierulf, P. 1992, "Compartmentalization of lipopolysaccharide production correlates with clinical presentation in meningococcal disease", *J.Infect.Dis.*, vol. 166,no. 3, pp. 650-652.

Brandtzaeg, P., Sandset, P. M., Joo, G. B., Ovstebo, R., Abildgaard, U., & Kierulf, P. 1989b, "The quantitative association of plasma endotoxin, antithrombin, protein C, extrinsic pathway inhibitor and fibrinopeptide A in systemic meningococcal disease", *Thromb.Res.*, vol. 55,no. 4, pp. 459-470.

Bredius, R. G., Derkx, B. H., Fijen, C. A., de, W. T., de, H. M., Weening, R. S., van-de, W. J., & Out, T. A. 1994, "Fc gamma receptor IIa (CD32) polymorphism in fulminant meningococcal septic shock in children", *J.Infect.Dis.*, vol. 170,no. 4, pp. 848-853.

Brightbill, H. D., Libraty, D. H., Krutzik, S. R., Yang, R. B., Belisle, J. T., Bleharski, J. R., Maitland, M., Norgard, M. V., Plevy, S. E., Smale, S. T., Brennan, P. J., Bloom, B. R., Godowski, P. J., & Modlin, R. L. 1999a, "Host defense mechanisms triggered by microbial lipoproteins through toll-like receptors", *Science*, vol. 285,no. 5428, pp. 732-736.

Buelens, C., Verhasselt, V., De, G. D., Thielemans, K., Goldman, M., & Willems, F. 1997, "Human dendritic cell responses to lipopolysaccharide and CD40 ligation are differentially regulated by interleukin-10.", *Eur.J.Immunol.*, vol. 27,no. 8, pp. 1848-1852.

Cannon, J. G., Tompkins, R. G., Gelfand, J. A., Michie, H. R., Stanford, G. G., van-der, M. J., Endres, S., Lonnemann, G., Corsetti, J., Chernow, B., & et, a. 1990, "Circulating interleukin-1 and tumor necrosis factor in septic shock and experimental endotoxin fever", *J.Infect.Dis.*, vol. 161,no. 1, pp. 79-84.

Capodici, C., Chen, S., Sidorczyk, Z., Elsbach, P., & Weiss, J. 1994, "Effect of lipopolysaccharide (LPS) chain length on interactions of bactericidal/permeability-increasing protein and its bioactive 23-kilodalton NH2-terminal fragment with isolated LPS and intact Proteus mirabilis and Escherichia coli.", *Infect.Immun.*, vol. 62,no. 1, pp. 259-265.

Carlos, T. M. & Harlan, J. M. 1994, "Leukocyte-endothelial adhesion molecules.", *Blood*, vol. 84,no. 7, pp. 2068-2101.

Caron, E. & Hall, A. 1998, "Identification of two distinct mechanisms of phagocytosis controlled by different Rho GTPases", *Science*, vol. 282,no. 5394, pp. 1717-1721.

Cartwright, K. 1995a, "Introduction and Historical Perpsectives," in *Meningococcal Disease*, 1st edn, K. Cartwright, ed., Chichester;John Willey and Sons, pp. 1-19.

Cartwright, K. 1995b, "Meningococcal Carriage and Disease," in *Meningococcal Disease*, K. Cartwright, ed., John Wiley and sons, Chichester, pp. 115-146.

Cartwright, K., Morris, R., Rumke, H., Fox, A., Borrow, R., Begg, N., Richmond, P., & Poolman, J. 1999, "Immunogenicity and reactogenicity in UK infants of a novel meningococcal vesicle vaccine containing multiple class 1 (PorA) outer membrane proteins", *Vaccine*, vol. 17,no. 20-21, pp. 2612-2619.

Cartwright, K. A., Jones, D. M., Smith, A. J., Stuart, J. M., Kaczmarski, E. B., & Palmer, S. R. 1991, "Influenza A and meningococcal disease", *Lancet*, vol. 338,no. 8766, pp. 554-557.

Cella, M., Sallusto, F., & Lanzavecchia, A. 1997, "Origin, maturation and antigen presenting function of dendritic cells.", *Curr.Opin.Immunol.*, vol. 9,no. 1, pp. 10-16.

Cella, M., Scheidegger, D., Palmer, L. K., Lane, P., Lanzavecchia, A., & Alber, G. 1996, "Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T-T help via APC activation", *J.Exp.Med.*, vol. 184,no. 2, pp. 747-752.

Clementz, T., Zhou, Z., & Raetz, C. R. 1997, "Function of the Escherichia coli msbB gene, a multicopy suppressor of htrB knockouts, in the acylation of lipid A. Acylation by MsbB follows laurate incorporation by HtrB", *J.Biol.Chem.*, vol. 272,no. 16, pp. 10353-10360.

Cohen, L., Henzel, W. J., & Baeuerle, P. A. 1998, "IKAP is a scaffold protein of the IkappaB kinase complex [see comments]", *Nature*, vol. 395,no. 6699, pp. 292-296.

Collins, T., Read, M. A., Neish, A. S., Whitley, M. Z., Thanos, D., & Maniatis, T. 1995, "Transcriptional regulation of endothelial cell adhesion molecules: NF-kappa B and cytokine-inducible enhancers", *FASEB J.*, vol. 9,no. 10, pp. 899-909.

Cooper, N. R. & Morrison, D. C. 1978, "Binding and activation of the first component of human complement by the lipid A region of lipopolysaccharides", *J.Immunol.*, vol. 120,no. 6, pp. 1862-1868.

Cotran, R. S., Gimbrone-MA, J., Bevilacqua, M. P., Mendrick, D. L., & Pober, J. S. 1986, "Induction and detection of a human endothelial activation antigen in vivo", *J.Exp.Med.*, vol. 164,no. 2, pp. 661-666.

D'Andrea, A., Aste-Amezaga, M., Valiante, N. M., Ma, X., Kubin, M., & Trinchieri, G. 1993, "Interleukin 10 (IL-10) inhibits human lymphocyte interferon gamma-production by suppressing natural killer cell stimulatory factor/IL-12 synthesis in accessory cells", *J.Exp.Med.*, vol. 178,no. 3, pp. 1041-1048.

de-la Paz H., Cooke, S. J., & Heckels, J. E. 1995, "Effect of sialylation of lipopolysaccharide of Neisseria gonorrhoeae on recognition and complement-mediated killing by monoclonal antibodies directed against different outer-membrane antigens", *Microbiology.*, vol. 141,no. Pt 4, pp. 913-920.

de Vries, F. P., van Der Ende, A., van Putten, J. P., & Dankert, J. 1996, "Invasion of primary nasopharyngeal epithelial cells by Neisseria meningitidis is controlled by phase variation of multiple surface antigens", *Infect.Immun.*, vol. 64,no. 8, pp. 2998-3006.

De Luca, L. G., Johnson, D. R., Whitley, M. Z., Collins, T., & Pober, J. S. 1994, "cAMP and tumor necrosis factor competitively regulate transcriptional activation through and nuclear factor binding to the cAMP-responsive element/activating transcription factor element of the endothelial leukocyte adhesion molecule-1 (E-selectin) promoter", *J.Biol.Chem.*, vol. 269,no. 30, pp. 19193-19196.

Delude, R. L., Savedra, R., Zhao, H., Thieringer, R., Yamamoto, S., Fenton, M. J., & Golenbock, D. T. 1995, "CD14 enhances cellular responses to endotoxin without imparting ligand-specific recognition", *Proc.Natl.Acad.Sci.U.S.A.*, vol. 92,no. 20, pp. 9288-9292.

Dent, C. L. a. L. D. S. 1993, "The DNA mobility shift assay," in *Transcription Factors-a practical approach*, 1st edn, D. S. Latchman, ed., Oxford University Press, Oxford, pp. 1-26.

Derkx, B., Marchant, A., Goldman, M., Bijlmer, R., & van Deventer, S. 1995, "High levels of interleukin-10 during the initial phase of fulminant meningococcal septic shock", *J.Infect.Dis.*, vol. 171,no. 1, pp. 229-232.

Detmers, P. A., Lo, S. K., Olsen, E. E., Walz, A., Baggiolini, M., & Cohn, Z. A. 1990, "Neutrophil-activating protein 1/interleukin 8 stimulates the binding activity of the leukocyte adhesion receptor CD11b/CD18 on human neutrophils", *J.Exp.Med.*, vol. 171,no. 4, pp. 1155-1162.

DeVoe, I. W. & Gilchrist, J. E. 1975, "Pili on meningococci from primary cultures of nasopharyngeal carriers and cerebrospinal fluid of patients with acute disease", *J.Exp.Med.*, vol. 141,no. 2, pp. 297-305.

Dignam, J. D., Lebovitz, R. M., & Roeder, R. G. 1983, "Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei.", *Nucleic.Acids.Res.*, vol. 11,no. 5, pp. 1475-1489.

Dinarello, C. A. 1996, "Biologic basis for interleukin-1 in disease", *Blood*, vol. 87,no. 6, pp. 2095-2147.

Donnelly, S. C., MacGregor, I., Zamani, A., Gordon, M. W., Robertson, C. E., Steedman, D. J., Little, K., & Haslett, C. 1995, "Plasma elastase levels and the development of the adult respiratory distress syndrome", *Am.J.Respir.Crit Care Med.*, vol. 151,no. 5, pp. 1428-1433.

Doukas, J. & Pober, J. S. 1990, "IFN-gamma enhances endothelial activation induced by tumor necrosis factor but not IL-1", *J.Immunol.*, vol. 145,no. 6, pp. 1727-1733.

Dubois, B., Vanbervliet, B., Fayette, J., Massacrier, C., Van, K. C., Briere, F., Banchereau, J., & Caux, C. 1997, "Dendritic cells enhance growth and differentiation of CD40-activated B lymphocytes [see comments]", *J.Exp.Med.*, vol. 185,no. 5, pp. 941-951.

Dustin, M. L. & Springer, T. A. 1988, "Lymphocyte function-associated antigen-1 (LFA-1) interaction with intercellular adhesion molecule-1 (ICAM-1) is one of at least three mechanisms for lymphocyte adhesion to cultured endothelial cells", *J.Cell Biol.*, vol. 107,no. 1, pp. 321-331.

Eden, C. S., Shahin, R., & Briles, D. 1988, "Host resistance to mucosal gram-negative infection. Susceptibility of lipopolysaccharide nonresponder mice", *J.Immunol.*, vol. 140,no. 9, pp. 3180-3185.

Edwards, M. S. & Baker, C. J. 1981, "Complications and sequelae of meningococcal infections in children", *J.Pediatr.*, vol. 99,no. 4, pp. 540-545.

Elsbach, P. 1998, "The bactericidal/permeability-increasing protein (BPI) in antibacterial host defense", *J.Leukoc.Biol.*, vol. 64,no. 1, pp. 14-18.

Estabrook, M. M., Griffiss, J. M., & Jarvis, G. A. 1997, "Sialylation of Neisseria meningitidis lipooligosaccharide inhibits serum bactericidal activity by masking lacto-N-neotetraose", *Infect.Immun.*, vol. 65,no. 11, pp. 4436-4444.

Evans, R. W., Glick, B., Kimball, F., & Bobell, M. 1969, "Fatal intravascular consumption coagulopathy in meningococcal sepsis", *Am.J.Med.*, vol. 46,no. 6, pp. 910-918.

Fan, S. T. & Edgington, T. S. 1993, "Integrin regulation of leukocyte inflammatory functions. CD11b/CD18 enhancement of the tumor necrosis factor-alpha responses of monocytes", *J.Immunol.*, vol. 150,no. 7, pp. 2972-2980.

Faure, E., Equils, O., Sieling, P. A., Thomas, L., Zhang, F. X., Kirschning, C. J., Polentarutti, N., Muzio, M., & Arditi, M. 2000, "Bacterial Lipopolysaccharide Activates NF-kappaB through Toll-like Receptor 4 (TLR-4) in Cultured Human Dermal Endothelial Cells. Differential expression of tlr-4 and tlr-2 in endothelial cells", *J.Biol.Chem.*, vol. 275,no. 15, pp. 11058-11063.

Fearon, D. T. 1997, "Seeking wisdom in innate immunity [news; comment]", *Nature*, vol. 388,no. 6640, pp. 323-324.

Feist, W., Ulmer, A. J., Musehold, J., Brade, H., Kusumoto, S., & Flad, H. D. 1989, "Induction of tumor necrosis factor-alpha release by lipopolysaccharide and defined lipopolysaccharide partial structures", *Immunobiology*, vol. 179,no. 4-5, pp. 293-307.

Fenton, M. J. & Golenbock, D. T. 1998, "LPS-binding proteins and receptors", *J.Leukoc.Biol.*, vol. 64,no. 1, pp. 25-32.

Figueroa, J. E. & Densen, P. 1991b, "Infectious diseases associated with complement deficiencies", *Clin.Microbiol.Rev.*, vol. 4,no. 3, pp. 359-395.

Figueroa, J. E. & Densen, P. 1991a, "Infectious diseases associated with complement deficiencies", *Clin.Microbiol.Rev.*, vol. 4,no. 3, pp. 359-395.

Fijen, C. A., Kuijper, E. J., Hannema, A. J., Sjoholm, A. G., & van, P. J. 1989, "Complement deficiencies in patients over ten years old with meningococcal disease due to uncommon serogroups [see comments]", *Lancet*, vol. 2,no. 8663, pp. 585-588.

Finne, J., Leinonen, M., & Makela, P. H. 1983, "Antigenic similarities between brain components and bacteria causing meningitis. Implications for vaccine development and pathogenesis", *Lancet*, vol. 2,no. 8346, pp. 355-357.

Flaherty, S. F., Golenbock, D. T., Milham, F. H., & Ingalls, R. R. 1997, "CD11/CD18 leukocyte integrins: new signaling receptors for bacterial endotoxin", *J.Surg.Res.*, vol. 73,no. 1, pp. 85-89.

Fox, A. J., Jones, D. M., Gray, S. J., Caugant, D. A., & Saunders, N. A. 1991, "An epidemiologically valuable typing method for Neisseria meningitidis by analysis of restriction fragment length polymorphisms", *J.Med.Microbiol.*, vol. 34,no. 5, pp. 265-270.

Frank, M. M., Joiner, K., & Hammer, C. 1987, "The function of antibody and complement in the lysis of bacteria", *Rev.Infect.Dis.*, vol. 9 Suppl 5, p. S537-S545.

Frasch, C. E. 1995, "Meningococcal Vaccines:Past, Present, and Future," in *Meningococcal Disease*, 1st edn, K. Cartwright, ed., John Wiley and Sons Ltd., Chichester, pp. 245-284.

Fredriksen, J. H., Rosenqvist, E., Wedege, E., Bryn, K., Bjune, G., Froholm, L. O., Lindbak, A. K., Mogster, B., Namork, E., Rye, U., & et, a. 1991, "Production, characterization and control of MenB-vaccine "Folkehelsa": an outer membrane vesicle vaccine against group B meningococcal disease", *NIPH.Ann.*, vol. 14,no. 2, pp. 67-79.

Frosch, M. & Meyer, T. F. 1992, "Transformation-mediated exchange of virulence determinants by co-cultivation of pathogenic Neisseriae", *FEMS Microbiol.Lett.*, vol. 79,no. 1-3, pp. 345-349.

Furie, M. B., Tancinco, M. C., & Smith, C. W. 1991, "Monoclonal antibodies to leukocyte integrins CD11a/CD18 and CD11b/CD18 or intercellular adhesion molecule-1 inhibit chemoattractant-stimulated neutrophil transendothelial migration in vitro", *Blood*, vol. 78,no. 8, pp. 2089-2097.

Gazzano, S. H., Parent, J. B., Grinna, L., Horwitz, A., Parsons, T., Theofan, G., Elsbach, P., Weiss, J., & Conlon, P. J. 1992, "High-affinity binding of the bactericidal/permeability-increasing protein and a recombinant amino-terminal fragment to the lipid A region of lipopolysaccharide.", *Infect.Immun.*, vol. 60,no. 11, pp. 4754-4761.

Gerard, C., Bruyns, C., Marchant, A., Abramowicz, D., Vandenabeele, P., Delvaux, A., Fiers, W., Goldman, M., & Velu, T. 1993, "Interleukin 10 reduces the release of tumor necrosis factor and prevents lethality in experimental endotoxemia", *J.Exp.Med.*, vol. 177,no. 2, pp. 547-550.

Ghersa, P., Whelan, J., Cambet, Y., DeLamarter, J. F., & Hooft-van, H. R. 1997, "Distamycin prolongs E-selectin expression by interacting with a specific NF-kappaB-HMG-I(Y) binding site in the promoter", *Nucleic.Acids.Res.*, vol. 25,no. 2, pp. 339-346.

Ghosh, S., May, M. J., & Kopp, E. B. Nf-κB AND REL PROTEINS: Evolutionarily Conserved Mediators of Immune Responses. Annu.Rev.Immunol.16,225-260.1998.

Gilbert, M., Watson, D. C., Cunningham, A. M., Jennings, M. P., Young, N. M., & Wakarchuk, W. W. 1996b, "Cloning of the lipooligosaccharide alpha-2,3-sialyltransferase from the bacterial pathogens Neisseria meningitidis and Neisseria gonorrhoeae", *J.Biol.Chem.*, vol. 271,no. 45, pp. 28271-28276.

Gilbert, M., Watson, D. C., Cunningham, A. M., Jennings, M. P., Young, N. M., & Wakarchuk, W. W. 1996a, "Cloning of the lipooligosaccharide alpha-2,3-sialyltransferase from the bacterial pathogens Neisseria meningitidis and Neisseria gonorrhoeae", *J.Biol.Chem.*, vol. 271,no. 45, pp. 28271-28276.

Gimbrone-MA, J., Cotran, R. S., & Folkman, J. 1974, "Human vascular endothelial cells in culture. Growth and DNA synthesis.", *J.Cell Biol.*, vol. 60,no. 3, pp. 673-684.

Gimbrone-MA, J., Obin, M. S., Brock, A. F., Luis, E. A., Hass, P. E., Hebert, C. A., Yip, Y. K., Leung, D. W., Lowe, D. G., Kohr, W. J., & et, a. 1989, "Endothelial interleukin-8: a novel inhibitor of leukocyte-endothelial interactions", *Science*, vol. 246,no. 4937, pp. 1601-1603.

Ginsberg, M. H., Du, X., & Plow, E. F. 1992, "Inside-out integrin signalling", *Curr.Opin.Cell Biol.*, vol. 4,no. 5, pp. 766-771.

Girardin, E., Grau, G. E., Dayer, J. M., Roux Lombard, P., & Lambert, P. H. 1988, "Tumor necrosis factor and interleukin-1 in the serum of children with severe infectious purpura", *N.Engl.J.Med.*, vol. 319,no. 7, pp. 397-400.

Giroir, B. P., Quint, P. A., Barton, P., Kirsch, E. A., Kitchen, L., Goldstein, B., Nelson, B. J., Wedel, N. J., Carroll, S. F., & Scannon, P. J. 1997, "Preliminary evaluation of recombinant amino-terminal fragment of human

bactericidal/permeability-increasing protein in children with severe meningococcal sepsis [see comments]", *Lancet*, vol. 350,no. 9089, pp. 1439-1443.

Glauser, M. P., Zanetti, G., Baumgartner, J. D., & Cohen, J. 1991b, "Septic shock: pathogenesis [see comments]", *Lancet*, vol. 338,no. 8769, pp. 732-736.

Glauser, M. P., Zanetti, G., Baumgartner, J. D., & Cohen, J. 1991a, "Septic shock: pathogenesis [see comments]", *Lancet*, vol. 338,no. 8769, pp. 732-736.

Gold, R., Goldschneider, I., Lepow, M. L., Draper, T. F., & Randolph, M. 1978, "Carriage of Neisseria meningitidis and Neisseria lactamica in infants and children", *J.Infect.Dis.*, vol. 137,no. 2, pp. 112-121.

Goldman, A. P., Kerr, S. J., Butt, W., Marsh, M. J., Murdoch, I. A., Paul, T., Firmin, R. K., Tasker, R. C., & Macrae, D. J. 1997, "Extracorporeal support for intractable cardiorespiratory failure due to meningococcal disease [see comments]", *Lancet*, vol. 349,no. 9050, pp. 466-469.

Goldschneider, I., Gotschlich, E. C., & Artenstein, M. S. 1969b, "Human immunity to the meningococcus. I. The role of humoral antibodies", *J.Exp.Med.*, vol. 129,no. 6, pp. 1307-1326.

Goldschneider, I., Gotschlich, E. C., & Artenstein, M. S. 1969a, "Human immunity to the meningococcus. II. Development of natural immunity", *J.Exp.Med.*, vol. 129,no. 6, pp. 1327-1348.

Goldschneider, I., Lepow, M. L., Gotschlich, E. C., Mauck, F. T., Bachl, F., & Randolph, M. 1973, "Immunogenicity of group A and group C meningococcal polysaccharides in human infants", *J.Infect.Dis.*, vol. 128,no. 6, pp. 769-776.

