The role of commensal bacteria in the development of protective immunity to meningococcal disease

by

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PhD

University of Edinburgh

April 2001

This thesis is written in the memory of

Lee McKiernan

who died of meningococcal sepicaemia and of those
who were affected by meningococcal disease

This thesis is dedicated to my mother
and my late aunt Helga Pili

Declaration

In accordance with the regulation 3.8.7 of the University of Edinburgh I hereby declare, that the presented thesis has been composed by myself, and that the entire work is my own. No part of this thesis has been submitted for any other degree or professional qualification.

I would like to acknowledge the technical assistance, training and support I received from the members of the Infection and Immunity research group, lead by Dr. Caroline C. Blackwell and Prof. Donald M. Weir.

Dr. Peter Christie, Dr. Crispin Best and Dr. Stuart Clark have kindly given permission to include unpublished data and information about meningococcal disease in Scotland into my thesis. All collaborators and joint authors have given permission to include the work into my thesis.

I pledge in accordance with the Pugwash conference on science and world affairs to work for a better world, where science and technology are used in socially responsible ways. I will not use my education for any purpose intended to harm human beings or the environment. Throughout my career, I will consider the ethical implications of my work before I take action. While the demands placed upon me might be great, I sign this declaration because I recognize that individual responsibility is the first step on the path to peace.

Jan Matthias Braun
Edinburgh, April 2001

Acknowledgements:

I would like to thank Dr. Caroline C. Blackwell, who was instrumental in my scientific career, for all her support and her input into the design and execution of my postgraduate education and for her friendship and hospitality. I would like to thank my second supervisor Prof. Ian R. Poxton and his research group for their support.

I would like to thank Prof. Donald M. Weir for may stimulating discussions, comments and ideas and his involvement in my scientific education. I am grateful to the members of the Infection and Immunity research group for all their support and friendship: Dr. Hamid Alkout, Dr. Omar El-Ahmer, Miss. Sonja Giersen, Dr. Ann Gordon, Mrs. Valerie James, Miss. Doris McKenzie, and Dr. Mohammed Raza.

I would like to thank especially Mrs. Valerie James for her technical expertise and her endless supply of delicious chocolate, and Mr. Bill Neil who shared his extensive knowledge of flow cytometry with me. Many thanks to Mr. Bob Brown and Mr. Mike Kerr for their technical advice, and to all the volunteers donating blood. I would like to thank Dr. Jonathan Bard and his team for their help with confocal microscopy, Mr. Derek Notman for his help with electron microscopy, Mr. Iain Brown at Medical Illustration, and Rob Elton for statistical support.

I would like to thank and acknowledge Prof. Denis Goldberg for all the advice and input he gave me throughout my scientific education. Many thanks to Prof. Josef Beuth for his friendship and many stimulating discussions and support since I started working in the biosciences.

I would like to thank and acknowledge our collaborators who kindly provided antibodies and bacterial strains: Dr. Wendel Zollinger, Washington D.C., USA; Dr. Paula Krizova, Prague, Czech Republic; Dr. Kristin Jónsdóttir, Reykjavik, Iceland; Dr. Georgina Tzanakaki, Athens, Greece; Dr. Stuart Clark, SMPRL Glasgow; Dr. Crispin Best, Yorkhill Glasgow; and Dr. Peter Christie, Scottish Centre for Infection and Environmental Health Glasgow.

I would like to thank the staff of the animal house and especially Loraine from the SNBTS for their help in producing polyclonal and monoclonal antibodies.

I would like to thank Audrey Nash, Paulski Higgins, Sarah and Jessica Bannerman, and Suzanne Boyce for their support and friendship, who made my stay in Scotland such a pleasure.

I would like to thank especially my mother and my family for all the support they gave me throughout my studies. Without their help I would not have been able to pursue my scientific career.

I would like to thank Eileen McKiernan and the members of The Meningitis Association of Scotland for their support and friendship, and the Lloyds TSB Foundation (Scotland) for funding this PhD project.

Abstract

Group B *Neisseria meningitidis* (NM) is a major pathogen associated with severe and often fatal meningococcal disease in Europe. Recently introduced group A or C polysaccharide vaccines do not provide any protection against this predominant form of bacterial meningitis and septicaemia in Scotland.