Gotschlich, E. C., Goldschneider, I., & Artenstein, M. S. 1969, "Human immunity to the meningococcus. IV. Immunogenicity of group A and group C meningococcal polysaccharides in human volunteers", *J.Exp.Med.*, vol. 129,no. 6, pp. 1367-1384.

Grados, O. & Ewing, W. H. 1970, "Antigenic relationship between Escherichia coli and Neisseria meningitidis", *J.Infect.Dis.*, vol. 122,no. 1, pp. 100-103.

Gregory, C. D. 2000, "CD14-dependent clearance of apoptotic cells: relevance to the immune system", *Curr.Opin.Immunol.*, vol. 12,no. 1, pp. 27-34.

Griffiss, J. M., Brandt, B. L., Broud, D. D., Goroff, D. K., & Baker, C. J. 1984, "Immune response of infants and children to disseminated infections with Neisseria meningitidis", *J.Infect.Dis.*, vol. 150,no. 1, pp. 71-79.

Grossman, N. & Leive, L. 1984, "Complement activation via the alternative pathway by purified Salmonella lipopolysaccharide is affected by its structure but not its O-antigen length", *J.Immunol.*, vol. 132,no. 1, pp. 376-385.

Gupta, D., Kirkland, T. N., Viriyakosol, S., & Dziarski, R. 1996, "CD14 is a cell-activating receptor for bacterial peptidoglycan.", *J.Biol.Chem.*, vol. 271,no. 38, pp. 23310-23316.

Hafezi, M. A. & Ley, K. 1999, "Relevance of L-selectin shedding for leukocyte rolling in vivo", *J.Exp.Med.*, vol. 189,no. 6, pp. 939-948.

Hailman, E., Lichenstein, H. S., Wurfel, M. M., Miller, D. S., Johnson, D. A., Kelley, M., Busse, L. A., Zukowski, M. M., & Wright, S. D. 1994, "Lipopolysaccharide (LPS)-binding protein accelerates the binding of LPS to CD14", *J.Exp.Med.*, vol. 179,no. 1, pp. 269-277.

Halstensen, A., Pedersen, S. H., Haneberg, B., Bjorvatn, B., & Solberg, C. O. 1987, "Case fatality of meningococcal disease in western Norway", *Scand.J.Infect.Dis.*, vol. 19,no. 1, pp. 35-42.

Halstensen, A., Sjursen, H., Vollset, S. E., Froholm, L. O., Naess, A., Matre, R., & Solberg, C. O. 1989, "Serum opsonins to serogroup B meningococci in meningococcal disease", *Scand.J.Infect.Dis.*, vol. 21,no. 3, pp. 267-276.

Hammerschmidt, S., Birkholz, C., Zahringer, U., Robertson, B. D., van Putten, J., Ebeling, O., & Frosch, M. 1994, "Contribution of genes from the capsule gene complex (cps) to lipooligosaccharide biosynthesis and serum resistance in Neisseria meningitidis", *Mol.Microbiol.*, vol. 11,no. 5, pp. 885-896.

Hammerschmidt, S., Hilse, R., van Putten, J. P., Gerardy Schahn, R., Unkmeir, A., & Frosch, M. 1996a, "Modulation of cell surface sialic acid expression in Neisseria meningitidis via a transposable genetic element", *EMBO J.*, vol. 15,no. 1, pp. 192-198.

Hammerschmidt, S., Muller, A., Sillmann, H., Muhlenhoff, M., Borrow, R., Fox, A., van Putten, J., Zollinger, W. D., Gerardy Schahn, R., & Frosch, M. 1996b, "Capsule phase variation in Neisseria meningitidis serogroup B by slipped-strand mispairing in the polysialyltransferase gene (siaD): correlation with bacterial invasion and the outbreak of meningococcal disease", *Mol.Microbiol.*, vol. 20,no. 6, pp. 1211-1220.

Harlan, J. M. 1993, "Leukocyte adhesion deficiency syndrome: insights into the molecular basis of leukocyte emigration", *Clin.Immunol.Immunopathol.*, vol. 67,no. 3 Pt 2, p. S16-S24.

Hatada, E. N., Krappmann, D., & Scheidereit, C. 2000, "NF-kappaB and the innate immune response", *Curr.Opin.Immunol.*, vol. 12,no. 1, pp. 52-58.

Havens, P. L., Garland, J. S., Brook, M. M., Dewitz, B. A., Stremski, E. S., & Troshynski, T. J. 1989, "Trends in mortality in children hospitalized with meningococcal infections, 1957 to 1987", *Pediatr.Infect.Dis.J.*, vol. 8,no. 1, pp. 8-11.

Hawrylowicz, C. M., Santoro, S. A., Platt, F. M., & Unanue, E. R. 1989, "Activated platelets express IL-1 activity", *J.Immunol.*, vol. 143,no. 12, pp. 4015-4018.

Heckels, J. E. 1989, "Structure and function of pili of pathogenic Neisseria species", *Clin.Microbiol.Rev.*, vol. 2 Suppl, p. S66-S73.

Heine, H., Kirschning, C. J., Lien, E., Monks, B. G., Rothe, M., & Golenbock, D. T. 1999, "Cutting edge: cells that carry A null allele for toll-like receptor 2 are capable of responding to endotoxin", *J.Immunol.*, vol. 162,no. 12, pp. 6971-6975.

Helmerhorst, E. J., Maaskant, J. J., & Appelmelk, B. J. 1998, "Anti-lipid A monoclonal antibody centoxin (HA-1A) binds to a wide variety of hydrophobic ligands", *Infect.Immun.*, vol. 66,no. 2, pp. 870-873.

Henson, P. M. & Johnston-RB, J. 1987, "Tissue injury in inflammation. Oxidants, proteinases, and cationic proteins", *J.Clin.Invest.*, vol. 79,no. 3, pp. 669-674.

Hermans, P. W., Hibberd, M. L., Booy, R., Daramola, O., Hazelzet, J. A., de Groot, R., & Levin, M. 1999, "4G/5G promoter polymorphism in the plasminogen-activator-inhibitor-1 gene and outcome of meningococcal disease. Meningococcal Research Group", *Lancet*, vol. 354,no. 9178, pp. 556-560.

Heyderman, R. S. 1993, "Sepsis and intravascular thrombosis", *Arch.Dis.Child*, vol. 68,no. 5, pp. 621-623.

Heyderman, R. S., Ison, C. A., Peakman, M., Levin, M., & Klein, N. J. 1999, "Neutrophil response to Neisseria meningitidis: inhibition of adhesion molecule expression and phagocytosis by recombinant bactericidal/permeability-increasing protein (rBPI21)", *J.Infect.Dis.*, vol. 179,no. 5, pp. 1288-1292.

Heyderman, R. S., Klein, N. J., Daramola, O. A., Hammerschmidt, S., Frosch, M., Robertson, B. D., Levin, M., & Ison, C. A. 1997, "Induction of human endothelial tissue factor expression by Neisseria meningitidis: the influence of bacterial killing and adherence to the endothelium.", *Microb.Pathog.*, vol. 22,no. 5, pp. 265-274.

Hill, W. a. K. T. 1947, "The cutaneous lesions in acute meningococcaemia", *JAMA*, vol. 134, pp. 513-518.

Hirschfeld, M., Kirschning, C. J., Schwandner, R., Wesche, H., Weis, J. H., Wooten, R. M., & Weis, J. J. 1999, "Cutting edge: inflammatory signaling by

Borrelia burgdorferi lipoproteins is mediated by toll-like receptor 2", *J.Immunol.*, vol. 163,no. 5, pp. 2382-2386.

Hooft, v. H., Whelan, J., Pescini, R., Becker-Andre, M., Schenk, A. M., & DeLamarter, J. F. 1992, "A T-cell enhancer cooperates with NF-kappa B to yield cytokine induction of E-selectin gene transcription in endothelial cells", *J.Biol.Chem.*, vol. 267,no. 31, pp. 22385-22391.

Hsieh, C. S., Macatonia, S. E., Tripp, C. S., Wolf, S. F., O'Garra, A., & Murphy, K. M. 1993, "Development of TH1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages [see comments]", *Science*, vol. 260,no. 5107, pp. 547-549.

Huang, A. J., Manning, J. E., Bandak, T. M., Ratau, M. C., Hanser, K. R., & Silverstein, S. C. 1993, "Endothelial cell cytosolic free calcium regulates neutrophil migration across monolayers of endothelial cells", *J.Cell Biol.*, vol. 120,no. 6, pp. 1371-1380.

Huang, K., Fishwild, D. M., Wu, H. M., & Dedrick, R. L. 1995, "Lipopolysaccharide-induced E-selectin expression requires continuous presence of LPS and is inhibited by bactericidal/permeability-increasing protein.", *Inflammation*, vol. 19,no. 3, pp. 389-404.

Huber, A. R., Kunkel, S. L., Todd, R. F., & Weiss, S. J. 1991, "Regulation of transendothelial neutrophil migration by endogenous interleukin-8 [published errata appear in Science 1991 Nov 1;254(5032):631 and 1991 Dec 6;254(5037):1435]", *Science*, vol. 254,no. 5028, pp. 99-102.

Hubert, B., Watier, L., Garnerin, P., & Richardson, S. 1992, "Meningococcal disease and influenza-like syndrome: a new approach to an old question", *J.Infect.Dis.*, vol. 166,no. 3, pp. 542-545.

Hunt, B. J. & Jurd, K. M. 1997, "Relation between endothelial-cell activation and infection, inflammation, and infarction [letter; comment]", *Lancet*, vol. 350,no. 9073, p. 293.

Ison, C. A., Heyderman, R. S., Klein, N. J., Peakman, M., & Levin, M. 1995, "Whole blood model of meningococcal bacteraemia--a method for exploring host-bacterial interactions", *Microb.Pathog.*, vol. 18,no. 2, pp. 97-107.

Issekutz, A. C. & Movat, H. Z. 1980, "The in vivo quantitation and kinetics of rabbit neutrophil leukocyte accumulation in the skin in response to chemotactic agents and Escherichia coli", *Lab.Invest.*, vol. 42,no. 3, pp. 310-317.

Iwanaga, S., Morita, T., Harada, T., Nakamura, S., Niwa, M., Takada, K., Kimura, T., & Sakakibara, S. 1978, "Chromogenic substrates for horseshoe crab clotting enzyme. Its application for the assay of bacterial endotoxins", *Haemostasis*, vol. 7,no. 2-3, pp. 183-188.

Jack, D. L. 1998, *Epidemiological and in vitro studies of mannose-binding lectin (MBL) in host defence*, PhD.

Jack, D. L., Dodds, A. W., Anwar, N., Ison, C. A., Law, A., Frosch, M., Turner, M. W., & Klein, N. J. 1998, "Activation of complement by mannose-binding lectin on isogenic mutants of Neisseria meningitidis serogroup B", *J.Immunol.*, vol. 160,no. 3, pp. 1346-1353.

Jacob, C. O., Fronek, Z., Lewis, G. D., Koo, M., Hansen, J. A., & McDevitt, H. O. 1990, "Heritable major histocompatibility complex class II-associated differences in production of tumor necrosis factor alpha: relevance to genetic predisposition to systemic lupus erythematosus", *Proc.Natl.Acad.Sci.U.S.A.*, vol. 87,no. 3, pp. 1233-1237.

Jahr, T. G., Ryan, L., Sundan, A., Lichenstein, H. S., Skjak, B. G., & Espevik, T. 1997, "Induction of tumor necrosis factor production from monocytes stimulated with mannuronic acid polymers and involvement of lipopolysaccharide-binding protein, CD14, and bactericidal/permeability-increasing factor.", *Infect.Immun.*, vol. 65,no. 1, pp. 89-94.

Jarvis, G. A. 1994, "Analysis of C3 deposition and degradation on Neisseria meningitidis and Neisseria gonorrhoeae", *Infect.Immun.*, vol. 62,no. 5, pp. 1755-1760.

Jarvis, G. A. & Vedros, N. A. 1987b, "Sialic acid of group B Neisseria meningitidis regulates alternative complement pathway activation", *Infect.Immun.*, vol. 55,no. 1, pp. 174-180.

Jarvis, G. A. & Vedros, N. A. 1987a, "Sialic acid of group B Neisseria meningitidis regulates alternative complement pathway activation", *Infect.Immun.*, vol. 55,no. 1, pp. 174-180.

Jennens, I. D., O'Reilley, M., & Yung, A. P. 1990, "Chronic meningococcal disease [see comments]", *Med.J.Aust.*, vol. 153,no. 9, pp. 556-559.

Jennings, H. J., Bhattacharjee, A. K., Bundle, D. R., Kenny, C. P., Martin, A., & Smith, I. C. 1977, "Strucutres of the capsular polysaccharides of Neisseria meningitidis as determined by 13C-nuclear magnetic resonance spectroscopy", *J.Infect.Dis.*, vol. 136 Suppl, p. S78-S83.

Jerse, A. E. & Rest, R. F. 1997, "Adhesion and invasion by the pathogenic neisseria", *Trends.Microbiol.*, vol. 5,no. 6, pp. 217-221.

Johnson, K., Choi, Y., DeGroot, E., Samuels, I., Creasey, A., & Aarden, L. 1998, "Potential mechanisms for a proinflammatory vascular cytokine response to coagulation activation", *J.Immunol.*, vol. 160,no. 10, pp. 5130-5135.

Jones, D. M. 1995, "Epidemiology of meningococcal disease in Europe and the USA," in *Meningococcal Disease*, K. Cartwright, ed., Chichester: John Wiley & Sons Ltd., pp. 71-114.

Jones, D. M., Borrow, R., Fox, A. J., Gray, S., Cartwright, K. A., & Poolman, J. T. 1992, "The lipooligosaccharide immunotype as a virulence determinant in Neisseria meningitidis", *Microb.Pathog.*, vol. 13,no. 3, pp. 219-224.

Jung, U. & Ley, K. 1999, "Mice lacking two or all three selectins demonstrate overlapping and distinct functions for each selectin", *J.Immunol.*, vol. 162,no. 11, pp. 6755-6762.

Jutila, M. A., Rott, L., Berg, E. L., & Butcher, E. C. 1989, "Function and regulation of the neutrophil MEL-14 antigen in vivo: comparison with LFA-1 and MAC-1", *J.Immunol.*, vol. 143,no. 10, pp. 3318-3324.

Kahler, C. M., Martin, L. E., Shih, G. C., Rahman, M. M., Carlson, R. W., & Stephens, D. S. 1998, "The (alpha2-->8)-linked polysialic acid capsule and lipooligosaccharide structure both contribute to the ability of serogroup B Neisseria meningitidis to resist the bactericidal activity of normal human serum.", *Infect.Immun.*, vol. 66,no. 12, pp. 5939-5947.

Kalinski, P., Schuitemaker, J. H., Hilkens, C. M., Wierenga, E. A., & Kapsenberg, M. L. 1999, "Final maturation of dendritic cells is associated with impaired responsiveness to IFN-gamma and to bacterial IL-12 inducers: decreased ability of mature dendritic cells to produce IL-12 during the interaction with Th cells", *J.Immunol.*, vol. 162,no. 6, pp. 3231-3236.

Kalter, E. S., Daha, M. R., ten, C. J., Verhoef, J., & Bouma, B. N. 1985, "Activation and inhibition of Hageman factor-dependent pathways and the complement system in uncomplicated bacteremia or bacterial shock", *J.Infect.Dis.*, vol. 151,no. 6, pp. 1019-1027.

Karin, M. & Delhase, M. 1998, "JNK or IKK, AP-1 or NF-kappaB, which are the targets for MEK kinase 1 action?", *Proc.Natl.Acad.Sci.U.S.A.*, vol. 95,no. 16, pp. 9067-9069.

Karmann, K., Hughes, C. C., Schechner, J., Fanslow, W. C., & Pober, J. S. 1995, "CD40 on human endothelial cells: inducibility by cytokines and functional regulation of adhesion molecule expression", *Proc.Natl.Acad.Sci.U.S.A.*, vol. 92,no. 10, pp. 4342-4346.

Karmann, K., Min, W., Fanslow, W. C., & Pober, J. S. 1996, "Activation and homologous desensitization of human endothelial cells by CD40 ligand, tumor necrosis factor, and interleukin 1.", *J.Exp.Med.*, vol. 184,no. 1, pp. 173-182.

Karp, C. L., Wysocka, M., Ma, X., Marovich, M., Factor, R. E., Nutman, T., Armant, M., Wahl, L., Cuomo, P., & Trinchieri, G. 1998, "Potent suppression of IL-12 production from monocytes and dendritic cells during endotoxin tolerance", *Eur.J.Immunol.*, vol. 28,no. 10, pp. 3128-3136.

Kaszubska, W., Hooft-van, H. R., Ghersa, P., DeRaemy, S. A., Chen, B. P., Hai, T., DeLamarter, J. F., & Whelan, J. 1993, "Cyclic AMP-independent ATF family members interact with NF-kappa B and function in the activation of the E-selectin promoter in response to cytokines", *Mol.Cell Biol.*, vol. 13,no. 11, pp. 7180-7190.

Kiniwa, M., Gately, M., Gubler, U., Chizzonite, R., Fargeas, C., & Delespesse, G. 1992, "Recombinant interleukin-12 suppresses the synthesis of immunoglobulin E by interleukin-4 stimulated human lymphocytes", *J.Clin.Invest.*, vol. 90,no. 1, pp. 262-266.

Kirsch, E. A., Barton, R. P., Kitchen, L., & Giroir, B. P. 1996, "Pathophysiology, treatment and outcome of meningococcemia: a review and recent experience.", *Pediatr.Infect.Dis.J.*, vol. 15,no. 11, pp. 967-978.

Kirschning, C. J., Wesche, H., Merrill, A. T., & Rothe, M. 1998, "Human toll-like receptor 2 confers responsiveness to bacterial lipopolysaccharide.", *J.Exp.Med.*, vol. 188,no. 11, pp. 2091-2097.

Kitchens, R. L., Ulevitch, R. J., & Munford, R. S. 1992, "Lipopolysaccharide (LPS) partial structures inhibit responses to LPS in a human macrophage cell line without inhibiting LPS uptake by a CD14-mediated pathway", *J.Exp.Med.*, vol. 176,no. 2, pp. 485-494.

Kjellen, L. & Lindahl, U. 1991, "Proteoglycans: structures and interactions [published erratum appears in Annu Rev Biochem 1992;61:following viii]", *Annu.Rev.Biochem.*, vol. 60, pp. 443-475.

Klein, N. J., Ison, C. A., Peakman, M., Levin, M., Hammerschmidt, S., Frosch, M., & Heyderman, R. S. 1996, "The influence of capsulation and lipooligosaccharide structure on neutrophil adhesion molecule expression and endothelial injury by Neisseria meningitidis", *J.Infect.Dis.*, vol. 173,no. 1, pp. 172-179.

Klein, N. J., Levin, M., Strobel, S., & Finn, A. 1993, "Degradation of glycosaminoglycans and fibronectin on endotoxin- stimulated endothelium by adherent neutrophils: relationship to CD11b/CD18 and L-selectin expression", *J.Infect.Dis.*, vol. 167,no. 4, pp. 890-898.

Klein, N. J., Shennan, G. I., Heyderman, R. S., & Levin, M. 1992, "Alteration in glycosaminoglycan metabolism and surface charge on human umbilical vein endothelial cells induced by cytokines, endotoxin and neutrophils", *J.Cell Sci.*, vol. 102,no. Pt 4, pp. 821-832.

Kopp, E. B. & Medzhitov, R. 1999a, "The Toll-receptor family and control of innate immunity", *Curr.Opin.Immunol.*, vol. 11,no. 1, pp. 13-18.

Kopp, E. B. & Medzhitov, R. 1999b, "The Toll-receptor family and control of innate immunity", *Curr.Opin.Immunol.*, vol. 11,no. 1, pp. 13-18.

Kornelisse, R. F., Hazelzet, J. A., Hop, W. C., Spanjaard, L., Suur, M. H., van-der, V. E., & de, G. R. 1997, "Meningococcal septic shock in children: clinical and laboratory features, outcome, and development of a prognostic score", *Clin.Infect.Dis.*, vol. 25,no. 3, pp. 640-646.

Kotowicz, K., Callard, R. E., Friedrich, K., Matthews, D. J., & Klein, N. 1996, "Biological activity of IL-4 and IL-13 on human endothelial cells: functional evidence that both cytokines act through the same receptor.", *Int.Immunol.*, vol. 8,no. 12, pp. 1915-1925.

Kulshin, V. A., Zahringer, U., Lindner, B., Frasch, C. E., Tsai, C. M., Dmitriev, B. A., & Rietschel, E. T. 1992, "Structural characterization of the lipid A component of pathogenic Neisseria meningitidis", *J.Bacteriol.*, vol. 174,no. 6, pp. 1793-1800.

Kumar, A., Thota, V., Dee, L., Olson, J., Uretz, E., & Parrillo, J. E. 1996, "Tumor necrosis factor alpha and interleukin 1beta are responsible for in vitro myocardial cell depression induced by human septic shock serum", *J.Exp.Med.*, vol. 183,no. 3, pp. 949-958.