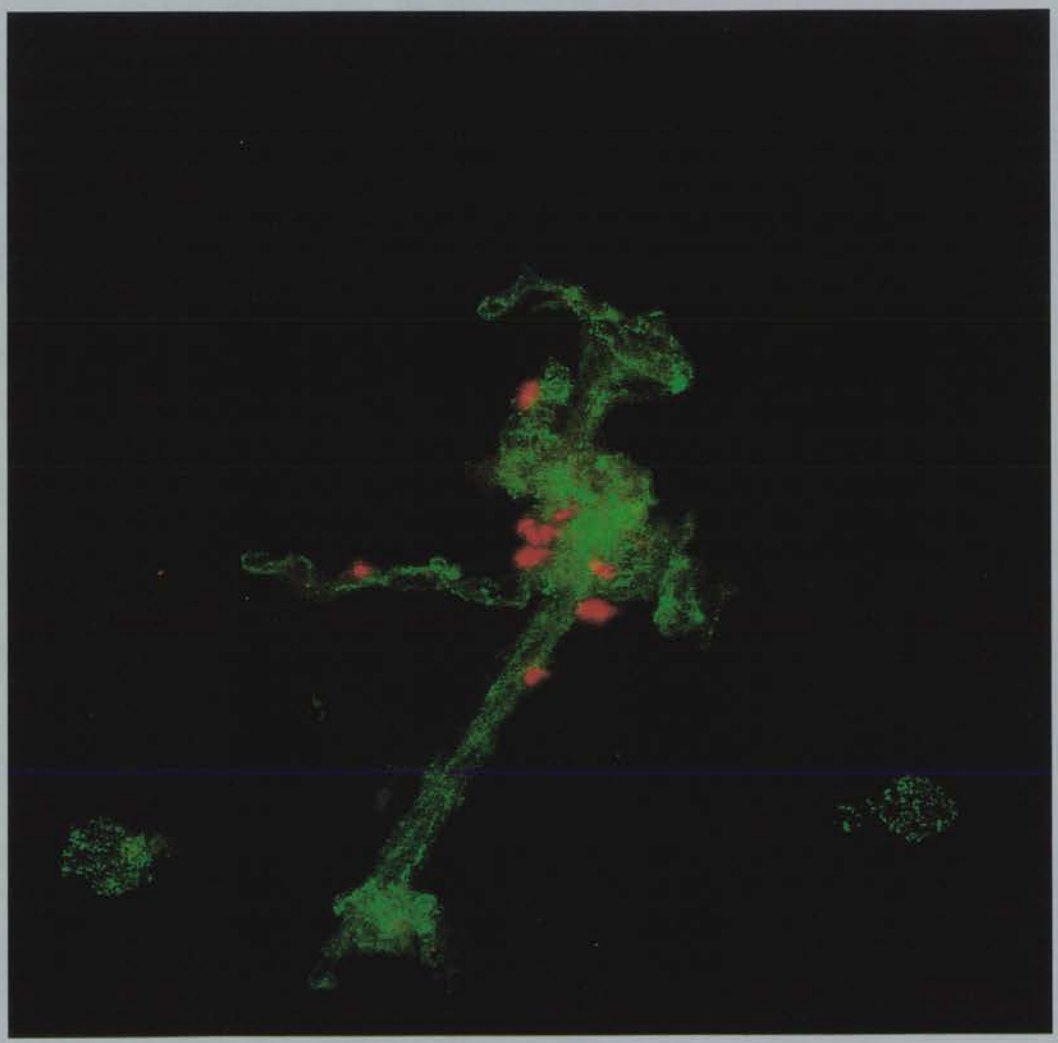
Carriage of the commensals *Neisseria lactamica* (NL) and *Moraxella catarrhalis* (MC) coincides with a progressive increase in the level of natural immunity against meningococci, and investigations to identify epitopes that might be responsible for the induction of antibodies that are cross-reactive between meningococci and these commensals might lead to the development of a vaccine against group B meningococci. The most promising candidate for cross-reactive antigens is lipooligosaccharide (LOS).

Absorption of normal human serum showing bactericidal activity against meningococcal isolates and reference strains by commensal isolates from different regions in Europe provided evidence for cross reactivity between epitopes shared by NL, MC and NL LOS. Investigations into the opsonic activity of normal human serum showed the presence of anti-meningococcal antibodies that were independent from bactericidal antibodies.

Meningococcal LOS is associated with virulence, and is a potent inducer of inflammatory responses to meningococcal LOS that contribute to the severity and fatality of disease. Its use as a vaccine needs to be assessed in relation to its ability to induce pro-inflammatory cytokines. Meningococcal meningitis and septicaemia are exclusively human diseases, and a suitable animal model for safety assessments of LOS vaccines does not exist. Because of the genetically controlled variability of the inflammatory response of individuals to bacterial antigens and toxins, an *in vitro* model using a human monocytic cell line was developed for initial screening of cytokine release induced by LOS preparations from meningococci and commensal species, providing evidence that endotoxin from commensal species was less toxic than meningococcal LOS, and that anti-meningococcal antibodies were present in normal human serum and immune mouse sera able to neutralise the bioactivity of LOS.

These findings provided evidence that some commensal species share cross-reactive antigens with pathogenic meningococci able to induce antibodies associated with the development of protective immunity to meningococcal disease. Additionally, due to their lower bioactivity compared to meningococcal LOS their use as a potential vaccine, or their incorporation into meningococcal protein vaccine candidates might provide an effective and safer alternative to meningococcal endotoxin.

Scanning confocal image of CD14 positive THP-1 (FITC, surface) cells of attached (red) immunotype L3 meningococci eliciting rhodamine-123 oxidative burst (green, internal) (670 x magnification)



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Chapter 1 Introduction

1.1 Meningococcal disease

Disease due to *Neisseria meningitidis* (NM) can kill a previously healthy child or young adult within hours of the first symptoms of illness. Meningococcal disease is the largest single cause of childhood death in the developed world. Worldwide over 350,000 fatalities caused by NM were registered by the World Health Organisation (WHO) per annum [Robbins & Freeman, 1988].

Incidences of meningococcal disease in Scotland increased from 200 to about 350 cases per year since 1995, with over 300 cases of meningococcal disease confirmed in Scotland in 2000; the fatality rate 6-10% with young children most at risk of meningococcal disease and death (Figures 1.1 & 1.2) [personal communications, S. Clark, Scottish Meningococcal and Pneumococcal Reference Laboratory, SMPRL, Glasgow; P. Christie, Scottish Centre for Infection and Environmental Health]. While the pathogen NM is the predominant causative agent of bacterial meningitis other capsulate bacteria can also lead to rapid progressive meningitis, *e.g. Streptococcus pneumoniae* and *Haemophilus influenzae* type B (Hib).

Meningococcal disease can manifest itself in two main forms, meningitis and septicaemia. Meningococcal meningitis is an inflammation of the meninges, the membrane lining the brain and the spinal cord. In both fulminant meningococcal septicaemia and meningococcal meningitis damage is caused by an uncontrolled localised or systemic host inflammatory response [Brandtzaeg *et al.*, 1989, Hodgetts *et al.*, 1998; Vieusseux, 1805].

Meningococcal septicaemia, or blood poisoning, is caused by invasion of meningococci into the blood system of the patient. The host's immune defence is unable to kill and clear the invading pathogen successfully. During the evasion of the immune response or due to treatment with antibiotics, meningococci shed endotoxin or lipooligosaccharide (LOS) into the blood system. In the absence of specific or cross-reactive neutralising antibodies, endotoxin induces a massive inflammatory

response characterised by increased secretion of inflammatory mediators such as interleukin 1 (IL-1), interleukin-6 (IL-6), interleukin 8 (IL-8), tumour necrosis factor alpha (TNF α), interferon gamma (IFN γ) and acute phase proteins. Severity and fatality of the disease has been correlated with levels of inflammatory mediators detected in the blood (Table 1.1).

Figure 1.1 Laboratory confirmed cases of meningococcal disease in Scotland 1993-2000 [S.Clark, SMPRL, unpublished data]