Kusunoki, T., Hailman, E., Juan, T. S., Lichenstein, H. S., & Wright, S. D. 1995b, "Molecules from Staphylococcus aureus that bind CD14 and stimulate innate immune responses", *J.Exp.Med.*, vol. 182,no. 6, pp. 1673-1682.

Kusunoki, T., Hailman, E., Juan, T. S., Lichenstein, H. S., & Wright, S. D. 1995a, "Molecules from Staphylococcus aureus that bind CD14 and stimulate innate immune responses", *J.Exp.Med.*, vol. 182,no. 6, pp. 1673-1682.

Lane, P. & Brocker, T. 1999, "Developmental regulation of dendritic cell function", *Curr.Opin.Immunol.*, vol. 11, pp. 308-313.

Lane, T. A., Lamkin, G. E., & Wancewicz, E. 1989, "Modulation of endothelial cell expression of intercellular adhesion molecule 1 by protein kinase C activation", *Biochem.Biophys.Res.Commun.*, vol. 161,no. 3, pp. 945-952.

Languino, L. R., Plescia, J., Duperray, A., Brian, A. A., Plow, E. F., Geltosky, J. E., & Altieri, D. C. 1993, "Fibrinogen mediates leukocyte adhesion to vascular endothelium through an ICAM-1-dependent pathway", *Cell*, vol. 73,no. 7, pp. 1423-1434.

Lanzavecchia, A. 1996, "Mechanisms of antigen uptake for presentation", *Curr.Opin.Immunol.*, vol. 8,no. 3, pp. 348-354.

Lanzavecchia, A. 1998, "Immunology. Licence to kill [news; comment]", *Nature*, vol. 393,no. 6684, pp. 413-414.

Lawrence, M. B., Bainton, D. F., & Springer, T. A. 1994, "Neutrophil tethering to and rolling on E-selectin are separable by requirement for L-selectin", *Immunity.*, vol. 1,no. 2, pp. 137-145.

Lawrence, M. B. & Springer, T. A. 1991, "Leukocytes roll on a selectin at physiologic flow rates: distinction from and prerequisite for adhesion through integrins", *Cell*, vol. 65,no. 5, pp. 859-873.

Lawrence, M. B. & Springer, T. A. 1993, "Neutrophils roll on E-selectin", *J.Immunol.*, vol. 151,no. 11, pp. 6338-6346.

Lehmann, A. K., Halstensen, A., Aaberge, I. S., Holst, J., Michaelsen, T. E., Sornes, S., Wetzler, L. M., & Guttormsen, H. 1999, "Human opsonins induced during meningococcal disease recognize outer membrane proteins PorA and PorB", *Infect.Immun.*, vol. 67,no. 5, pp. 2552-2560.

Lentschat, A., El, S. V., Schletter, J., Kusumoto, S., Brade, L., Rietschel, E. T., Gerdes, J., Ernst, M., Flad, H., & Ulmer, A. J. 1999, "The internalization time course of a given lipopolysaccharide chemotype does not correspond to its activation kinetics in monocytes", *Infect.Immun.*, vol. 67,no. 5, pp. 2515-2521.

Levi, M., ten Cate, H., van der Poll, T., & van Deventer, S. J. 1993, "Pathogenesis of disseminated intravascular coagulation in sepsis [see comments]", *JAMA*, vol. 270,no. 8, pp. 975-979.

Levy, O. 1996, "Antibiotic proteins of polymorphonuclear leukocytes", *Eur.J.Haematol.*, vol. 56,no. 5, pp. 263-277.

Lien, E., Means, T. K., Heine, H., Yoshimura, A., Kusumoto, S., Fukase, K., Fenton, M. J., Oikawa, M., Qureshi, N., Monks, B., Finberg, R. W., Ingalls, R. R., & Golenbock, D. T. 2000, "Toll-like receptor 4 imparts ligand-specific recognition of bacterial lipopolysaccharide", *J.Clin.Invest*, vol. 105,no. 4, pp. 497-504.

Lindahl, U. & Hook, M. 1978, "Glycosaminoglycans and their binding to biological macromolecules", *Annu.Rev.Biochem.*, vol. 47, pp. 385-417.

Liu, T. Y., Gotschlich, E. C., Dunne, F. T., & Jonssen, E. K. 1971a, "Studies on the meningococcal polysaccharides. II. Composition and chemical properties of the group B and group C polysaccharide", *J.Biol.Chem.*, vol. 246,no. 15, pp. 4703-4712.

Liu, T. Y., Gotschlich, E. C., Jonssen, E. K., & Wysocki, J. R. 1971b, "Studies on the meningococcal polysaccharides. I. Composition and chemical properties of the group A polysaccharide", *J.Biol.Chem.*, vol. 246,no. 9, pp. 2849-2858.

Liu, Y. J., Grouard, G., de, B. O., & Banchereau, J. 1996, "Follicular dendritic cells and germinal centers", *Int.Rev.Cytol.* , vol. 166, pp. 139-179.

Lo, S. K., Lee, S., Ramos, R. A., Lobb, R., Rosa, M., Chi, R. G., & Wright, S. D. 1991, "Endothelial-leukocyte adhesion molecule 1 stimulates the adhesive activity of leukocyte integrin CR3 (CD11b/CD18, Mac-1, alpha m beta 2) on human neutrophils", *J.Exp.Med.*, vol. 173,no. 6, pp. 1493-1500.

Loggie, B. W. & Hinchey, E. J. 1986, "Does splenectomy predispose to meningococcal sepsis? An experimental study and clinical review", *J.Pediatr.Surg.*, vol. 21,no. 4, pp. 326-330.

Lore, K., Sonnerborg, A., Spetz, A. L., Andersson, U., & Andersson, J. 1998, "Immunocytochemical detection of cytokines and chemokines in Langerhans cells and in vitro derived dendritic cells.", *J.Immunol.Methods*, vol. 214,no. 1-2, pp. 97-111.

Lorenzen, D. R., F. Dux, U. Wolk, A. Tsirpouchtsidis, G. Haas, and T. F. Meyer. 1999. Immunoglobulin A1 protease, an exoenzyme of pathogenic Neisseriae, is a potent inducer of proinflammatory cytokines. J.Exp.Med. 190:1049-1058.

Luderitz, O., Tanamoto, K., Galanos, C., McKenzie, G. R., Brade, H., Zahringer, U., Rietschel, E. T., Kusumoto, S., & Shiba, T. 1984, "Lipopolysaccharides: structural principles and biologic activities", *Rev.Infect.Dis.*, vol. 6,no. 4, pp. 428-431.

Luscinskas, F. W., Cybulsky, M. I., Kiely, J. M., Peckins, C. S., Davis, V. M., & Gimbrone-MA, J. 1991, "Cytokine-activated human endothelial monolayers support enhanced neutrophil transmigration via a mechanism involving both endothelial-leukocyte adhesion molecule-1 and intercellular adhesion molecule-1", *J.Immunol.*, vol. 146,no. 5, pp. 1617-1625.

Macatonia, S. E., Hosken, N. A., Litton, M., Vieira, P., Hsieh, C. S., Culpepper, J. A., Wysocka, M., Trinchieri, G., Murphy, K. M., & O'Garra, A. 1995, "Dendritic cells produce IL-12 and direct the development of Th1 cells from naive CD4+ T cells", *J.Immunol.*, vol. 154,no. 10, pp. 5071-5079.

Mackman, N. 1995, "Regulation of the tissue factor gene", *FASEB J.*, vol. 9,no. 10, pp. 883-889.

Mandrell, R. E., Kim, J. J., John, C. M., Gibson, B. W., Sugai, J. V., Apicella, M. A., Griffiss, J. M., & Yamasaki, R. 1991, "Endogenous sialylation of the lipooligosaccharides of Neisseria meningitidis", *J.Bacteriol.*, vol. 173,no. 9, pp. 2823-2832.

Mandrell, R. E. & Zollinger, W. D. 1977, "Lipopolysaccharide serotyping of Neisseria meningitidis by hemagglutination inhibition", *Infect.Immun.*, vol. 16,no. 2, pp. 471-475.

Manetti, R., Gerosa, F., Giudizi, M. G., Biagiotti, R., Parronchi, P., Piccinni, M. P., Sampognaro, S., Maggi, E., Romagnani, S., Trinchieri, G., & et, a. 1994, "Interleukin 12 induces stable priming for interferon gamma (IFN-gamma) production during differentiation of human T helper (Th) cells and transient IFN-gamma production in established Th2 cell clones", *J.Exp.Med.*, vol. 179,no. 4, pp. 1273-1283.

Manger, I. D. & Relman, D. A. 2000, "How the host 'sees' pathogens: global gene expression responses to infection [In Process Citation]", *Curr.Opin.Immunol.*, vol. 12,no. 2, pp. 215-218.

Marcum, J. A. & Rosenberg, R. D. 1984, "Anticoagulantly active heparin-like molecules from vascular tissue", *Biochemistry*, vol. 23,no. 8, pp. 1730-1737.

Marks, M. I., Frasch, C. E., & Shapera, R. M. 1979, "Meningococcal colonization and infection in children and their household contacts", *Am.J.Epidemiol.*, vol. 109,no. 5, pp. 563-571.

Marks, R. M., Todd, R. F., & Ward, P. A. 1989, "Rapid induction of neutrophil-endothelial adhesion by endothelial complement fixation", *Nature*, vol. 339,no. 6222, pp. 314-317.

Marra, M. N., Thornton, M. B., Snable, J. L., Wilde, C. G., & Scott, R. W. 1994, "Endotoxin-binding and -neutralizing properties of recombinant bactericidal/permeability-increasing protein and monoclonal antibodies HA-1A and E5 [see comments]", *Crit.Care Med.*, vol. 22,no. 4, pp. 559-565.

Marra, M. N., Wilde, C. G., Griffith, J. E., Snable, J. L., & Scott, R. W. 1990, "Bactericidal/permeability-increasing protein has endotoxin-neutralizing activity.", *J.Immunol.*, vol. 144,no. 2, pp. 662-666.

Marriott, I., Hammond, T. G., Thomas, E. K., & Bost, K. L. 1999, "Salmonella efficiently enter and survive within cultured CD11c+ dendritic cells initiating cytokine expression", *Eur.J.Immunol.*, vol. 29,no. 4, pp. 1107-1115.

Marui, N., Offermann, M. K., Swerlick, R., Kunsch, C., Rosen, C. A., Ahmad, M., Alexander, R. W., & Medford, R. M. 1993, "Vascular cell adhesion molecule-1 (VCAM-1) gene transcription and expression are regulated through an antioxidant-sensitive mechanism in human vascular endothelial cells", *J.Clin.Invest.*, vol. 92,no. 4, pp. 1866-1874.

Masson, L. & Holbein, B. E. 1985, "Influence of nutrient limitation and low pH on serogroup B Neisseria meningitidis capsular polysaccharide levels: correlation with virulence for mice", *Infect.Immun.*, vol. 47,no. 2, pp. 465-471.

May, M. J. & Ghosh, S. 1998, "Signal transduction through NF-kappa B.", *Immunol.Today*, vol. 19,no. 2, pp. 80-88.

McCloskey, R. V., Straube, R. C., Sanders, C., Smith, S. M., & Smith, C. R. 1994, "Treatment of septic shock with human monoclonal antibody HA-1A. A randomized, double-blind, placebo-controlled trial. CHESS Trial Study Group [see comments]", *Ann.Intern.Med.*, vol. 121,no. 1, pp. 1-5.

McGee, Z. A. & Stephens, D. S. 1984, "Common pathways of invasion of mucosal barriers by Neisseria gonorrhoeae and Neisseria meningitidis", *Surv.Synth.Pathol.Res.*, vol. 3,no. 1, pp. 1-10.

McKnight, A. J., Zimmer, G. J., Fogelman, I., Wolf, S. F., & Abbas, A. K. 1994, "Effects of IL-12 on helper T cell-dependent immune responses in vivo", *J.Immunol.*, vol. 152,no. 5, pp. 2172-2179.

McLeod Griffiths, J. 1995, "Mechanims of Host Immunity," in *Meningococcal Disease*, K. Cartwright, ed., John Wiley and sons, Chichester, pp. 35-70.

McNeil, G. & Virji, M. 1997, "Phenotypic variants of meningococci and their potential in phagocytic interactions: the influence of opacity proteins, pili, PilC and surface sialic acids.", *Microb.Pathog.*, vol. 22,no. 5, pp. 295-304.

McWilliam, A. S., Napoli, S., Marsh, A. M., Pemper, F. L., Nelson, D. J., Pimm, C. L., Stumbles, P. A., Wells, T. N., & Holt, P. G. 1996, "Dendritic cells are recruited into the airway epithelium during the inflammatory response to a broad spectrum of stimuli", *J.Exp.Med.*, vol. 184,no. 6, pp. 2429-2432.

Means, T. K., Lien, E., Yoshimura, A., Wang, S., Golenbock, D. T., & Fenton, M. J. 1999, "The CD14 ligands lipoarabinomannan and lipopolysaccharide differ in their requirement for Toll-like receptors", *J.Immunol.*, vol. 163,no. 12, pp. 6748-6755.

Medzhitov, R. & Janeway-CA, J. 1997, "Innate immunity: impact on the adaptive immune response", *Curr.Opin.Immunol.*, vol. 9,no. 1, pp. 4-9.

Medzhitov, R. & Janeway-CA, J. 1998, "An ancient system of host defense", *Curr.Opin.Immunol.*, vol. 10,no. 1, pp. 12-15.

Medzhitov, R., Preston, H. P., & Janeway-CA, J. 1997, "A human homologue of the Drosophila Toll protein signals activation of adaptive immunity [see comments]", *Nature*, vol. 388,no. 6640, pp. 394-397.

Mercier, J. C., Beaufils, F., Hartmann, J. F., & Azema, D. 1988, "Hemodynamic patterns of meningococcal shock in children.", *Crit.Care Med.*, vol. 16,no. 1, pp. 27-33.

Montgomery, K. F., Osborn, L., Hession, C., Tizard, R., Goff, D., Vassallo, C., Tarr, P. I., Bomsztyk, K., Lobb, R., Harlan, J. M., & et, a. 1991, "Activation of endothelial-leukocyte adhesion molecule 1 (ELAM-1) gene transcription.", *Proc.Natl.Acad.Sci.U.S.A.*, vol. 88,no. 15, pp. 6523-6527.

Moore, K. W., O'Garra, A., de-Waal, M. R., Vieira, P., & Mosmann, T. R. 1993, "Interleukin-10", *Annu.Rev.Immunol.*, vol. 11, pp. 165-190.

Morrison, D. C. & Kline, L. F. 1977, "Activation of the classical and properdin pathways of complement by bacterial lipopolysaccharides (LPS)", *J.Immunol.*, vol. 118,no. 1, pp. 362-368.

Morrison, D. C. & Ulevitch, R. J. 1978, "The effects of bacterial endotoxins on host mediation systems. A review", *Am.J.Pathol.*, vol. 93,no. 2, pp. 526-617.

Mulks, M. H. & Plaut, A. G. 1978, "IgA protease production as a characteristic distinguishing pathogenic from harmless neisseriaceae", *N.Engl.J.Med.*, vol. 299,no. 18, pp. 973-976.

Muller, W. A., Ratti, C. M., McDonnell, S. L., & Cohn, Z. A. 1989, "A human endothelial cell-restricted, externally disposed plasmalemmal protein enriched in intercellular junctions", *J.Exp.Med.*, vol. 170,no. 2, pp. 399-414.

Muller, W. A., Weigl, S. A., Deng, X., & Phillips, D. M. 1993, "PECAM-1 is required for transendothelial migration of leukocytes", *J.Exp.Med.*, vol. 178,no. 2, pp. 449-460.

Munford, R. S. & Hall, C. L. 1986, "Detoxification of bacterial lipopolysaccharides (endotoxins) by a human neutrophil enzyme", *Science*, vol. 234,no. 4773, pp. 203-205.

Muzio, M., Bosisio, D., Polentarutti, N., D'Amico, G., Stoppacciaro, A., Mancinelli, R., van't Veer, C., Penton-Rol, G., Ruco, L. P., Allavena, P., & Mantovani, A. 2000b, "Differential Expression and Regulation of Toll-Like Receptors (TLR) in Human Leukocytes: Selective Expression of TLR3 in Dendritic Cells", *J.Immunol.*, vol. 164,no. 11, pp. 5998-6004.

Muzio, M., Natoli, G., Saccani, S., Levrero, M., & Mantovani, A. 1998, "The human toll signaling pathway: divergence of nuclear factor kappaB and JNK/SAPK activation upstream of tumor necrosis factor receptor-associated factor 6 (TRAF6)", *J.Exp.Med.*, vol. 187,no. 12, pp. 2097-2101.

Muzio, M., Polentarutti, N., Bosisio, D., Prahladan, M. K., & Mantovani, A. 2000a, "Toll-like receptors: a growing family of immune receptors that are differentially expressed and regulated by different leukocytes [In Process Citation]", *J.Leukoc.Biol.*, vol. 67,no. 4, pp. 450-456.

Naess, L. M., Oftung, F., Aase, A., Wetzler, L. M., Sandin, R., & Michaelsen, T. E. 1998, "Human T-cell responses after vaccination with the Norwegian group B meningococcal outer membrane vesicle vaccine", *Infect.Immun.*, vol. 66,no. 3, pp. 959-965.

Natanson, C., Hoffman, W. D., Suffredini, A. F., Eichacker, P. Q., & Danner, R. L. 1994, "Selected treatment strategies for septic shock based on proposed mechanisms of pathogenesis", *Ann.Intern.Med.*, vol. 120,no. 9, pp. 771-783.

Naumann, M., Rudel, T., Wieland, B., Bartsch, C., & Meyer, T. F. 1998, "Coordinate activation of activator protein 1 and inflammatory cytokines in

response to Neisseria gonorrhoeae epithelial cell contact involves stress response kinases.", *J.Exp.Med.*, vol. 188,no. 7, pp. 1277-1286.

Naumann, M., Wessler, S., Bartsch, C., Wieland, B., & Meyer, T. F. 1997, "Neisseria gonorrhoeae epithelial cell interaction leads to the activation of the transcription factors nuclear factor kappaB and activator protein 1 and the induction of inflammatory cytokines.", *J.Exp.Med.*, vol. 186,no. 2, pp. 247-258.

Nawroth, P. P. & Stern, D. M. 1986, "Modulation of endothelial cell hemostatic properties by tumor necrosis factor", *J.Exp.Med.*, vol. 163,no. 3, pp. 740-745.

Neal, K. R., Nguyen-Van-Tam, J. S., Jeffrey, N., Slack, R. C., Madeley, R. J., Ait-Tahar, K., Job, K., Wale, M. C., & Ala'Aldeen, D. A. 2000, "Changing carriage rate of Neisseria meningitidis among university students during the first week of term: cross sectional study", *BMJ*, vol. 320,no. 7238, pp. 846-849.

Nelson, J. D. 1996, "Jails, microbes, and the three-foot barrier [editorial; comment]", *N.Engl.J.Med.*, vol. 335,no. 12, pp. 885-886.

Netter, A. & Salanier, M. 1917, "The presence of meningococci in the purpuric elements of meningococcal infection", *Br.J.Child.Dis*, vol. 14, pp. 101-104.

Noble, K. E., Panayiotidis, P., Collins, P. W., Hoffbrand, A. V., & Yong, K. L. 1996, "Monocytes induce E-selectin gene expression in endothelial cells: role of CD11/CD18 and extracellular matrix proteins", *Eur.J.Immunol.*, vol. 26,no. 12, pp. 2944-2951.

Noel-RF, J., Sato, T. T., Mendez, C., Johnson, M. C., & Pohlman, T. H. 1995, "Activation of human endothelial cells by viable or heat-killed gram-negative bacteria requires soluble CD14", *Infect.Immun.*, vol. 63,no. 10, pp. 4046-4053.

Nugent, S. K., Bausher, J. A., Moxon, E. R., & Rogers, M. C. 1979, "Raised intracranial pressure: its management in Neisseria meningitidis meningoencephalitis", *Am.J.Dis.Child*, vol. 133,no. 3, pp. 260-262.

Odegaard, T. J., Kaltashov, I. A., Cotter, R. J., Steeghs, L., van der, L. P., Khan, S., Maskell, D. J., & Raetz, C. R. 1997, "Shortened hydroxyacyl chains on lipid A of Escherichia coli cells expressing a foreign UDP-N-acetylglucosamine O-acyltransferase", *J.Biol.Chem.*, vol. 272,no. 32, pp. 19688-19696.

Oftung, F., Naess, L. M., Wetzler, L. M., Korsvold, G. E., Aase, A., Hoiby, E. A., Dalseg, R., Holst, J., Michaelsen, T. E., & Haneberg, B. 1999, "Antigen-specific T-cell responses in humans after intranasal immunization with a meningococcal serogroup B outer membrane vesicle vaccine", *Infect.Immun.*, vol. 67,no. 2, pp. 921-927.

op de Coul, M. E. & Kardos, G. 1996, "[An unusual cause of fever: chronic meningococcemia] Een ongewone oorzaak van koorts: chronische meningokokkemie", *Ned.Tijdschr.Geneeskd.*, vol. 140,no. 14, pp. 785-787.

Osterud, B. & Flaegstad, T. 1983, "Increased tissue thromboplastin activity in monocytes of patients with meningococcal infection: related to an unfavourable prognosis", *Thromb.Haemost.*, vol. 49,no. 1, pp. 5-7.

Parrillo, J. E. 1993, "Pathogenetic mechanisms of septic shock [see comments]", *N.Engl.J.Med.*, vol. 328,no. 20, pp. 1471-1477.

Pavliak, V., Brisson, J. R., Michon, F., Uhrin, D., & Jennings, H. J. 1993, "Structure of the sialylated L3 lipopolysaccharide of Neisseria meningitidis", *J.Biol.Chem.*, vol. 268,no. 19, pp. 14146-14152.

Perona, R., Montaner, S., Saniger, L., Sanchez, P. I., Bravo, R., & Lacal, J. C. 1997a, "Activation of the nuclear factor-kappaB by Rho, CDC42, and Rac-1 proteins", *Genes Dev.*, vol. 11,no. 4, pp. 463-475.

Perona, R., Montaner, S., Saniger, L., Sanchez, P. I., Bravo, R., & Lacal, J. C. 1997b, "Activation of the nuclear factor-kappaB by Rho, CDC42, and Rac-1 proteins", *Genes Dev.*, vol. 11,no. 4, pp. 463-475.

Peters, M. J., Dixon, G., Kotowicz, K. T., Hatch, D. J., Heyderman, R. S., & Klein, N. J. 1999, "Circulating platelet-neutrophil complexes represent a subpopulation of activated neutrophils primed for adhesion, phagocytosis and intracellular killing", *Br.J.Haematol.*, vol. 106,no. 2, pp. 391-399.

Plaksin, D., Baeuerle, P. A., & Eisenbach, L. 1993, "KBF1 (p50 NF-kappa B homodimer) acts as a repressor of H-2Kb gene expression in metastatic tumor cells", *J.Exp.Med.*, vol. 177,no. 6, pp. 1651-1662.

Pober, J. S. 1988, "Warner-Lambert/Parke-Davis award lecture. Cytokine-mediated activation of vascular endothelium. Physiology and pathology", *Am.J.Pathol.*, vol. 133,no. 3, pp. 426-433.

Pober, J. S., Bevilacqua, M. P., Mendrick, D. L., Lapierre, L. A., Fiers, W., & Gimbrone-MA, J. 1986, "Two distinct monokines, interleukin 1 and tumor necrosis factor, each independently induce biosynthesis and transient expression of the same antigen on the surface of cultured human vascular endothelial cells", *J.Immunol.*, vol. 136,no. 5, pp. 1680-1687.

Poltorak, A., He, X., Smirnova, I., Liu, M. Y., Huffel, C. V., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., Freudenberg, M., Ricciardi, C. P., Layton, B., & Beutler, B. 1998, "Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene", *Science*, vol. 282,no. 5396, pp. 2085-2088.

Poltorak, A., Ricciardi-Castagnoli, P., Citterio, S., & Beutler, B. 2000, "Physical contact between lipopolysaccharide and Toll-like receptor 4 revealed by genetic complementation", *Proc.Natl.Acad.Sci.U.S.A*, vol. 97,no. 5, pp. 2163-2167.

Poolman, J., van der Ley, P., & Tommassen, J. 1995, "Surface Structures and Secreted Products of Meningococci," in *Meningococcal Disease*, K. Cartwright, ed., John Wiley and Sons, Chichester, pp. 21-34.

Pugin, J., Schurer Maly, C. C., Leturcq, D., Moriarty, A., Ulevitch, R. J., & Tobias, P. S. 1993, "Lipopolysaccharide activation of human endothelial and epithelial cells

is mediated by lipopolysaccharide-binding protein and soluble CD14", *Proc.Natl.Acad.Sci.U.S.A.*, vol. 90,no. 7, pp. 2744-2748.

Raetz, C. R., Ulevitch, R. J., Wright, S. D., Sibley, C. H., Ding, A., & Nathan, C. F. 1991, "Gram-negative endotoxin: an extraordinary lipid with profound effects on eukaryotic signal transduction", *FASEB J.*, vol. 5,no. 12, pp. 2652-2660.

Ram, S., Sharma, A. K., Simpson, S. D., Gulati, S., McQuillen, D. P., Pangburn, M. K., & Rice, P. A. 1998, "A novel sialic acid binding site on factor H mediates serum resistance of sialylated Neisseria gonorrhoeae", *J.Exp.Med.*, vol. 187,no. 5, pp. 743-752.

Ramsay, M., Kaczmarski, E., Rush, M., Mallard, R., Farrington, P., & White, J. 1997, "Changing patterns of case ascertainment and trends in meningococcal disease in England and Wales", *Commun.Dis.Rep.CDR.Rev.*, vol. 7,no. 4, p. R49-R54.

Read, M. A., Whitley, M. Z., Gupta, S., Pierce, J. W., Best, J., Davis, R. J., & Collins, T. 1997, "Tumor necrosis factor alpha-induced E-selectin expression is activated by the nuclear factor-kappaB and c-JUN N-terminal kinase/p38 mitogen-activated protein kinase pathways", *J.Biol.Chem.*, vol. 272,no. 5, pp. 2753-2761.

Reimold, A. M., Grusby, M. J., Kosaras, B., Fries, J. W., Mori, R., Maniwa, S., Clauss, I. M., Collins, T., Sidman, R. L., Glimcher, M. J., & Glimcher, L. H. 1996, "Chondrodysplasia and neurological abnormalities in ATF-2-deficient mice", *Nature*, vol. 379,no. 6562, pp. 262-265.

Rescigno, M., Citterio, S., Thery, C., Rittig, M., Medaglini, D., Pozzi, G., Amigorena, S., & Ricciardi, C. P. 1998a, "Bacteria-induced neo-biosynthesis, stabilization, and surface expression of functional class I molecules in mouse dendritic cells", *Proc.Natl.Acad.Sci.U.S.A.*, vol. 95,no. 9, pp. 5229-5234.

Rescigno, M., Granucci, F., Citterio, S., foti, M., & Ricciardi, C. P. 1999, "Coordinated events during bacteria-induced DC maturation", *Immunol.Today*, vol. 20,no. 5, pp. 200-203.

Rescigno, M., Martino, M., Sutherland, C. L., Gold, M. R., & Ricciardi, C. P. 1998b, "Dendritic cell survival and maturation are regulated by different signaling pathways", *J.Exp.Med.*, vol. 188,no. 11, pp. 2175-2180.

Ria, F., Penna, G., & Adorini, L. 1998, "Th1 cells induce and Th2 inhibit antigen-dependent IL-12 secretion by dendritic cells", *Eur.J.Immunol.*, vol. 28,no. 6, pp. 2003-2016.

Richmond, P., Borrow, R., Miller, E., Clark, S., Sadler, F., Fox, A., Begg, N., Morris, R., & Cartwright, K. 1999, "Meningococcal serogroup C conjugate vaccine is immunogenic in infancy and primes for memory", *J.Infect.Dis.*, vol. 179,no. 6, pp. 1569-1572.

Richter, J., Olsson, I., & Andersson, T. 1990, "Correlation between spontaneous oscillations of cytosolic free Ca2+ and tumor necrosis factor-induced degranulation in adherent human neutrophils.", *J.Biol.Chem.*, vol. 265,no. 24, pp. 14358-14363.

Ridge, J. P., Di, R. F., & Matzinger, P. 1998, "A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell [see comments]", *Nature*, vol. 393,no. 6684, pp. 474-478.

Rissoan, M. C., Soumelis, V., Kadowaki, N., Grouard, G., Briere, F., de-Waal, M. R., & Liu, Y. J. 1999, "Reciprocal control of T helper cell and dendritic cell differentiation [see comments]", *Science*, vol. 283,no. 5405, pp. 1183-1186.

Roake, J. A., Rao, A. S., Morris, P. J., Larsen, C. P., Hankins, D. F., & Austyn, J. M. 1995, "Systemic lipopolysaccharide recruits dendritic cell progenitors to nonlymphoid tissues", *Transplantation*, vol. 59,no. 9, pp. 1319-1324.

Rock, F. L., Hardiman, G., Timans, J. C., Kastelein, R. A., & Bazan, J. F. 1998, "A family of human receptors structurally related to Drosophila Toll", *Proc.Natl.Acad.Sci.U.S.A.*, vol. 95,no. 2, pp. 588-593.

Ross, S. C., Rosenthal, P. J., Berberich, H. M., & Densen, P. 1987, "Killing of Neisseria meningitidis by human neutrophils: implications for normal and complement-deficient individuals", *J.Infect.Dis.*, vol. 155,no. 6, pp. 1266-1275.

Rot, A. 1992, "Endothelial cell binding of NAP-1/IL-8: role in neutrophil emigration", *Immunol.Today*, vol. 13,no. 8, pp. 291-294.

Rothstein, J. L. & Schreiber, H. 1988, "Synergy between tumor necrosis factor and bacterial products causes hemorrhagic necrosis and lethal shock in normal mice", *Proc.Natl.Acad.Sci.U.S.A.*, vol. 85,no. 2, pp. 607-611.

Rudel, T., Scheurerpflug, I., & Meyer, T. F. 1995, "Neisseria PilC protein identified as type-4 pilus tip-located adhesin", *Nature*, vol. 373,no. 6512, pp. 357-359.

Ruoslahti, E. 1988, "Fibronectin and its receptors", *Annu.Rev.Biochem.*, vol. 57, pp. 375-413.

Ruoslahti, E. & Pierschbacher, M. D. 1987, "New perspectives in cell adhesion: RGD and integrins", *Science*, vol. 238,no. 4826, pp. 491-497.

Sallusto, F., Cella, M., Danieli, C., & Lanzavecchia, A. 1995, "Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products [see comments]", *J.Exp.Med.*, vol. 182,no. 2, pp. 389-400.

Sallusto, F. & Lanzavecchia, A. 1999, "Mobilizing dendritic cells for tolerance, priming, and chronic inflammation [comment]", *J.Exp.Med.*, vol. 189,no. 4, pp. 611-614.

Sallusto, F., Lanzavecchia, A., & Mackay, C. R. 1998, "Chemokines and chemokine receptors in T-cell priming and Th1/Th2-mediated responses", *Immunol.Today*, vol. 19,no. 12, pp. 568-574.

Sallusto, F., Schaerli, P., Loetscher, P., Schaniel, C., Lenig, D., Mackay, C. R., Qin, S., & Lanzavecchia, A. 1998, "Rapid and coordinated switch in chemokine receptor expression during dendritic cell maturation.", *Eur.J.Immunol.*, vol. 28,no. 9, pp. 2760-2769.

Sander, B., Andersson, J., & Andersson, U. 1991, "Assessment of cytokines by immunofluorescence and the paraformaldehyde-saponin procedure.", *Immunol.Rev.*, vol. 119, pp. 65-93.

Sato, T. T., Kovacich, J. C., Boyle-EM, J., Haddix, T. L., Weintraub, A., & Pohlman, T. H. 1998, "CD14-dependent activation of human endothelial cells by Bacteroides fragilis outer membrane.", *J.Surg.Res.*, vol. 74,no. 2, pp. 103-111.

Scheicher, C., Mehlig, M., Dienes, H. P., & Reske, K. 1995, "Uptake of bead-adsorbed versus soluble antigen by bone marrow derived dendritic cells triggers their activation and increases their antigen presentation capacity.", *Adv.Exp.Med.Biol.*, vol. 378, pp. 253-255.

Schleiffenbaum, B., Fehr, J., Odermatt, B., & Sperb, R. 1998, "Inhibition of leukocyte emigration induced during the systemic inflammatory reaction in vivo is not due to IL-8", *J.Immunol.*, vol. 161,no. 7, pp. 3631-3638.

Schleimer, R. P. & Rutledge, B. K. 1986, "Cultured human vascular endothelial cells acquire adhesiveness for neutrophils after stimulation with interleukin 1, endotoxin, and tumor-promoting phorbol diesters", *J.Immunol.*, vol. 136,no. 2, pp. 649-654.

Schoenberger, S. P., Toes, R. E., van-der, V. E., Offringa, R., & Melief, C. J. 1998, "T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions [see comments]", *Nature*, vol. 393,no. 6684, pp. 480-483.

Schumann, R. R., Leong, S. R., Flaggs, G. W., Gray, P. W., Wright, S. D., Mathison, J. C., Tobias, P. S., & Ulevitch, R. J. 1990, "Structure and function of lipopolysaccharide binding protein", *Science*, vol. 249,no. 4975, pp. 1429-1431.

Schwandner, R., Dziarski, R., Wesche, H., Rothe, M., & Kirschning, C. J. 1999, "Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by toll-like receptor 2", *J.Biol.Chem.*, vol. 274,no. 25, pp. 17406-17409.

Schwartz, J. D., Monea, S., Marcus, S. G., Patel, S., Eng, K., Galloway, A. C., Mignatti, P., & Shamamian, P. 1998, "Soluble factor(s) released from neutrophils activates endothelial cell matrix metalloproteinase-2", *J.Surg.Res.*, vol. 76,no. 1, pp. 79-85.

Schwarzer, N., Nost, R., Seybold, J., Parida, S. K., Fuhrmann, O., Krull, M., Schmidt, R., Newton, R., Hippenstiel, S., Domann, E., Chakraborty, T., & Suttorp, N. 1998, "Two distinct phospholipases C of Listeria monocytogenes induce ceramide generation, nuclear factor-kappa B activation, and E-selectin expression in human endothelial cells", *J.Immunol.*, vol. 161,no. 6, pp. 3010-3018.

Schweitzer, K. M., Drager, A. M., van-der, V. P., Thijsen, S. F., Zevenbergen, A., Theijsmeijer, A. P., van-der, S. C., & Langenhuijsen, M. M. 1996, "Constitutive expression of E-selectin and vascular cell adhesion molecule-1 on endothelial cells of hematopoietic tissues", *Am.J.Pathol.*, vol. 148,no. 1, pp. 165-175.

Shappell, S. B., Toman, C., Anderson, D. C., Taylor, A. A., Entman, M. L., & Smith, C. W. 1990, "Mac-1 (CD11b/CD18) mediates adherence-dependent hydrogen peroxide production by human and canine neutrophils.", *J.Immunol.*, vol. 144,no. 7, pp. 2702-2711.

Sher, A. & Coffman, R. L. 1992, "Regulation of immunity to parasites by T cells and T cell-derived cytokines", *Annu.Rev.Immunol.*, vol. 10, pp. 385-409.

Shibata, Y., Metzger, W. J., & Myrvik, Q. N. 1997, "Chitin particle-induced cell-mediated immunity is inhibited by soluble mannan: mannose receptor-mediated phagocytosis initiates IL-12 production", *J.Immunol.*, vol. 159,no. 5, pp. 2462-2467.

Shimazu, R., Akashi, S., Ogata, H., Nagai, Y., Fukudome, K., Miyake, K., & Kimoto, M. 1999, "MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4", *J.Exp.Med.*, vol. 189,no. 11, pp. 1777-1782.

Shimizu, Y., Newman, W., Tanaka, Y., & Shaw, S. 1992, "Lymphocyte interactions with endothelial cells", *Immunol.Today*, vol. 13,no. 3, pp. 106-112.

Sierra, G. V., Campa, H. C., Varcacel, N. M., Garcia, I. L., Izquierdo, P. L., Sotolongo, P. F., Casanueva, G. V., Rico, C. O., Rodriguez, C. R., & Terry, M. H. 1991, "Vaccine against group B Neisseria meningitidis: protection trial and mass vaccination results in Cuba", *NIPH.Ann.*, vol. 14,no. 2, pp. 195-207.

Smedly, L. A., Tonnesen, M. G., Sandhaus, R. A., Haslett, C., Guthrie, L. A., Johnston-RB, J., Henson, P. M., & Worthen, G. S. 1986, "Neutrophil-mediated injury to endothelial cells. Enhancement by endotoxin and essential role of neutrophil elastase.", *J.Clin.Invest.*, vol. 77,no. 4, pp. 1233-1243.

Smith, C. W., Marlin, S. D., Rothlein, R., Toman, C., & Anderson, D. C. 1989b, "Cooperative interactions of LFA-1 and Mac-1 with intercellular adhesion molecule-1 in facilitating adherence and transendothelial migration of human neutrophils in vitro", *J.Clin.Invest.*, vol. 83,no. 6, pp. 2008-2017.

Smith, C. W., Marlin, S. D., Rothlein, R., Toman, C., & Anderson, D. C. 1989a, "Cooperative interactions of LFA-1 and Mac-1 with intercellular adhesion molecule-1 in facilitating adherence and transendothelial migration of human neutrophils in vitro", *J.Clin.Invest.*, vol. 83,no. 6, pp. 2008-2017.

Smith, W. B., Gamble, J. R., Clark, L. I., & Vadas, M. A. 1991, "Interleukin-8 induces neutrophil transendothelial migration", *Immunology*, vol. 72,no. 1, pp. 65-72.

Smyth, S. S., Joneckis, C. C., & Parise, L. V. 1993, "Regulation of vascular integrins [published erratum appears in Blood 1994 Apr 1;83(7):2013]", *Blood*, vol. 81,no. 11, pp. 2827-2843.

Snijders, A., Kalinski, P., Hilkens, C. M., & Kapsenberg, M. L. 1998, "High-level IL-12 production by human dendritic cells requires two signals", *Int.Immunol.*, vol. 10,no. 11, pp. 1593-1598.

Sotto, M. N., Langer, B., Hoshino, S. S., & de, B. T. 1976, "Pathogenesis of cutaneous lesions in acute meningococcemia in humans: light, immunofluorescent, and electron microscopic studies of skin biopsy specimens.", *J.Infect.Dis.*, vol. 133,no. 5, pp. 506-514.

Sozzani, S., Allavena, P., D'Amico, G., Luini, W., Bianchi, G., Kataura, M., Imai, T., Yoshie, O., Bonecchi, R., & Mantovani, A. 1998, "Differential regulation of chemokine receptors during dendritic cell maturation: a model for their trafficking properties.", *J.Immunol.*, vol. 161,no. 3, pp. 1083-1086.

Springer, T. A. 1990, "Adhesion receptors of the immune system.", *Nature*, vol. 346,no. 6283, pp. 425-434.

Springer, T. A. 1994, "Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm.", *Cell*, vol. 76,no. 2, pp. 301-314.

Stahl, P. D. & Ezekowitz, R. A. 1998, "The mannose receptor is a pattern recognition receptor involved in host defense", *Curr.Opin.Immunol.*, vol. 10,no. 1, pp. 50-55.

Steeghs, L., den, H. R., den, B. A., Zomer, B., Roholl, P., & van-der, L. P. 1998, "Meningitis bacterium is viable without endotoxin [letter]", *Nature*, vol. 392,no. 6675, pp. 449-450.

Steeghs, L., Kuipers, B., Hamstra, H. J., Kersten, G., van Alphen, L., & van der, L. P. 1999, "Immunogenicity of outer membrane proteins in a lipopolysaccharide-deficient mutant of Neisseria meningitidis: influence of adjuvants on the immune response", *Infect.Immun.*, vol. 67,no. 10, pp. 4988-4993.

Steinman, R. M. 1991, "The dendritic cell system and its role in immunogenicity", *Annu.Rev.Immunol.*, vol. 9, pp. 271-296.

Steinman, R. M. & Swanson, J. 1995, "The endocytic activity of dendritic cells [comment]", *J.Exp.Med.*, vol. 182,no. 2, pp. 283-288.

Stephens, D. S. & McGee, Z. A. 1981, "Attachment of Neisseria meningitidis to human mucosal surfaces: influence of pili and type of receptor cell", *J.Infect.Dis.*, vol. 143,no. 4, pp. 525-532.

Stephens, D. S., Spellman, P. A., & Swartley, J. S. 1993a, "Effect of the (alpha 2--
>8)-linked polysialic acid capsule on adherence of Neisseria meningitidis to human mucosal cells", *J.Infect.Dis.*, vol. 167,no. 2, pp. 475-479.

Stuart, J. M., Cartwright, K. A., Robinson, P. M., & Noah, N. D. 1989, "Effect of smoking on meningococcal carriage", *Lancet*, vol. 2,no. 8665, pp. 723-725.

Suffredini, A. F., Harpel, P. C., & Parrillo, J. E. 1989, "Promotion and subsequent inhibition of plasminogen activation after administration of intravenous endotoxin to normal subjects [see comments]", *N.Engl.J.Med.*, vol. 320,no. 18, pp. 1165-1172.

Sullivan, T. D. & LaScolea-LJ, J. 1987, "Neisseria meningitidis bacteremia in children: quantitation of bacteremia and spontaneous clinical recovery without antibiotic therapy", *Pediatrics*, vol. 80,no. 1, pp. 63-67.

Swain, S. L., Weinberg, A. D., English, M., & Huston, G. 1990, "IL-4 directs the development of Th2-like helper effectors", *J.Immunol.*, vol. 145,no. 11, pp. 3796-3806.