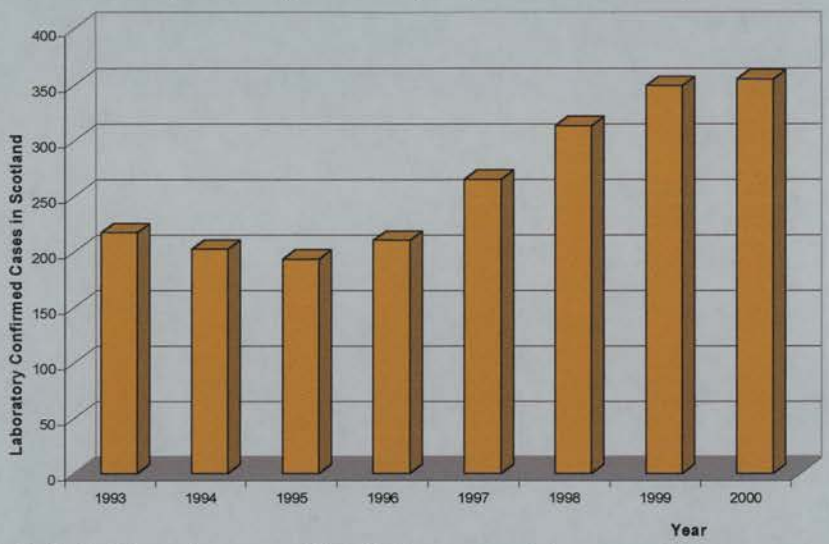


Figure 1.2 Fatality rate per 100.000 caused by meningococcal disease in Scotland 1999-2000 [P. Christie, Scottish Centre for Infection and Environmental Health, unpublished data].

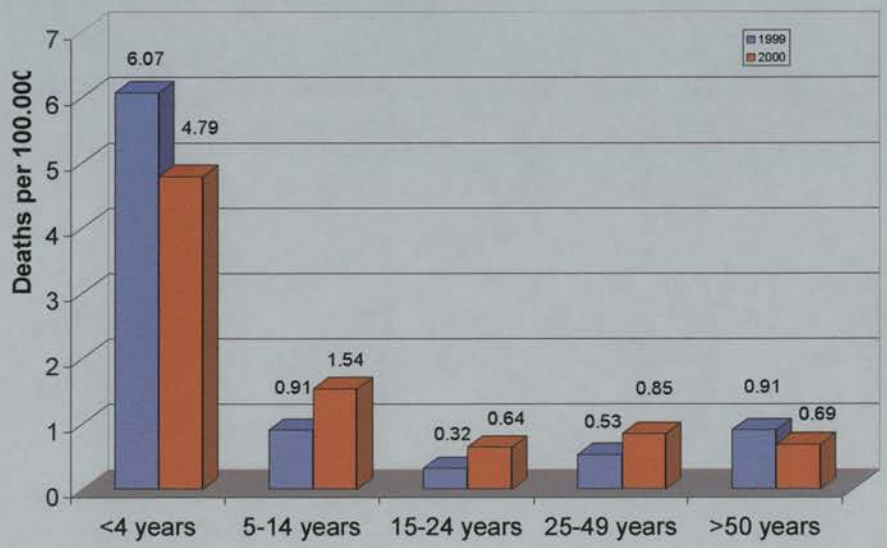


Table 1.1 Association between pathology of meningococcal disease and cytokine mediators

Cytokine concentration										
[LOS]	[IL-1]	[IL-6]	[IL-8]	[IL-10]	[TNFα]	[IFNγ]	[C3]	other	Associated pathology	Reference
		?			↓				Favourable patient outcome	Martin, 1994
		↑ [IL6sR]						↑ [neo]	Septic shock	Delogu, 1995
	✓	✓	✓		✓				Septic shock	Rixen, 1996
		✓	✓						Patient outcome	Holzheimer, 2000
		✓	✓					[GSF]	Predicts outcome	Slotman, 2000
✓		✓	✓	✓					Sepsis	Damas, 1997
-		↑	✓						Neonatal infection	Edgar, 1994
		✓			✓			[lac]	Severity of sepsis	Hazelzet, 1994
		↑			↑		↑		Severity and mortality [IL-6]>1000 pg/ml	Damas, 1992
	-	✓			-			ANC	Predict outcome (0-36 months)	Strait, 1999
		✓			✓				Sepsis	Waage, 1989
		↑						↑ [TPO]	Early and fulminant septicaemia	Bjerre, 2000
		-	-	-		↑		↑[IL12p40]	Associated with meningitis in CSF	Kornelisse, 1997
		-						↑ [IL6sR]	Sepsis	Frieling, 1995
			✓						Bactericaemia	Engel, 1999
✓					✓				Severity	Brandtzaeg, 1994
					✓				Sepsis	Waage, 1997
					↑				Severity and fatality	Nuernberger, 1995
					↑			[NO]	Early meningococcal disease	Padron, 1999
					↑				In CSF meningitis without shock	Van Deuren, 1995
					↑				Shock and death [TNF] 100-5000 pg/ml	Damas, 1989
					✓				Fatality	Waage, 1987
	✓				✓	-			Patient outcome	Calandra, 2000
	✓						✓	[fib]	Predicts fatality	Hazelzet, 1994
	↑								Fatality	Van Deuren, 1995
	✓								IL-1 gene	Read, 2000
	✓			✓					Outcome	Waage, 1989