Swartley, J. S., Marfin, A. A., Edupuganti, S., Liu, L. J., Cieslak, P., Perkins, B., Wenger, J. D., & Stephens, D. S. 1997, "Capsule switching of Neisseria meningitidis", *Proc.Natl.Acad.Sci.U.S.A.*, vol. 94,no. 1, pp. 271-276.

Taha, M. K., Giorgini, D., & Nassif, X. 1996, "The pilA regulatory gene modulates the pilus-mediated adhesion of Neisseria meningitidis by controlling the transcription of pilC1", *Mol.Microbiol.*, vol. 19,no. 5, pp. 1073-1084.

Takada, H. & Kotani, S. 1989, "Structural requirements of lipid A for endotoxicity and other biological activities", *Crit.Rev.Microbiol.*, vol. 16,no. 6, pp. 477-523.

Takahashi, M., Masuyama, J., Ikeda, U., Kitagawa, S., Kasahara, T., Saito, M., Kano, S., & Shimada, K. 1995b, "Effects of endogenous endothelial interleukin-8 on neutrophil migration across an endothelial monolayer", *Cardiovasc.Res.*, vol. 29,no. 5, pp. 670-675.

Takahashi, M., Masuyama, J., Ikeda, U., Kitagawa, S., Kasahara, T., Saito, M., Kano, S., & Shimada, K. 1995a, "Effects of endogenous endothelial interleukin-8 on neutrophil migration across an endothelial monolayer", *Cardiovasc.Res.*, vol. 29,no. 5, pp. 670-675.

Takeuchi, O., Hoshino, K., Kawai, T., Sanjo, H., Takada, H., Ogawa, T., Takeda, K., & Akira, S. 1999b, "Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components", *Immunity.*, vol. 11,no. 4, pp. 443-451.

Takeuchi, O., Kawai, T., Sanjo, H., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., Takeda, K., & Akira, S. 1999a, "TLR6: A novel member of an expanding toll-like receptor family", *Gene*, vol. 231,no. 1-2, pp. 59-65.

Tamaru, M. & Narumi, S. 1999, "E-selectin gene expression is induced synergistically with the coexistence of activated classic protein kinase C and signals elicited by interleukin-1beta but not tumor necrosis factor-alpha", *J.Biol.Chem.*, vol. 274,no. 6, pp. 3753-3763.

Tanamoto, K., Azumi, S., Haishima, Y., Kumada, H., & Umemoto, T. 1997, "The lipid A moiety of Porphyromonas gingivalis lipopolysaccharide specifically mediates the activation of C3H/HeJ mice", *J.Immunol.*, vol. 158,no. 9, pp. 4430-4436.

Tappero, J. W., Lagos, R., Ballesteros, A. M., Plikaytis, B., Williams, D., Dykes, J., Gheesling, L. L., Carlone, G. M., Hoiby, E. A., Holst, J., Nokleby, H., Rosenqvist, E., Sierra, G., Campa, C., Sotolongo, F., Vega, J., Garcia, J., Herrera, P., Poolman,

J. T., & Perkins, B. A. 1999, "Immunogenicity of 2 serogroup B outer-membrane protein meningococcal vaccines: a randomized controlled trial in Chile [see comments]", *JAMA*, vol. 281,no. 16, pp. 1520-1527.

Tappero, J. W., Reporter, R., Wenger, J. D., Ward, B. A., Reeves, M. W., Missbach, T. S., Plikaytis, B. D., Mascola, L., & Schuchat, A. 1996, "Meningococcal disease in Los Angeles County, California, and among men in the county jails [see comments]", *N.Engl.J.Med.*, vol. 335,no. 12, pp. 833-840.

Tedder, T. F., Steeber, D. A., Chen, A., & Engel, P. 1995, "The selectins: vascular adhesion molecules", *FASEB J.*, vol. 9,no. 10, pp. 866-873.

Tettelin, H., Saunders, N. J., Heidelberg, J., Jeffries, A. C., Nelson, K. E., Eisen, J. A., Ketchum, K. A., Hood, D. W., Peden, J. F., Dodson, R. J., Nelson, W. C., Gwinn, M. L., DeBoy, R., Peterson, J. D., Hickey, E. K., Haft, D. H., Salzberg, S. L., White, O., Fleischmann, R. D., Dougherty, B. A., Mason, T., Ciecko, A., Parksey, D. S., Blair, E., Cittone, H., & Clark, E. B. 2000, "Complete genome sequence of neisseria meningitidis serogroup B strain MC58 [In Process Citation]", *Science*, vol. 287,no. 5459, pp. 1809-1815.

Thieblemont, N., Thieringer, R., & Wright, S. D. 1998, "Innate immune recognition of bacterial lipopolysaccharide: dependence on interactions with membrane lipids and endocytic movement", *Immunity.*, vol. 8,no. 6, pp. 771-777.

Thompson, J. E., Phillips, R. J., Erdjument-Bromage, H., Tempst, P., & Ghosh, S. 1995, "I kappa B-beta regulates the persistent response in a biphasic activation of NF-kappa B", *Cell*, vol. 80,no. 4, pp. 573-582.

Thornhill, M. H. & Haskard, D. O. 1990, "IL-4 regulates endothelial cell activation by IL-1, tumor necrosis factor, or IFN-gamma", *J.Immunol.*, vol. 145,no. 3, pp. 865-872.

Thornhill, M. H., Kyan Aung, U., & Haskard, D. O. 1990, "IL-4 increases human endothelial cell adhesiveness for T cells but not for neutrophils", *J.Immunol.*, vol. 144,no. 8, pp. 3060-3065.

Tobias, P. S., Soldau, K., & Ulevitch, R. J. 1986, "Isolation of a lipopolysaccharide-binding acute phase reactant from rabbit serum", *J.Exp.Med.*, vol. 164,no. 3, pp. 777-793.

Tracey, K. J., Beutler, B., Lowry, S. F., Merryweather, J., Wolpe, S., Milsark, I. W., Hariri, R. J., Fahey, T. J., Zentella, A., Albert, J. D., & et, a. 1986, "Shock and tissue injury induced by recombinant human cachectin", *Science*, vol. 234,no. 4775, pp. 470-474.

Trinchieri, G. 1993, "Interleukin-12 and its role in the generation of TH1 cells", *Immunol.Today*, vol. 14,no. 7, pp. 335-338.

Trinchieri, G. 1995, "Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity", *Annu.Rev.Immunol.*, vol. 13, pp. 251-276.

Tsai, C. M., Frasch, C. E., Rivera, E., Holstein, & H.D. Measurement of lipopolysaccharide (endotoxin) in meningococcal protein and polysaccharide preparations for vaccines usage. J.Biol.Stand 17, 249-258. 1989. Ref Type: Journal (Full)

Turner, M. W. 1996, "Mannose-binding lectin: the pluripotent molecule of the innate immune system", *Immunol.Today*, vol. 17,no. 11, pp. 532-540.

Ulevitch, R. J. 1999, "Endotoxin opens the Tollgates to innate immunity [news]", *Nat.Med.*, vol. 5,no. 2, pp. 144-145.

Ulevitch, R. J. & Tobias, P. S. 1995, "Receptor-dependent mechanisms of cell stimulation by bacterial endotoxin", *Annu.Rev.Immunol.*, vol. 13, pp. 437-457.

Underhill, D. M., Ozinsky, A., Hajjar, A. M., Stevens, A., Wilson, C. B., Bassetti, M., & Aderem, A. 1999, "The Toll-like receptor 2 is recruited to macrophage phagosomes and discriminates between pathogens [see comments]", *Nature*, vol. 401,no. 6755, pp. 811-815.

van-der, V. E., van, D. H., Kuipers, B., van-der, B. J., van-der, L. P., Meylis, J., Claassen, I., & Poolman, J. 1997, "Human B- and T-cell responses after immunization with a hexavalent PorA meningococcal outer membrane vesicle vaccine", *Infect.Immun.*, vol. 65,no. 12, pp. 5184-5190.

van der Poll, T., Buller, H. R., ten Cate, H., Wortel, C. H., Bauer, K. A., van Deventer, S. J., Hack, C. E., Sauerwein, H. P., Rosenberg, R. D., & ten Cate, J. W. 1990, "Activation of coagulation after administration of tumor necrosis factor to normal subjects [see comments]", *N.Engl.J.Med.*, vol. 322,no. 23, pp. 1622-1627.

van Deuren, M. 1998, "Meningococcal infections, an overview," in *Acute meningococal disease. A study of clinical management, cytokine activation and regulation*, Katholieke Universiteit, Nijmegen, pp. 13-42.

van Deuren, M., Neeleman, C., Van-'t, H. L., & van-der, M. J. 1998, "A normal platelet count at admission in acute meningococcal disease does not exclude a fulminant course", *Intensive.Care Med.*, vol. 24,no. 2, pp. 157-161.

van Deuren, M., Santman, F. W., van, D. R., Sauerwein, R. W., Span, L. F., & van-der, M. J. 1992, "Plasma and whole blood exchange in meningococcal sepsis", *Clin.Infect.Dis.*, vol. 15,no. 3, pp. 424-430.

van Deuren, M., van-der-Ven, J. J., Bartelink, A. K., van, D. R., Sauerwein, R. W., & van-der, M. J. 1995, "Correlation between proinflammatory cytokines and antiinflammatory mediators and the severity of disease in meningococcal infections", *J.Infect.Dis.*, vol. 172,no. 2, pp. 433-439.

van Deuren, M., van-der-Ven, J. J., Demacker, P. N., Bartelink, A. K., van, D. R., Sauerwein, R. W., Gallati, H., Vannice, J. L., & van-der, M. J. 1994, "Differential expression of proinflammatory cytokines and their inhibitors during the course of meningococcal infections", *J.Infect.Dis.*, vol. 169,no. 1, pp. 157-161.

van Deuren, M., van, d., V, Vannier, E., van Dalen, R., Pesman, G., Bartelink, A. K., Dinarello, C. A., & van der Meer, J. W. 1997, "The pattern of interleukin-1beta (IL-1beta) and its modulating agents IL-1 receptor antagonist and IL-1 soluble

receptor type II in acute meningococcal infections", *Blood*, vol. 90,no. 3, pp. 1101-1108.

van Deuren, M., van, D. B., Koopman, R. J., Horrevorts, A. M., Meis, J. F., Santman, F. W., & van-der, M. J. 1993, "Rapid diagnosis of acute meningococcal infections by needle aspiration or biopsy of skin lesions [see comments]", *BMJ.*, vol. 306,no. 6887, pp. 1229-1232.

van Dissel, J. T., van Langervelde, P., Westendorp, R. G., Kwappenberg, K., & Frolich, M. 1998, "Anti-inflammatory cytokine profile and mortality in febrile patients", *Lancet*, vol. 351, pp. 950-953.

van, S. G., Newman, W., Shimizu, Y., Nutman, T. B., Tanaka, Y., Horgan, K. J., Gopal, T. V., Ennis, E., O'Sullivan, D., Grey, H., & et, a. 1991, "Analysis of T cell stimulation by superantigen plus major histocompatibility complex class II molecules or by CD3 monoclonal antibody: costimulation by purified adhesion ligands VCAM-1, ICAM-1, but not ELAM-1", *J.Exp.Med.*, vol. 174,no. 4, pp. 901-913.

Varki, A. 1997, "Selectin ligands: will the real ones please stand up?", *J.Clin.Invest.*, vol. 100,no. 11 Suppl, p. S31-S35.

Vercellotti, G. M., McCarthy, J., Furcht, L. T., Jacob, H. S., & Moldow, C. F. 1983, "Inflamed fibronectin: an altered fibronectin enhances neutrophil adhesion", *Blood*, vol. 62,no. 5, pp. 1063-1069.

Verhasselt, V., Buelens, C., Willems, F., De, G. D., Haeffner, C. N., & Goldman, M. 1997, "Bacterial lipopolysaccharide stimulates the production of cytokines and the expression of costimulatory molecules by human peripheral blood dendritic cells: evidence for a soluble CD14-dependent pathway.", *J.Immunol.*, vol. 158,no. 6, pp. 2919-2925.

Vernier, G. A., al, O. S., Gourieux, B., Klein, J. P., & Wachsmann, D. 1998, "Protein I/II of oral viridans streptococci increases expression of adhesion

molecules on endothelial cells and promotes transendothelial migration of neutrophils in vitro", *Cell Immunol.*, vol. 187,no. 2, pp. 145-150.

Vik, M. H., Lote, K., & Nordoy, A. 1978, "Disseminated intravascular coagulation in patients with meningococcal infection: laboratory diagnosis and prognostic factors", *Scand.J.Infect.Dis.*, vol. 10,no. 3, pp. 187-191.

Virji, M., Alexandrescu, C., Ferguson, D. J., Saunders, J. R., & Moxon, E. R. 1992b, "Variations in the expression of pili: the effect on adherence of Neisseria meningitidis to human epithelial and endothelial cells", *Mol.Microbiol.*, vol. 6,no. 10, pp. 1271-1279.

Virji, M., Kayhty, H., Ferguson, D. J., Alexandrescu, C., Heckels, J. E., & Moxon, E. R. 1991, "The role of pili in the interactions of pathogenic Neisseria with cultured human endothelial cells", *Mol.Microbiol.*, vol. 5,no. 8, pp. 1831-1841.

Virji, M., Makepeace, K., Ferguson, D. J., Achtman, M., & Moxon, E. R. 1993a, "Meningococcal Opa and Opc proteins: their role in colonization and invasion of human epithelial and endothelial cells", *Mol.Microbiol.*, vol. 10,no. 3, pp. 499-510.

Virji, M., Makepeace, K., Ferguson, D. J., Achtman, M., Sarkari, J., & Moxon, E. R. 1992a, "Expression of the Opc protein correlates with invasion of epithelial and endothelial cells by Neisseria meningitidis", *Mol.Microbiol.*, vol. 6,no. 19, pp. 2785-2795.

Virji, M., Makepeace, K., & Moxon, E. R. 1994, "Distinct mechanisms of interactions of Opc-expressing meningococci at apical and basolateral surfaces of human endothelial cells; the role of integrins in apical interactions", *Mol.Microbiol.*, vol. 14,no. 1, pp. 173-184.

Virji, M., Makepeace, K., Peak, I. R., Ferguson, D. J., Jennings, M. P., & Moxon, E. R. 1995, "Opc- and pilus-dependent interactions of meningococci with human endothelial cells: molecular mechanisms and modulation by surface polysaccharides", *Mol.Microbiol.*, vol. 18,no. 4, pp. 741-754.

Virji, M., Saunders, J. R., Sims, G., Makepeace, K., Maskell, D., & Ferguson, D. J. 1993b, "Pilus-facilitated adherence of Neisseria meningitidis to human epithelial and endothelial cells: modulation of adherence phenotype occurs concurrently with changes in primary amino acid sequence and the glycosylation status of pilin", *Mol.Microbiol.*, vol. 10,no. 5, pp. 1013-1028.

Vogel, S. N., Johnson, D., Perera, P. Y., Medvedev, A., Lariviere, L., Qureshi, S. T., & Malo, D. 1999b, "Cutting edge: functional characterization of the effect of the C3H/HeJ defect in mice that lack an Lpsn gene: in vivo evidence for a dominant negative mutation", *J.Immunol.*, vol. 162,no. 10, pp. 5666-5670.

Vogel, U., Claus, H., & Frosch, M. 2000, "Rapid serogroup switching in Neisseria meningitidis [letter]", *N.Engl.J.Med.*, vol. 342,no. 3, pp. 219-220.

Vogel, U., Claus, H., Heinze, G., & Frosch, M. 1997, "Functional characterization of an isogenic meningococcal alpha-2,3-sialyltransferase mutant: the role of lipooligosaccharide sialylation for serum resistance in serogroup B meningococci.", *Med.Microbiol.Immunol.Berl.*, vol. 186,no. 2-3, pp. 159-166.

Vogel, U., Claus, H., Heinze, G., & Frosch, M. Role of Lipopolysaccharide Sialylation in Serum Resistance of Serogroup B and C Meningococcal Disease Isolates.*Infect.Immun.*67[2].1999.

Vogel, U., Hammerschmidt, S., & Frosch, M. 1996, "Sialic acids of both the capsule and the sialylated lipooligosaccharide of Neisseria meningitis serogroup B are prerequisites for virulence of meningococci in the infant rat", *Med.Microbiol.Immunol.Berl.*, vol. 185,no. 2, pp. 81-87.

von Asmuth, E. J., Dentener, M. A., Bazil, V., Bouma, M. G., Leeuwenberg, J. F., & Buurman, W. A. 1993, "Anti-CD14 antibodies reduce responses of cultured human endothelial cells to endotoxin", *Immunology*, vol. 80,no. 1, pp. 78-83.

von Adrian U.H, Berger, E. M., Ramezani, L., Chambers, J. D., Ochs, H. D., Harlan, J. M., Paulson, J. C., Etzioni, A., & Arfors, K. E. 1993, "In vivo behavior of

of neutrophils from two patients with distinct inherited leukocyte adhesion deficiency syndromes", *J.Clin.Invest.*, vol. 91,no. 6, pp. 2893-2897.

von Andrian, U. U., Chambers, J. D., McEvoy, L. M., Bargatze, R. F., Arfors, K. E., & Butcher, E. C. 1991, "Two-step model of leukocyte-endothelial cell interaction in inflammation: distinct roles for LECAM-1 and the leukocyte beta 2 integrins in vivo", *Proc.Natl.Acad.Sci.U.S.A.*, vol. 88,no. 17, pp. 7538-7542.

von Asmuth, E.J., Smeets, E. F., Ginsel, L. A., Onderwater, J. J., Leeuwenberg, J. F., & Buurman, W. A. 1992, "Evidence for endocytosis of E-selectin in human endothelial cells", *Eur.J.Immunol.*, vol. 22,no. 10, pp. 2519-2526.

Waage, A. & Espevik, T. 1988, "Interleukin 1 potentiates the lethal effect of tumor necrosis factor alpha/cachectin in mice", *J.Exp.Med.*, vol. 167,no. 6, pp. 1987-1992.

Waage, A., Halstensen, A., & Espevik, T. 1987, "Association between tumour necrosis factor in serum and fatal outcome in patients with meningococcal disease.", *Lancet*, vol. 1,no. 8529, pp. 355-357.

Wachtfogel, Y. T., Abrams, W., Kucich, U., Weinbaum, G., Schapira, M., & Colman, R. W. 1988, "Fibronectin degradation products containing the cytoadhesive tetrapeptide stimulate human neutrophil degranulation", *J.Clin.Invest.*, vol. 81,no. 5, pp. 1310-1316.

Wagner, J. G. & Roth, R. A. 1999, "Neutrophil migration during endotoxemia", *J.Leukoc.Biol.*, vol. 66,no. 1, pp. 10-24.

Wahl, S. M., Feldman, G. M., & McCarthy, J. B. 1996, "Regulation of leukocyte adhesion and signaling in inflammation and disease", *J.Leukoc.Biol.*, vol. 59,no. 6, pp. 789-796.

Weiss, J., Beckerdite, Q. S., & Elsbach, P. 1980, "Resistance of gram-negative bacteria to purified bactericidal leukocyte proteins: relation to binding and bacterial lipopolysaccharide structure", *J.Clin.Invest.*, vol. 65,no. 3, pp. 619-628.

Weiss, J., Elsbach, P., Shu, C., Castillo, J., Grinna, L., Horwitz, A., & Theofan, G. 1992, "Human bactericidal/permeability-increasing protein and a recombinant NH2-terminal fragment cause killing of serum-resistant gram-negative bacteria in whole blood and inhibit tumor necrosis factor release induced by the bacteria.", *J.Clin.Invest.*, vol. 90,no. 3, pp. 1122-1130.

Westendorp, R. G., Hottenga, J. J., & Slagboom, P. E. 1999, "Variation in plasminogen-activator-inhibitor-1 gene and risk of meningococcal septic shock", *Lancet*, vol. 354,no. 9178, pp. 561-563.

Westendorp, R. G., Langermans, J. A., de Bel, C. E., Meinders, A. E., Vandenbroucke, J. P., van Furth, R., & van Dissel, J. T. 1995, "Release of tumor necrosis factor: an innate host characteristic that may contribute to the outcome of meningococcal disease", *J.Infect.Dis.*, vol. 171,no. 4, pp. 1057-1060.

Westendorp, R. G., Langermans, J. A., Huizinga, T. W., Elouali, A. H., Verweij, C. L., Boomsma, D. I., & Vandenbrouke, J. P. 1997, "Genetic influence on cytokine production and fatal meningococcal disease [see comments]", *Lancet*, vol. 349,no. 9046, pp. 170-173.

Westphal, O., Westphal, U., & Sommer, T. 1977, in *Microbiology*, D. Schlessinger, ed., American Society of Microbilogy, Washington DC, pp. 221-238.

Whelan, J., Ghersa, P., Hooft-van, H. R., Gray, J., Chandra, G., Talabot, F., & DeLamarter, J. F. 1991, "An NF kappa B-like factor is essential but not sufficient for cytokine induction of endothelial leukocyte adhesion molecule 1 (ELAM-1) gene transcription", *Nucleic.Acids.Res.*, vol. 19,no. 10, pp. 2645-2653.

Wiertz, E. J., Delvig, A., Donders, E. M., Brugghe, H. F., van, U. L., Timmermans, H. A., Achtman, M., Hoogerhout, P., & Poolman, J. T. 1996, "T-cell responses to outer membrane proteins of Neisseria meningitidis: comparative study of the Opa, Opc, and PorA proteins", *Infect.Immun.*, vol. 64,no. 1, pp. 298-304.

Wilson, A. G., Gordon, C., di, G. F., de, V. N., van-de, P. L., Emery, P., & Duff, G. W. 1994, "A genetic association between systemic lupus erythematosus and tumor necrosis factor alpha", *Eur.J.Immunol.*, vol. 24,no. 1, pp. 191-195.

Woodhead, V. E., Binks, M. H., Chain, B. M., & Katz, D. R. 1998, "From sentinel to messenger: an extended phenotypic analysis of the monocyte to dendritic cell transition", *Immunology*, vol. 94,no. 4, pp. 552-559.

Woods, J. P., Kersulyte, D., Tolan-RW, J., Berg, C. M., & Berg, D. E. 1994, "Use of arbitrarily primed polymerase chain reaction analysis to type disease and carrier strains of Neisseria meningitidis isolated during a university outbreak", *J.Infect.Dis.*, vol. 169,no. 6, pp. 1384-1389.

Wright, S. D. Toll, A new Piece in the puzzle of Innate Immunity. J.Exp.Med. 189, 605-609.15-2-1999.

Wright, S. D., Ramos, R. A., Tobias, P. S., Ulevitch, R. J., & Mathison, J. C. 1990, "CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein [see comments]", *Science*, vol. 249,no. 4975, pp. 1431-1433.

Wright, S. D., Weitz, J. I., Huang, A. J., Levin, S. M., Silverstein, S. C., & Loike, J. D. 1988, "Complement receptor type three (CD11b/CD18) of human polymorphonuclear leukocytes recognizes fibrinogen", *Proc.Natl.Acad.Sci.U.S.A.*, vol. 85,no. 20, pp. 7734-7738.

Wu, L., Li, C. L., & Shortman, K. 1996, "Thymic dendritic cell precursors: relationship to the T lymphocyte lineage and phenotype of the dendritic cell progeny", *J.Exp.Med.*, vol. 184,no. 3, pp. 903-911.

Yanagihara, S., Komura, E., Nagafune, J., Watarai, H., & Yamaguchi, Y. 1998, "EBI1/CCR7 is a new member of dendritic cell chemokine receptor that is up-regulated upon maturation.", *J.Immunol.*, vol. 161,no. 6, pp. 3096-3102.

Yang, R. B., Mark, M. R., Gray, A., Huang, A., Xie, M. H., Zhang, M., Goddard, A., Wood, W. I., Gurney, A. L., & Godowski, P. J. 1998, "Toll-like receptor-2 mediates lipopolysaccharide-induced cellular signalling [see comments]", *Nature*, vol. 395,no. 6699, pp. 284-288.

Yoshimura, A., Lien, E., Ingalls, R. R., Tuomanen, E., Dziarski, R., & Golenbock, D. 1999, "Cutting edge: recognition of Gram-positive bacterial cell wall components by the innate immune system occurs via Toll-like receptor 2", *J.Immunol.*, vol. 163,no. 1, pp. 1-5.

Yu, B., Hailman, E., & Wright, S. D. 1997, "Lipopolysaccharide binding protein and soluble CD14 catalyze exchange of phospholipids", *J.Clin.Invest.*, vol. 99,no. 2, pp. 315-324.

Zhang, F. X., Kirschning, C. J., Mancinelli, R., Xu, X. P., Jin, Y., Faure, E., Mantovani, A., Rothe, M., Muzio, M., & Arditi, M. 1999, "Bacterial lipopolysaccharide activates nuclear factor-kappaB through interleukin-1 signaling mediators in cultured human dermal endothelial cells and mononuclear phagocytes", *J.Biol.Chem.*, vol. 274,no. 12, pp. 7611-7614.

Zhou, L. J. & Tedder, T. F. 1996, "CD14+ blood monocytes can differentiate into functionally mature CD83+ dendritic cells", *Proc.Natl.Acad.Sci.U.S.A.*, vol. 93,no. 6, pp. 2588-2592.

Ziegler, E. J., Fisher, C. J., Jr., Sprung, C. L., Straube, R. C., Sadoff, J. C., Foulke, G. E., Wortel, C. H., Fink, M. P., Dellinger, R. P., Teng, N. N., & et al 1991, "Treatment of gram-negative bacteremia and septic shock with HA- 1A human monoclonal antibody against endotoxin. A randomized, double-blind, placebo-controlled trial. The HA-1A Sepsis Study Group [see comments]", *N.Engl.J.Med.*, vol. 324,no. 7, pp. 429-436.

Zimmerman, G. A., McIntyre, T. M., & Prescott, S. M. 1996, "Adhesion and signaling in vascular cell--cell interactions", *J.Clin.Invest.*, vol. 98,no. 8, pp. 1699-1702.

Zimmerman, G. A., Prescott, S. M., & McIntyre, T. M. 1992, "Endothelial cell interactions with granulocytes: tethering and signaling molecules.", *Immunol.Today*, vol. 13,no. 3, pp. 93-100.

Zollinger, W. D., Moran, E. E., Devi, S. J., & Frasch, C. E. 1997, "Bactericidal antibody responses of juvenile rhesus monkeys immunized with group B Neisseria meningitidis capsular polysaccharide-protein conjugate vaccines", *Infect.Immun.*, vol. 65,no. 3, pp. 1053-1060.

Zollner, O. & Vestweber, D. 1996, "The E-selectin ligand-1 is selectively activated in Chinese hamster ovary cells by the alpha(1,3)-fucosyltransferases IV and VII", *J.Biol.Chem.*, vol. 271,no. 51, pp. 33002-33008.

Zwahlen, A. & Waldvogel, F. A. 1984, "Magnitude of bacteremia and complement activation during Neisseria meningitidis infection: study of two co-primary cases with different clinical presentations", *Eur.J.Clin.Microbiol.*, vol. 3,no. 5, pp. 439-441.

Publications

The following publications have arisen from the work described in the result sections of this thesis

1. Dixon, G.L.J, Heyderman,R., Kotowicz,K, Jack,D.L, Andersen,S.R, Vogel,U., Frosch,M, and Klein,N.J. Endothelial Adhesion Molecule Expression and Its Inhibition by Recombinant Bactericidal/Permeability-Increasing Protein are influenced by the Capsulation and Lipooligosaccharide Structure of Neisseria meningitidis. Infect.Immun. 1999; 67: (11)5626-5633.
2. Dixon, G.L.J., Heyderman R. S., Vogel U., Frosch M., and Klein, N. J. The Influence of Bacterial Structure on Endothelial Cell Adhesion Molecule Expression by *Neisseria meningitidis.*"__Abstract presented at the 11th International Pathogenic *Neisseria* conference, Nice, 1998.
3. Dixon G. L.J., Peters M.J.,. Andersen S.R, van der Ley, P, and Klein N.J. Endotoxin dependent and independent activation of vascular endothelium by *Neisseria meningitidis.* Abstract accepted for poster presentation at 3rd World Congress of Paediatric Intensive Care, Montreal, June 2000.
4. Dixon, G.L.J, Heyderman, R.S., Andersen, S.R, Vogel, U., Frosch, M, and Klein,N.J. Endothelial Adhesion Molecule Expression and its Inhibition by Recombinant Bactericidal/Permeability Increasing Protein ($rBPI_{21}$) is Influenced by the Capsulation and LOS Structure of *Neisseria meningitidis.*". Oral presentation at the Annual General Meeting of the Infectious Disease Society of America, Philadelphia, U.S.A. November 1999.
5. G.L.J. Dixon, P. Newton, S.R. Andersen, P. van der Ley, B. Chain, N.J. Klein and R.E. Callard. Endotoxin independent activation of Dendritic cells by

meningococci". Accepted for poster presentation at the 18th International Congress of Biochemistry and Molecular Biology, Satellite meeting on Innate Immunity, Stevenage, UK, July 2000.

The following publications contain flow cytometry data performed by the candidate.

1. Peters, M.J., G.L.J. Dixon, K.T. Kotowicz, D.J. Hatch, R.S. Heyderman, and N.J. Klein. 1999. Circulating platelet-neutrophil complexes represent a subpopulation of activated neutrophils primed for adhesion, phagocytosis and intracellular killing. *Br.J.Haematol.* 106:391.

2. Peters, M.J., A.. Petros, G.L.J. Dixon, D. Inwald, and N.J. Klein. 1999. Acquired immunoparalysis in paediatric intensive care: prospective observational study. *BMJ.* 319:609.

Candles

Days to come stand in front of us
Like a row of burning candles-
Golden, warm and vivid candles.

Days past fall behind us,
A gloomy line of burnt-out candles;
The nearest are still smoking,
Cold melted and bent.

I don't want to look at them: their shape saddens me,
And it saddens me to remember their original light.
I look ahead at my burning candles.

I don't want to turn, don't want to see, terrified,
How quickly that dark line gets longer,
How quickly one more candle joins another

C.P. Cavafis

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Endothelial Adhesion Molecule Expression and Its Inhibition by Recombinant Bactericidal/Permeability-Increasing Protein Are Influenced by the Capsulation and Lipooligosaccharide Structure of *Neisseria meningitidis*

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Vascular endothelial injury is responsible for many of the clinical manifestations of severe meningococcal disease. Binding and migration of activated host inflammatory cells is a central process in vascular damage. The expression and function of adhesion molecules regulate interactions between leukocytes and endothelial cells. Little is known about how meningococci directly influence these receptors. In this study we have explored the effect of *Neisseria meningitidis* on endothelial adhesion molecule expression and found this organism to be a potent inducer of the adhesion molecules CD62E, ICAM-1, and VCAM-1. Exposure of endothelium to a serogroup B strain of *Neisseria meningitidis*, B1940, and a range of isogenic mutants revealed that lipooligosaccharide (LOS) structure and capsulation influence the expression of adhesion molecules. Following only a brief exposure (15 min) to the bacteria, there were large differences in the capacity of the different mutants to induce vascular cell adhesion molecules, with the unencapsulated and truncated LOS strains being most potent ($P < 0.05$). Furthermore, the pattern of cell adhesion molecule expression was different with purified endotoxin from that with intact bacteria. Meningococci were more potent stimuli of CD62E expression than was endotoxin, whereas endotoxin was at least as effective as meningococci in inducing ICAM-1 and VCAM-1. The effect of bactericidal/permeability increasing protein ($rBPI_{21}$), an antibacterial molecule with antiendotoxin properties, was also dependent on LOS structure. The strains which possessed a truncated or nonsialylated LOS, whether capsulated or not, were more sensitive to the inhibitory effects of $rBPI_{21}$. These findings could have important implications for the use of antiendotoxin therapy in meningococcal disease.

Infections caused by *Neisseria meningitidis* remain an important cause of mortality and morbidity worldwide (14). It is the organism responsible for the majority of childhood cases of bacterial meningitis in the United Kingdom, and in patients presenting with severe shock, mortality may be as high as 50%. Although prompt recognition, early treatment and intensive care has reduced this figure in recent years (18), survivors may have extensive tissue injury, sometimes requiring amputation and/or skin grafting.

Capillary leak and intravascular thrombosis are serious consequences of meningococcal sepsis and are indicative of widespread vascular endothelial injury (23). Histological studies of meningococcal disease show that cutaneous lesions contain large numbers of organisms that are associated with the vascular endothelium (11). Recent studies have also shown that meningococci have the capacity to bind endothelial cells in a receptor-ligand-specific fashion (33, 34) and indicate that bacterium-endothelium contact may itself be critical in mediating the vascular injury seen in this disease (29). There is evidence that meningococci, both alone and in the presence of neutrophils, can lead to endothelium damage (20, 32). However, there is still very little information on how meningococci may themselves modulate the influx of neutrophils into inflammatory sites.

Expression of adhesion molecules by the vascular endothelium is a critical step in the inflammatory response. Leukocyte adhesion occurs through a complex and multistep process involving initial tethering and then rolling of leukocytes by low-avidity interactions with mainly the selectin family of cell adhesion molecules (e.g., CD62E/E-selectin). This is followed by firmer adhesion, which is mediated largely by higher-affinity interactions involving the members of the immunoglobulin Ig superfamily (e.g., ICAM-1 and VCAM-1) on endothelial cells (30). After firm adhesion, transendothelial and subendothelial migration may occur, a process also involving leukocyte integrins and complex cross talk among leukocytes, the endothelium, cytokines, and chemokines. The initial inflammatory stimulus to this activation cascade is critical since it determines which leukocytes will participate in the subsequent inflammatory response (4). The pattern of endothelial activation seen in response to the proinflammatory cytokines tumor necrosis factor alpha, interleukin-1, and CD40 (17) and bacterial endotoxin has been described (5, 39). There is very limited information on the response of endothelial adhesion molecules to live meningococci.

We have previously shown that encapsulation and lipooligosaccharide (LOS) structure influence the host inflammatory response to *N. meningitidis* (20). In this study, we have used isogenic mutants of *N. meningitidis* B1940 to investigate the relationship between bacterial structure and the expression of

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adhesion molecules on cultured human endothelial cells. We have also explored the effect of human recombinant bacterial/permeability increasing protein ($rBPI_{21}$) (2) to modulate endothelial activation by these organisms.

MATERIALS AND METHODS

Bacterial strains. The parent organism, *N. meningitidis* B1940, and three isogenic mutants derived from it have been described previously (7). The capsule-deficient (*siaD*), mutant of B1940 was constructed by using insertional inactivation of the polysialyltransferase gene. Inactivation of the *galE* gene by replacement of the *cpsD* region with a chloramphenicol resistance marker produces a capsulated mutant that expresses a truncated LOS that cannot be sialylated. In the *cps* mutant, the whole *cps* gene complex is missing, and so it has both defective capsule expression and a truncated LOS. A fourth mutant, the *lst* mutant of B1940, has a deleted α-2,3-sialyltransferase gene and cannot sialylate terminal lacto-*N*-neotetraose of its LOS (36). The parent bacterium, B1940, and its derived mutants express pili and both Opa and Opc as indicated by electron microscopy and immunoreactivity with specific monoclonal antibodies (20).

Materials. $rBPI_{21}$, a recombinant, modified amino-terminal fragment of bactericidal/permeability increasing factor, was a kind gift from XOMA (US) LLC, Berkeley, Calif. *Escherichia coli* lipopolysaccharide (LPS) (protein content, <1%), serotype O111:B4, was purchased from Sigma, Poole, United Kingdom. LOS from *N. meningitidis* serogroup B (strain 44/76) was prepared as previously described (1). Briefly, LPS was extracted by hot aqueous phenol extraction, ultracentrifugation, gel filtration, and cold-ethanol–NaCl precipitation (31). The final product contained <0.3% protein and was without detectable nucleic acids.

Bacterial culture. All the above strains were grown on gonococcal agar (Difco) supplemented with Vitox (Oxoid) and cultured in 6% CO_2 in air at 36°C. In all experiments, the bacteria used were subcultured at least once and were used after 18 h. Suspensions of bacteria were prepared in RPMI 1640 medium with no phenol red (Gibco, Paisley, United Kingdom), and their optical density was measured at 540 nm. Bacterial viability counts were measured by a modification of Miles and Misra technique (24). In some experiments, nonviable bacteria were used. These were killed either with 0.5% paraformaldehyde or by heating at 56°C for 30 min.

Endothelial-cell culture. Human umbilical vein endothelial cells (HUVEC) were obtained by collagenase type 2 (Gibco) digestion as described previously with some modifications (21). Cells in primary culture were grown in MCDB 131 medium (Gibco) supplemented with 2 mM L-glutamine, penicillin, streptomycin, and 20% heat-inactivated fetal calf serum (Gibco) in 25-cm^2 tissue culture flasks (Becton Dickinson, Oxford, United Kingdom). The cells were then passaged into 24-well plates, previously treated with endothelial attachment factor, by using trypsin-EDTA (Sigma). The cells were grown to confluence and then washed thoroughly, 24 to 48 h prior to experiments, in antibiotic-free RPMI 1640 medium supplemented with 25 mM HEPES buffer, 2 mM L-glutamine (Gibco), and 20% heat-inactivated fetal calf serum (Gibco).

Incubation of *N. meningitidis* with HUVEC. Initial experiments were conducted with either heat-inactivated or 0.5% paraformaldehyde-fixed bacteria. In subsequent experiments, HUVEC were stimulated with endotoxin or live meningococci and then incubated for up to 24 h at 37°C. Each well of confluent HUVEC contained approximately 10^5 cells, so that the ratio of bacteria to HUVEC ranged from 1:0.01 to 1:100. In some experiments, HUVEC were exposed to bacteria or LPS before being washed in fresh RPMI medium containing 20% fetal calf serum, and incubated for a further 5 h. The cells were then removed from culture plates by incubation in Puck's A saline followed by gentle mechanical scraping. Adhesion molecule expression was detected by incubation with mouse monoclonal antibodies to human ICAM-1, CD62E, and VCAM-1 (Serotec, Oxford, United Kingdom) followed by goat anti-mouse $F(ab')_2$-phycoerythrin conjugate (Dako, High Wycombe, United Kingdom). Nonspecific binding of antibodies was controlled for by inclusion of an irrelevant isotype-matched control (Dako). Samples were washed, resuspended in Cellfix (Becton Dickinson), and analyzed by flow cytometry (FACScalibur; Becton Dickinson) with Cell Quest software (Becton Dickinson). The endothelial-cell population was identified by its forward-scatter and side-scatter position and by expression of CD31 (Serotec). For each sample, 5,000 events were collected within the Endothelial gate.

Statistics. All experiments were done at least three times on independent primary cell cultures from different sources. Where statistical analysis is shown, the results are expressed as mean median fluorescence intensity and standard error of the mean. A comparison of ranking order of cellular adhesion molecule expression by parent and *cpsD*, *siaD*, and *cps* mutant strains was analyzed by a Kruskall-Wallis one-way analysis of variance.

RESULTS

HUVEC adhesion molecule expression induced by *N. meningitidis* B1940. Incubation of endothelial cells with the parent organism induced the expression of CD62E, VCAM-1, and ICAM-1. With 10^6 organisms per ml, CD62E expression was first detected at 2 h, reached maximal levels at 5 h, and diminished markedly by 24 h. VCAM-1 expression was not detected until 4 h, and it reached maximal levels between 12 and 18 h. Similar levels were still seen at 24 h. ICAM-1 was expressed on resting HUVEC, but the levels were seen to increase by 4 h and were still rising by 24 h. Similar levels and kinetics of these adhesion molecules were seen with 10 ng of *E. coli* LPS per ml.

Influence of capsulation and LOS structure on endothelial cell adhesion molecule expression. To examine the influence of capsulation and LOS structure on HUVEC adhesion molecule expression, HUVEC were exposed to the B1940 isogenic mutant organisms. When the endothelial cells were incubated with bacteria for 5 h, the magnitude of CD62E, ICAM-1, and VCAM-1 expression induced by the mutants was similar to that seen with the parent (capsulated, sialylated LOS) organism. However, when HUVEC were exposed to bacteria for shorter periods, differences in adhesion molecule expression became apparent. This was most marked after only 15 min of exposure. Under these conditions, the *siaD* (unencapsulated, sialylated LOS) and *cps* (unencapsulated, truncated LOS, nonsialylated) mutants consistently induced higher levels of all three adhesion molecules than did the parent. This was particularly marked for CD62E and VCAM-1 (Fig. 1). Expression induced by the *cpsD* (capsulated, truncated LOS, nonsialylated) organism was always higher than that induced by the parent but not usually to the levels observed with the *siaD* and *cps* mutants (Fig. 1). Interestingly, in separate experiments comparing the parent and the *cpsD* and *lst* (capsulated, nonsialylated LOS) mutants, the level of cell adhesion molecules seen with the *lst* organisms was between those seen with the parent and the *cpsD* mutant. Similar results were obtained when live meningococci were replaced with bacteria that were either heat inactivated or fixed in 0.5% paraformaldehyde (results not shown).

Once the patterns of adhesion molecule expression under these conditions had been established, the influence of bacterial concentration was investigated. Figure 2 shows the effect of bacterial concentration on CD62E expression. The pattern of adhesion molecule expression as shown in Fig. 1 was preserved at all the concentrations tested. There was a threshold bacterial concentration at which no adhesion molecule expression was observed. This differed between the parent and *siaD* mutant by at least 1 log unit in bacterial concentration (Fig. 2). Similar results were seen with ICAM-1 and VCAM-1 (results not shown).

***E. coli* LPS and meningococcal LOS cause a different pattern of adhesion molecule expression to *N. meningitidis* B1940 and isogenic mutants.** When HUVEC were incubated with purified *E. coli* LPS or meningococcal LOS, the profiles of CD62E, ICAM-1, and VCAM-1 expression observed by flow cytometry were similar (Fig. 3). In comparison, *N. meningitidis* induced greater expression of CD62E than did purified endotoxin from either bacterial species, even when very high endotoxin doses (100 ng/ml) were used. Endotoxin from either source was at least as effective at up-regulating ICAM-1 or VCAM-1 as were the bacteria (Fig. 3).

Results shown in Fig. 1 demonstrated that a short exposure of the HUVEC to unencapsulated *siaD* and *cps* meningococcal mutants induced higher levels of CD62E expression than did continuous exposure to purified endotoxin, whereas endotoxin induced higher levels of ICAM-1 and VCAM-1 expression. To investigate this further, HUVEC were incubated with either *E. coli* LPS or the *N. meningitidis* B1940 parent or *siaD* mutant for increasing lengths of time, ranging from 1 min to 3 h, before being washed thoroughly to remove free LPS and/or nonadherent organisms. Cultures were incubated for a further 5 h,

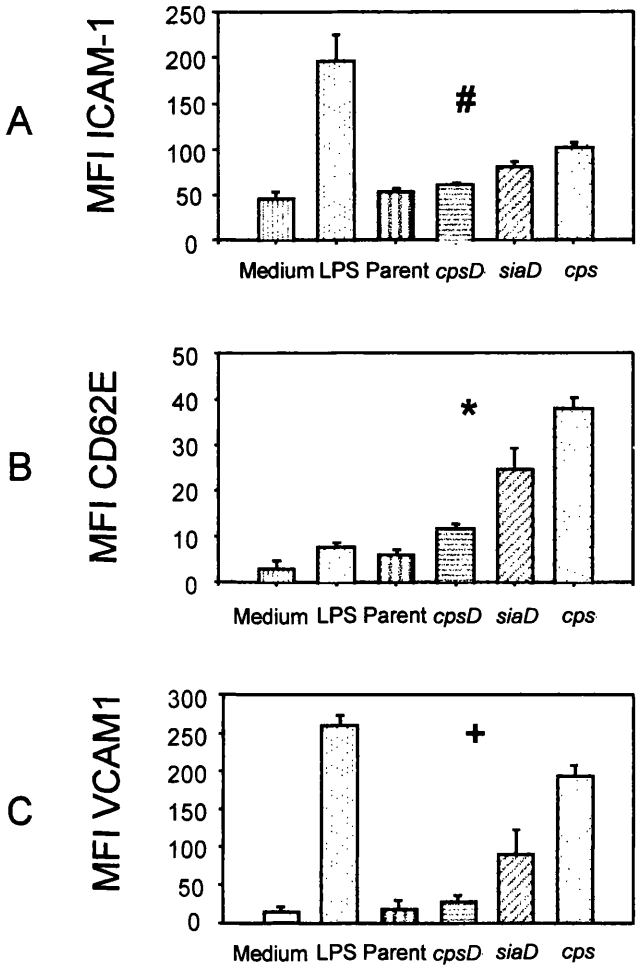

FIG. 1. Expression of ICAM-1 (A), E-selectin (B), and VCAM-1 (C) on HUVEC at 5 h after incubation with the *N. meningitidis* parent strain and *cps*, *siaD*, and *cpsD* mutants at 10^6 organisms per ml. HUVEC were washed thoroughly in fresh medium after 15 min of incubation with organisms. *E. coli* LPS was added at 10 ng per ml, and the cells were left unwashed. Results here are expressed as means and standard errors of the mean. #, $P = 0.03$; *, $P = 0.015$; +, $P = 0.045$.

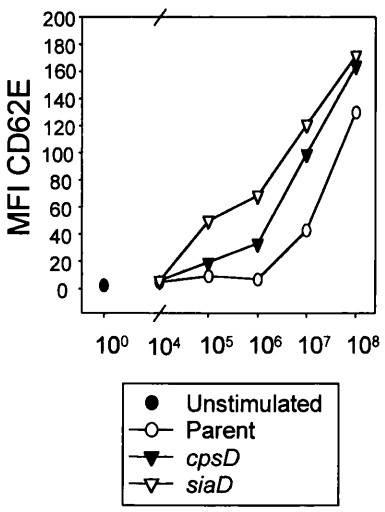

FIG. 2. Differential induction of CD62E on HUVEC by the *N. meningitidis* B1940 parent and the *cpsD* and *siaD* mutants occurs over a range of bacterial concentrations. Bacteria were added at 10^8 to 10^4 organisms per ml. After 15 min of incubation, HUVEC were washed thoroughly in fresh medium and incubated for a further 5 h. CD62E expression was determined at 5 h by flow cytometry. This is representative of at least three experiments yielding similar results.

after which time adhesion molecule expression was measured by flow cytometry. There was minimal induction of CD62E expression on HUVEC exposed to LPS for less than 15 min (Fig. 4A). For exposures of 15 min or more, CD62E expression could be detected. Similar results were obtained with ICAM-1 and VCAM-1 (data not shown). Increasing the duration of HUVEC exposure to LPS also increased the level of adhesion molecule expression, so that maximal expression was seen at 5 h. When the kinetics of adhesion molecule expression were examined by using the parent organism, the profiles were similar to that seen with LPS (Fig. 4B). The pattern of expression was very different when the experiments were performed with the *siaD* mutant. CD62E expression was detected after only a 5-min exposure (Fig. 4B), reached a peak at 60 min, and declined progressively by 5 h. Similar differences were observed with ICAM-1 and VCAM-1 (results not shown).

Bacterial concentration, LOS structure, and capsulation influence the inhibitory effect of $rBPI_{21}$ on the induction of HUVEC adhesion molecule expression by meningococci. The effects of the endotoxin antagonist $rBPI_{21}$ on the induction of HUVEC adhesion molecule expression were investigated. When 10 μg of rBPI per ml was added prior to or at the same time as 100 ng of *E. coli* LPS per ml, adhesion molecule expression was completely inhibited (Fig. 5). However, when $rBPI_{21}$ was added to meningococci, the pattern of cell adhesion molecule expression was more complicated. First, the bacterial concentration was found to be important. When 10 μg of $rBPI_{21}$ per ml was added to either the parent B1940 or *siaD* mutant at 10^4 organisms/ml, there was a marked reduction in adhesion molecule expression. However, when the bacterium was present at 10^5 organisms/ml, inhibition was only between 20 and 50%. Little effect was observed at 10^6 organisms/ml (Fig. 6). Second, LOS structure and encapsulation also influenced the efficacy of $rBPI_{21}$. When 10^5 organisms of *N. meningitidis* B1940 or the isogenic mutants per ml were incubated with $rBPI_{21}$, the levels of CD62E expression were more efficiently inhibited for the *cps* and *cpsD* mutants than for either the parent or *siaD* mutant (Fig. 7A). In separate experiments, the level of inhibition seen with the *lst* mutant was similar to that observed with the *cps* and *cpsD* mutants (Fig. 7B), indicating that LOS sialylation may be the major determinant of this effect. $rBPI_{21}$-mediated inhibition of either ICAM-1 or VCAM-1 up-regulation by meningococci was similar to that seen with CD62E expression (results not shown).

The influence of $rBPI_{21}$ on induction of HUVEC adhesion molecule expression after exposure to *N. meningitidis* or endotoxin. The capacity of $rBPI_{21}$ to influence adhesion molecule expression after bacterial exposure was investigated. HUVEC were exposed to either the parent (nonadherent) or the *siaD* mutant (adherent) for 15 min before washing. $rBPI_{21}$ was then added at 15, 60, and 120 min after bacterial exposure. A reduction of between 40 and 60% in the expression of CD62E was observed when $rBPI_{21}$ was added after a 15-min exposure to the bacteria (Fig. 8). This level of inhibition was similar even if $rBPI_{21}$ was added at 60 or 120 min. While the *siaD* mutant induced higher levels of CD62E than the parent organism, the relative effect of $rBPI_{21}$ was similar for both organisms.

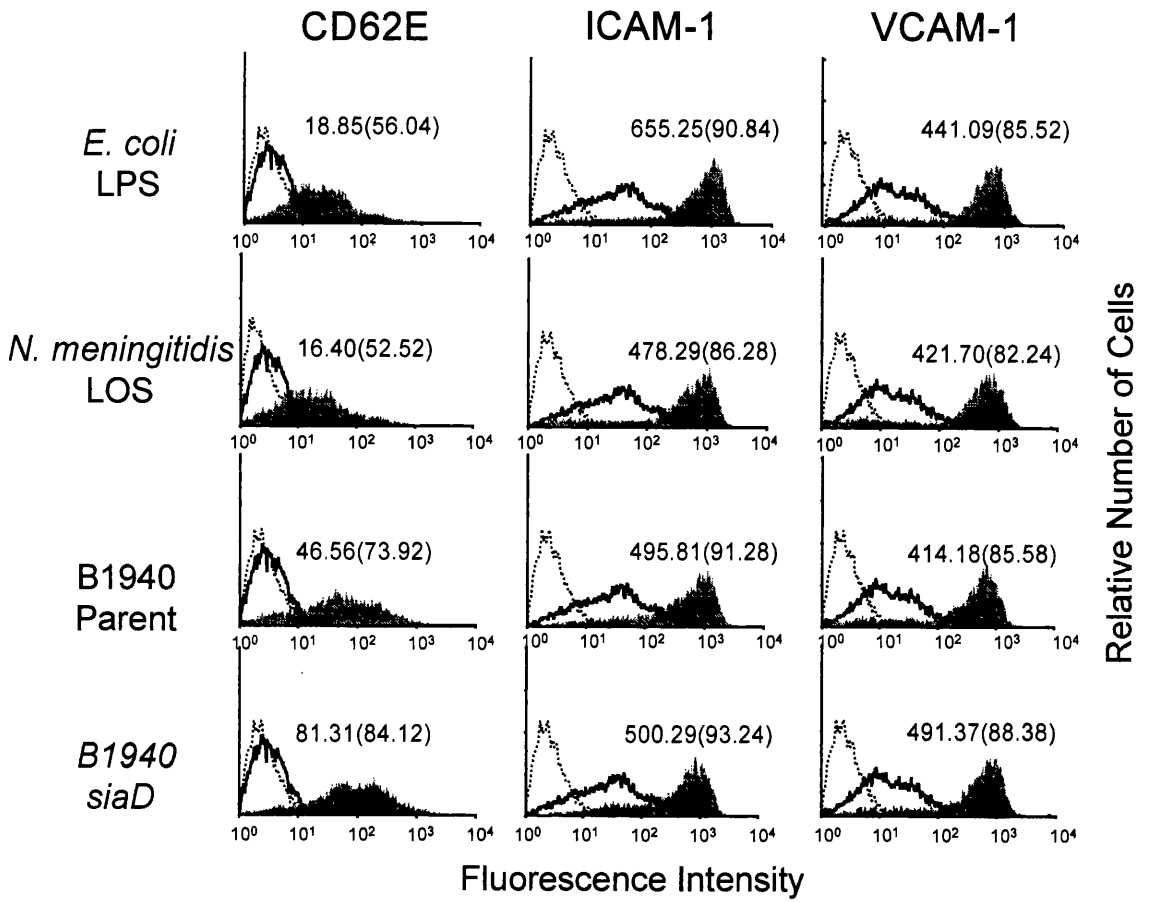

FIG. 3. Flow cytometric analysis of expression of CD62E, ICAM-1, and VCAM-1 on HUVEC following 5 h of incubation with 100 ng of *E. coli* O111:B4 LPS, 100 ng of meningococcal LOS, and 10^7 *N. meningitidis* B1940 parent and *siaD* mutant organisms per ml. Shaded areas represent CD62E, ICAM-1, and VCAM-1 staining in response to stimuli; the solid line represents cell adhesion molecule staining in unstimulated cells; and the dotted line represents staining with an irrelevant mouse immunoglobulin G1 antibody. Numbers on each plot are median fluorescent intensities and percent positive events for stimulated cells. Data presented here is representative of three experiments yielding similar results.

DISCUSSION

The nature and degree of the host inflammatory response to *N. meningitidis* may determine the fate of an infected individual. While host factors such as cytokine gene polymorphisms are likely to be determinants of this response, there is increasing recognition that bacterial properties are also important. This study provides evidence that bacterial composition can influence host endothelial cell responses. In view of the importance of vascular injury in this condition, these findings may be pertinent to understanding the pathophysiology of meningococcal disease.

In this study, we have used HUVEC to investigate the expression of adhesion molecules in response to *N. meningitidis*. We found this organism to be a potent inducer of the major vascular endothelial cell adhesion molecules, CD62E, ICAM-1, and VCAM-1, even after only limited periods of exposure. Our previous studies have shown that meningococci also markedly enhance the expression of the β_2-integrin CD11b/CD18 and diminish the expression of the selectin CD62L on neutrophils (20). Since these counterreceptors on both cell types are responsible for neutrophil adhesion and transmigration through vascular endothelia, these findings provide a mechanism to account for the presence of neutrophils within meningococcal lesions (29).

Capsulation and LOS structure have been shown to be determinants of meningococcal survival in human serum, in whole blood, and in the infant-rat model of meningococcal disease (16, 36–38). We have been able to show that these bacterial properties can also influence the degree and pattern of endothelial adhesion molecule expression. Interestingly, the organisms that induced the highest levels of adhesion molecules in this study were also those that we have previously shown to cause the largest reduction in neutrophil CD62L expression (20). These experiments were performed with whole blood and appeared to be related to the degree of bacterial killing. In the present study and in a previous study of meningococcal induction of endothelial cell tissue factor (10), bacterial killing was not the explanation for the differing effects of LOS structure and capsulation on endothelial cell activation. The reasons for the influence of these bacterial structures on endothelial cell activation are complex.

Capsulation is an important determinant of B1940 adhesion to HUVEC. Capsule-deficient mutants are more adherent to endothelial cells than are the capsulated parent strain and the nonsialylated, capsulated organism, B1940 *cpsD* (20). Possession of a truncated, nonsialylated LOS also influences bacterial adhesion to HUVEC, as indicated by enhanced binding of the B1940 *cps* mutant compared to the B1940 *siaD* mutant. In this study we show that adhesion molecule expression is directly correlated with the adherence of these bacteria to endothelial

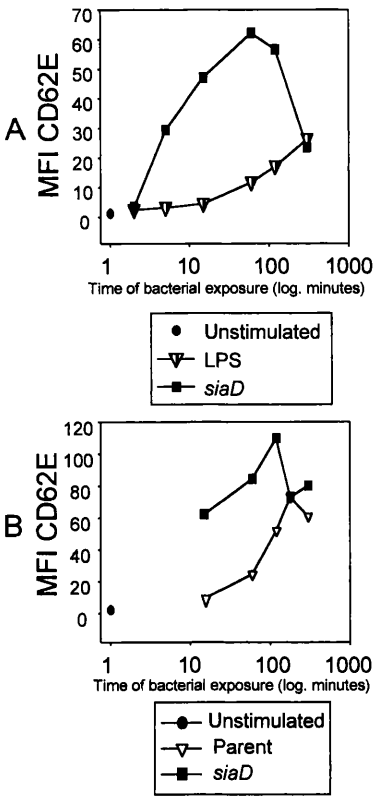

FIG. 4. Brief exposure of the unencapsulated *siaD* mutant to HUVEC is a potent inducer of expression of CD62E. (A) *N. meningitidis* B1940 *siaD* mutant at 10^6 organisms per ml or 10 ng of *E. coli* LPS was added to HUVEC, which were then washed thoroughly with fresh medium at 5, 15, 60, 120, and 240 min. (B) *N. meningitidis* B1940 *siaD* and parent organisms were incubated at 10^6 organisms per ml and washed at 15, 60, 120, and 240 min. CD62E expression was determined by flow cytometry after 5 h of incubation. The results shown here are representative experiments from at least three separate experiments that yielded similar results.

cells, which indicates that the adhesive capacity of *N. meningitidis* is likely to be a major factor in determining endothelial adhesion molecule expression.

One interpretation of our data is that the more adherent organisms effectively provide a higher dose of endotoxin to the endothelial cells. This appeared to be the explanation for the enhanced tissue factor expression seen in response to the *siaD* mutant compared to the parent strain (10). However, the dose of endotoxin does not fully account for the differences in adhesion molecule expression seen in this study. Meningococci, especially the unencapsulated *cps* and *siaD* strains, were more potent inducers of CD62E expression on HUVEC than were high doses of endotoxin. Indeed, the level of CD62E caused by these unencapsulated mutants after only 15 min of exposure was higher than that seen when HUVEC were continually exposed to endotoxin for a full 5 h. This was not observed with either ICAM-1 or VCAM-1, where endotoxin was at least as effective as bacteria at inducing the expression of these two cell adhesion molecules. Our results show that this differential activation is true for both purified, smooth *E. coli* O111:B4 endotoxin and purified group B meningococcal endotoxin, indicating that this effect is not simply due to structural differences between the endotoxins from the two sources. Why this differential activation should occur is not clear, but it does suggest that the dose of endotoxin may not be the sole determinant of vascular endothelial adhesion molecule expression.

There are a number of bacterial factors that contribute to the attachment of meningococci to endothelial cells (35). Pili appear to be important for initial endothelial-cell adhesion of encapsulated organisms, while firm attachment may be mediated by the outer membrane proteins Opa and Opc, particularly in unencapsulated strains (33). These bacterial components that mediate attachment to and invasion of host cells may also cause activation of important signal transduction pathways involved in transcriptional regulation of genes in the inflammatory response. It has been shown that both pili and Opa from *N. gonorrhoeae* can activate epithelial cells via the transcription factors NF-κB and AP-1 to produce inflammatory cytokines independently of endotoxin (26). These studies also showed no differences in NF-κB activation between invading and noninvading strains of *N. gonorrhoeae* (25). If this were the case, it would provide a putative mechanism by which the more strongly adherent organisms were also capable of inducing the highest levels of adhesion molecule expression. This is presumably because, in unencapsulated strains, there may be enhanced interaction between opacity proteins and their ligands. It may also explain the differential patterns of CD62E, ICAM-1, and VCAM-1 seen in response to the bacteria and purified endotoxin. It has been demonstrated recently that invasive strains of *N. gonorrhoeae* cause greater expression of epithelial ICAM-1 than do noninvasive strains. Interestingly, this was not associated with an increase in transcription of ICAM-1 mRNA, indicating that ICAM-1 expression can be modulated by bacteria at the translational or post-translational level (13). We suggest that there are multiple signalling mechanisms that occur when meningococci interact with vascular endothelial cells. Variations in bacterial structure could affect these mechanisms. We are currently undertaking studies to investigate the signal transduction pathways induced in activation of endothelial cells by the meningococcal mutants.

A further explanation for our results comes from the recent discovery of human Toll-like receptors that transduce signals in response to endotoxin in both $CD14^+$ and CD14− cells (43). There are at least five human homologues of the Toll receptor, and two of these, TLR2 and TLR4, transduce endo-

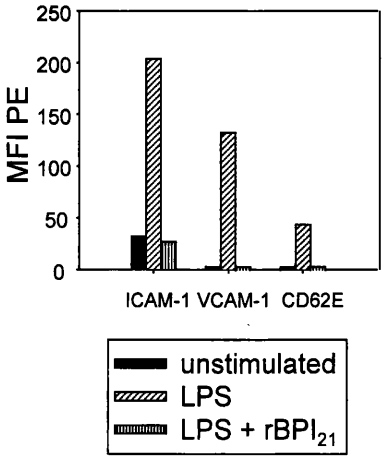

FIG. 5. Effect of $rBPI_{21}$ on *E. coli* O111:B4 LPS-induced HUVEC expression of CD62E, ICAM-1, and VCAM-1. *E. coli* LPS (100 ng/ml) was added to HUVEC preincubated with 10 μg of $rBPI_{21}$ per ml. Cell adhesion molecule determination was determined by flow cytometry after 5 h of incubation. The results shown are representative of multiple experiments with same level of inhibition.

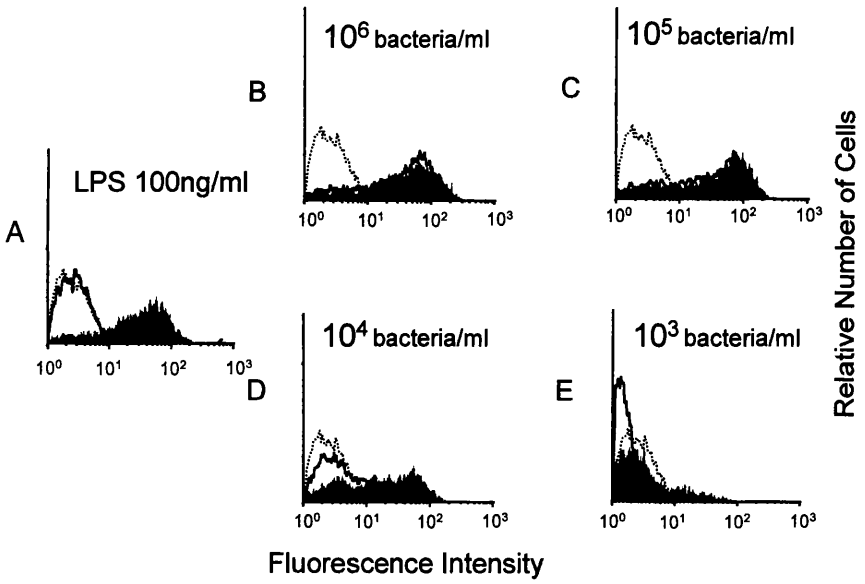

FIG. 6. Effect of $rBPI_{21}$ on HUVEC expression of CD62E by various doses of the *N. meningitidis* B1940 *siaD* mutant. $rBPI_{21}$ (10 μg/ml) or medium was added to HUVEC, and then either 100 ng of *E. coli* O111:B4 LPS or various concentrations of bacteria were added as shown. CD62E expression was determined after 5 h. The data presented here are histograms of fluorescence intensity of phycoerythrin fluorochrome plotted against the number of events. The dotted line represents resting CD62E expression; the shaded area represents CD62E expression with LPS or bacteria; and the continuous black line represents CD62E expression when LPS or bacteria were preincubated with $rBPI_{21}$.

toxin signals (28). The demonstration of these receptors has highlighted the potential complexity of endotoxin signalling to host cells. Initial findings indicate that different Toll receptors may have different properties and may relate to observed differences in signal transduction (19). It is becoming clear that the location and form of endotoxin are central to how it interacts with host endotoxin recognition molecules (42). This may be critical in how host cells interact with whole bacteria such as *N. meningitidis*. Our results demonstrate that LOS structure is an important factor in determining the pattern of vascular endothelial adhesion molecule expression. The *cpsD* mutant, which possesses a truncated LOS, and the *lst* mutant, which lacks just the terminal sialic acid of the α-oligosaccharide chain, induced higher levels of adhesion molecules than did the parent organism. This indicates that even subtle changes in LOS structure can influence endothelial-cell activation, possibly by influencing the interactions between the bacteria and endothelial-cell endotoxin recognition receptors.

In the light of these findings, we investigated the capacity of $rBPI_{21}$, a recombinant form of BPI, a host defense protein with antibacterial and antiendotoxin activities, to modulate the endothelial adhesion molecule response to these different bacterial strains. $rBPI_{21}$ kills gram-negative bacteria and decreases tumor necrosis factor alpha production induced by gram-negative bacteria or LPS in whole blood (41). $rBPI_{21}$ also binds to purified endotoxin and abrogates the degree of endothelial-cell activation (2). In our study, we found that 10 μg of $rBPI_{21}$ per ml could completely abolish the induction of cell adhesion molecules on HUVEC when given prior to or very early following exposure to high doses of purified *E. coli* endotoxin.

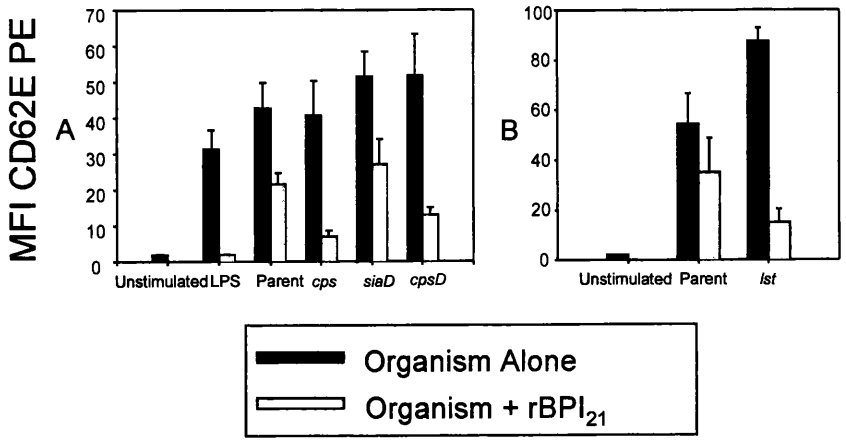

FIG. 7. Effect of $rBPI_{21}$ on induction of CD62E expression on HUVEC in response to *N. meningitidis* B1940 parent and *cps*, *siaD*, and *cpsD* mutants (A) and B1940 parent and the *lst* mutant (7B). HUVEC were pre-incubated with 10 μg of $rBPI_{21}$ per ml, and then 10^5 organisms/ml were added. CD62E expression was measured after 5 h by flow cytometry. The results shown are the mean median fluorescence intensity (MFI) and standard error of the mean from three separate experiments.

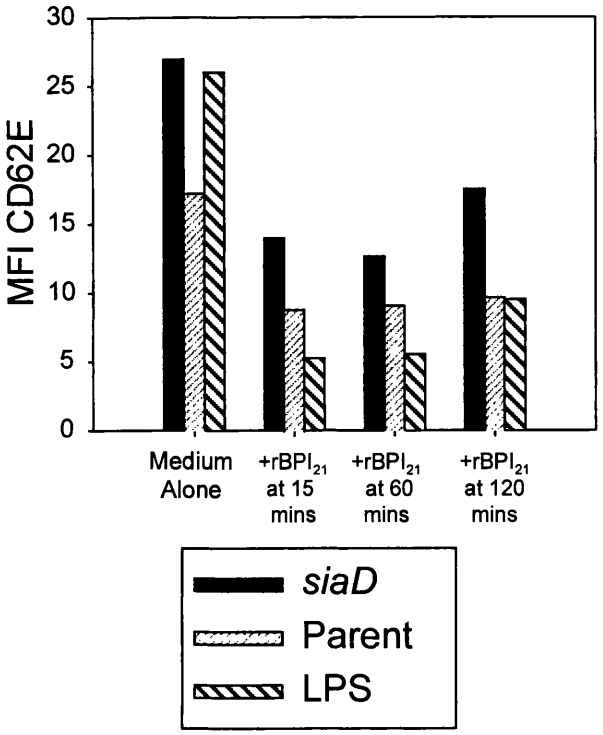

FIG. 8. Effect of time exposure of $rBPI_{21}$ on induction of CD62E on HUVEC after 5 h of stimulation with 10 ng of *E. coli* O111:B4 LPS or *N. meningitidis* B1940 parent and *siaD* mutant strains. Bacteria were added at 10^6 per ml and washed off after 15 min. Then 5 μg of $rBPI_{21}$ was added at 15, 60, and 120 min. *E. coli* O111:B4 LPS (10 ng) was added, and 5 μg of $rBPI_{21}$ was added at the same time points. The results shown are a representative of three experiments with similar results.

However, the pattern seen with organisms was more complex. Our results show that the effectiveness of $rBPI_{21}$ in blocking the induction of cell adhesion molecules by bacteria was dependent not only on the dose of infecting bacteria but also on the structure of the LOS; in particular, LOS sialylation may influence $rBPI_{21}$ activity. Previous studies have shown that BPI and its N-terminal fragment bind to the lipid A portion of endotoxin, resulting in endotoxin neutralization (6, 22). How LOS sialylation could influence $rBPI_{21}$ binding to the lipid A component of LPS is not known. Structure of polysaccharide can have an effect on the inhibitory effects of $rBPI_{21}$ on some LPSs (3). It has been demonstrated that mutants of *E. coli* that have incomplete LPS are much more sensitive to bactericidal permeability-increasing protein than are *E. coli* strains with intact LPS. It has been proposed that charged sugar moieties could impede the action of cationic proteins like BPI (40). Our results indicate that just the presence of sialic acid on the terminal lacto-*N*-neotetraose is sufficient to influence the inhibitory effects of BPI. This is presumably due to the effect of sialic acid on LOS-BPI interactions. This could be important in vivo, since most pathogenic meningococci have LOS structures (immunotypes) that can be sialylated (15). These findings may also indicate that the dose of rBPI required to inhibit the inflammatory effects of gram-negative bacteria may have to be optimized for different bacteria strains.

One of the disappointing features of most anti-inflammatory agents is that they are effective only when administered prior to or at the same time as the inflammatory stimulus. Our results indicate that this was not the case with $rBPI_{21}$ in vitro. We found that $rBPI_{21}$ could influence adhesion molecule expression even when added several hours after the organisms. This was true whether the organism was adherent or nonadherent. These results are consistent with previous studies involving *E. coli* LPS, which have shown that $rBPI_{21}$ can reverse the LPS-induced expression of CD62E (E-selectin) after several hours of continuous exposure (12).

Taken together, these results indicate that LOS structure and the presence of a capsule can influence the level, kinetics, and profile of endothelial adhesion molecule expression induced by *N. meningitidis*. The apparent ability of pathogenic *Neisseria* strains to down-regulate their capsule and desialylate the LOS in natural meningococcal infections would indicate that the endothelial-cell response to invading organisms may be variable even for a single strain (8, 9). It would appear that even limited exposure to some organisms might be able to induce a rapid influx of inflammatory cells. This may be beneficial in killing invading organisms but could influence the degree and extent of endothelial cell injury from activated inflammatory cells (27). Strategies to combat meningococcal disease should take into account the multiple signalling pathways that may be activated during the course of this potentially fatal disease.

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REFERENCES

1. **Andersen, S. R., K. Bry, K. Thorseng, and E. Jantzen.** 1996. Heterogeneity of lipopolysaccharides of *Neisseria meningitidis* revealed by thin-layer chromatography combined with monoclonal antibodies. J. Microbiol. Methods **25**:187.
2. **Arditi, M., J. Zhou, S. H. Huang, P. M. Luckett, M. N. Marra, and K. S. Kim.** 1994. Bactericidal/permeability-increasing protein protects vascular endothelial cells from lipopolysaccharide-induced activation and injury. Infect. Immun. **62**:3930–3936.
3. **Capodici, C., S. Chen, Z. Sidorczyk, P. Elsbach, and J. Weiss.** 1994. Effect of lipopolysaccharide (LPS) chain length on interactions of bactericidal/permeability-increasing protein and its bioactive 23-kilodalton NH_2-terminal fragment with isolated LPS and intact *Proteus mirabilis* and *Escherichia coli*. Infect. Immun. **62**:259–265.
4. **Carlos, T. M., and J. M. Harlan.** 1994. Leukocyte-endothelial adhesion molecules. Blood **84**:2068–2101.
5. **Dustin, M. L., and T. A. Springer.** 1988. Lymphocyte function-associated antigen-1 (LFA-1) interaction with intercellular adhesion molecule-1 (ICAM-1) is one of at least three mechanisms for lymphocyte adhesion to cultured endothelial cells. J. Cell Biol. **107**:321–331.
6. **Gazzano-Santoro, H., J. B. Parent, L. Grinna, A. Horwitz, T. Parsons, G. Theofan, P. Elsbach, J. Weiss, and P. J. Conlon.** 1992. High-affinity binding of the bactericidal/permeability-increasing protein and a recombinant amino-terminal fragment to the lipid A region of lipopolysaccharide. Infect. Immun. **60**:4754–4761.
7. **Hammerschmidt, S., C. Birkholz, U. Zahringer, B. D. Robertson, J. van Putten, O. Ebeling, and M. Frosch.** 1994. Contribution of genes from the capsule gene complex (cps) to lipooligosaccharide biosynthesis and serum resistance in *Neisseria meningitidis*. Mol. Microbiol. **11**:885–896.
8. **Hammerschmidt, S., R. Hilse, J. P. van Putten, R. Gerardy-Schahn, A. Unkmeir, and M. Frosch.** 1996. Modulation of cell surface sialic acid expression in *Neisseria meningitidis* via a transposable genetic element. EMBO J. **15**:192–198.
9. **Hammerschmidt, S., A. Muller, H. Sillmann, M. Muhlenhoff, R. Borrow, A. Fox, J. van Putten, W. D. Zollinger, R. Gerardy-Schahn, and M. Frosch.** 1996. Capsule phase variation in *Neisseria meningitidis* serogroup B by slipped-strand mispairing in the polysialyltransferase gene (siaD): correlation with bacterial invasion and the outbreak of meningococcal disease. Mol. Microbiol. **20**:1211–1220.
10. **Heyderman, R. S., N. J. Klein, O. A. Daramola, S. Hammerschmidt, M. Frosch, B. D. Robertson, M. Levin, and C. A. Ison.** 1997. Induction of human

endothelial tissue factor expression by *Neisseria meningitidis*: the influence of bacterial killing and adherence to the endothelium. Microb. Pathog. **22:**265–276.
11. **Hill, W. R., and T. D. Kinney.** 1947. The cutaneous lesions in acute meningococcaemia. JAMA **134:**513.
12. **Huang, K., D. M. Fishwild, H. M. Wu, and R. L. Dedrick.** 1995. Lipopolysaccharide-induced E-selectin expression requires continuous presence of LPS and is inhibited by bactericidal/permeability-increasing protein. Inflammation **19:**389–404.
13. **Jarvis, G. A., J. Li, and K. Swanson.** 1999. Invasion of human mucosal epithelial cells by *Neisseria gonorrhoeae* upregulates expression of intercellular adhesion molecule 1 (ICAM-1). Infect. Immun. **67:**1149–1156.
14. **Jones, D. M.** 1995. Epidemiology of meningococcal disease in Europe and the USA, p. 71–114. *In* K. Cartwright (ed.), Meningococcal disease. John Wiley & Sons Ltd., Chichester, United Kingdom.
15. **Jones, D. M., R. Borrow, A. J. Fox, S. Gray, K. A. Cartwright, and J. T. Poolman.** 1992. The lipooligosaccharide immunotype as a virulence determinant in *Neisseria meningitidis*. Microb. Pathog. **13:**219–224.
16. **Kahler, C. M., L. E. Martin, G. C. Shih, M. M. Rahman, R. W. Carlson, and D. S. Stephens.** 1998. The (alpha2→8)-linked polysialic acid capsule and lipooligosaccharide structure both contribute to the ability of serogroup B *Neisseria meningitidis* to resist the bactericidal activity of normal human serum. Infect. Immun. **66:**5939–5947.
17. **Karmann, K., W. Min, W. C. Fanslow, and J. S. Pober.** 1996. Activation and homologous desensitization of human endothelial cells by CD40 ligand, tumor necrosis factor, and interleukin 1. J. Exp. Med. **184:**173–182.
18. **Kirsch, E. A., R. P. Barton, L. Kitchen, and B. P. Giroir.** 1996. Pathophysiology, treatment and outcome of meningococcemia: a review and recent experience. Pediatr. Infect. Dis. J. **15:**967–978.
19. **Kirschning, C. J., H. Wesche, A. T. Merrill, and M. Rothe.** 1998. Human toll-like receptor 2 confers responsiveness to bacterial lipopolysaccharide. J. Exp. Med. **188:**2091–2097.
20. **Klein, N. J., C. A. Ison, M. Peakman, M. Levin, S. Hammerschmidt, M. Frosch, and R. S. Heyderman.** 1996. The influence of capsulation and lipooligosaccharide structure on neutrophil adhesion molecule expression and endothelial injury by *Neisseria meningitidis*. J. Infect. Dis. **173:**172–179.
21. **Kotowicz, K., R. E. Callard, K. Friedrich, D. J. Matthews, and N. Klein.** 1996. Biological activity of IL-4 and IL-13 on human endothelial cells: functional evidence that both cytokines act through the same receptor. Int. Immunol. **8:**1915–1925.
22. **Marra, M. N., C. G. Wilde, J. E. Griffith, J. L. Snable, and R. W. Scott.** 1990. Bactericidal/permeability-increasing protein has endotoxin-neutralizing activity. J. Immunol. **144:**662–666.
23. **Mercier, J. C., F. Beaufils, J. F. Hartmann, and D. Azema.** 1988. Hemodynamic patterns of meningococcal shock in children. Crit. Care Med. **16:**27–33.
24. **Miles, A. A., S. S. Misia, and J. O. Irwin.** 1932. The estimation of the bactericidal power of blood. Journal Hyg. Camb. **38:**732.
25. **Naumann, M., T. Rudel, B. Wieland, C. Bartsch, and T. F. Meyer.** 1998. Coordinate activation of activator protein 1 and inflammatory cytokines in response to *Neisseria gonorrhoeae* epithelial cell contact involves stress response kinases. J. Exp. Med. **188:**1277–1286.
26. **Naumann, M., S. Wessler, C. Bartsch, B. Wieland, and T. F. Meyer.** 1997. *Neisseria gonorrhoeae* epithelial cell interaction leads to the activation of the transcription factors nuclear factor kappaB and activator protein 1 and the induction of inflammatory cytokines. J. Exp. Med. **186:**247–258.
27. **Richter, J., I. Olsson, and T. Andersson.** 1990. Correlation between spontaneous oscillations of cytosolic free Ca^{2+} and tumor necrosis factor-induced degranulation in adherent human neutrophils. J. Biol. Chem. **265:**14358–14363.
28. **Rock, F. L., G. Hardiman, J. C. Timans, R. A. Kastelein, and J. F. Bazan.** 1998. A family of human receptors structurally related to *Drosophila* Toll. Proc. Natl. Acad. Sci. USA **95:**588–593.
29. **Sotto, M. N., B. Langer, S. S. Hoshino, and T. de Brito.** 1976. Pathogenesis of cutaneous lesions in acute meningococcemia in humans: light, immunofluorescent, and electron microscopic studies of skin biopsy specimens. J. Infect. Dis. **133:**506–514.
30. **Springer, T. A.** 1994. Traffic signals for lymphocyte recirculation and leukocyte migration: the multistep paradigm. Cell **76:**301–314.
31. **Tsai, C. M., C. E. Frasch, E. Rivera, and H. D. Hochstein.** 1989. Measurement of lipopolysaccharide (endotoxin) in meningococcal protein and polysaccharide preparations for vaccines usage. J. Biol. Stand. **17:**249–258.
32. **Virji, M., H. Kayhty, D. J. Ferguson, C. Alexandrescu, J. E. Heckels, and E. R. Moxon.** 1991. The role of pili in the interactions of pathogenic *Neisseria* with cultured human endothelial cells. Mol. Microbiol. **5:**1831–1841.
33. **Virji, M., K. Makepeace, D. J. Ferguson, M. Achtman, and E. R. Moxon.** 1993. Meningococcal Opa and Opc proteins: their role in colonization and invasion of human epithelial and endothelial cells. Mol. Microbiol. **10:**499–510.
34. **Virji, M., K. Makepeace, D. J. Ferguson, M. Achtman, J. Sarkari, and E. R. Moxon.** 1992. Expression of the Opc protein correlates with invasion of epithelial and endothelial cells by *Neisseria meningitidis*. Mol. Microbiol. **6:**2785–2795.
35. **Virji, M., K. Makepeace, I. R. Peak, D. J. Ferguson, M. P. Jennings, and E. R. Moxon.** 1995. Opc- and pilus-dependent interactions of meningococci with human endothelial cells: molecular mechanisms and modulation by surface polysaccharides. Mol. Microbiol. **18:**741–754.
36. **Vogel, U., H. Claus, G. Heinze, and M. Frosch.** 1997. Functional characterization of an isogenic meningococcal alpha-2,3-sialyltransferase mutant: the role of lipooligosaccharide sialylation for serum resistance in serogroup B meningococci. Med. Microbiol. Immunol. Berlin **186:**159–166.
37. **Vogel, U., H. Claus, G. Heinze, and M. Frosch.** 1999. Role of lipopolysaccharide sialylation in serum resistance of serogroup B and C meningococcal disease isolates. Infect. Immun. **67:**954–957.
38. **Vogel, U., S. Hammerschmidt, and M. Frosch.** 1996. Sialic acids of both the capsule and the sialylated lipooligosaccharide of *Neisseria meningitidis* serogroup B are prerequisites for virulence of meningococci in the infant rat. Med. Microbiol. Immunol. Berlin **185:**81–87.
39. **von Asmuth, E. J., M. A. Dentener, V. Bazil, M. G. Bouma, J. F. Leeuwenberg, and W. A. Buurman.** 1993. Anti-CD14 antibodies reduce responses of cultured human endothelial cells to endotoxin. Immunology **80:**78–83.
40. **Weiss, J., Q. S. Beckerdite, and P. Elsbach.** 1980. Resistance of gram-negative bacteria to purified bactericidal leukocyte proteins: relation to binding and bacterial lipopolysaccharide structure. J. Clin. Investig. **65:**619–628.
41. **Weiss, J., P. Elsbach, C. Shu, J. Castillo, L. Grinna, A. Horwitz, and G. Theofan.** 1992. Human bactericidal/permeability-increasing protein and a recombinant NH_2-terminal fragment cause killing of serum-resistant gram-negative bacteria in whole blood and inhibit tumor necrosis factor release induced by the bacteria. J. Clin. Investig. **90:**1122–1130.
42. **Wright, S. D.** 1999. Toll, a new piece in the puzzle of innate immunity. J. Exp. Med. **189:**605–609.
43. **Yang, R. B., M. R. Mark, A. Gray, A. Huang, M. H. Xie, M. Zhang, A. Goddard, W. I. Wood, A. L. Gurney, and P. J. Godowski.** 1998. Toll-like receptor-2 mediates lipopolysaccharide-induced cellular signalling. Nature **395:**284–288.

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