✓, associated with pathology; ↑, increased concentration associated with pathology; -, not associated with pathology; ANC, absolute neutrophile count; GSF, granulocyte stimulating factor; neo, neopterin; lac, lactate; fib, fibrinogen; NO, nitric oxide; IL6sR, IL-6 soluble receptor; IL12sR, IL-12 soluble receptor; IL12p40, biological active unit of IL-12

In susceptible patients, this release of endotoxin and the results of inflammatory responses lead to rapid deterioration and failure of the normal homeostatic mechanisms. The removal of free endotoxin and the intervention in controlling the inflammatory response to endotoxin are crucial in preventing further damage to the host. Disseminated intravascular coagulation or blood platelet aggregation can result in the loss of limbs. Bleeding or leaking of peripheral blood into the surrounding tissue of blood vessels is recognised by the typical spots under the skin. The loss of perfusion may lead to the patient falling into a coma. Myocardial depression and multiple organ failure can lead to death.

Because meningococci are transmitted by aerosols or close ("kissing") contact immunisation is the only effective means for prevention of disease in individuals lacking protective immunity. This chapter reviews:

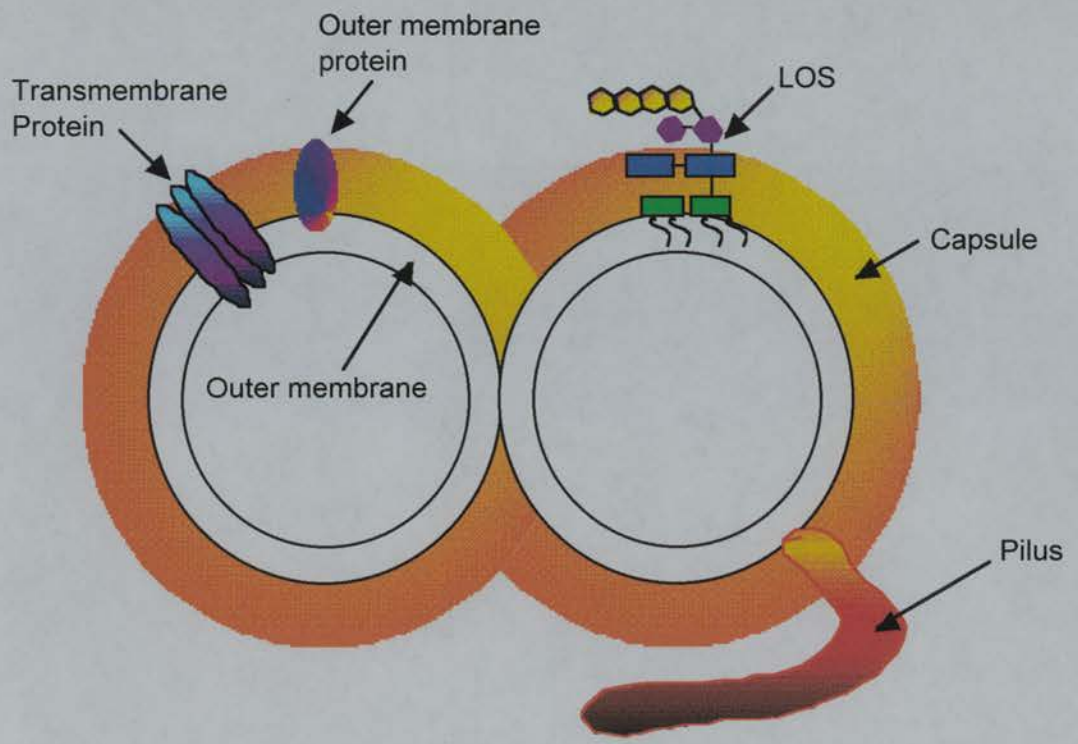
1. the success achieved to date in development of vaccines against meningococcal disease;
2. the problems that remain;
3. the way in which assessment of development of 'natural' immunity through exposure to commensal flora could provide a new approach to meningococcal vaccines.

1.2 Cell surface structure of meningococci in relation to vaccine development

Named after the Passau microbiologist Albert L.S. Neisser (1855-1916), Neisser's diplococci, NM is an exclusively human pathogen. It is Gram-negative, 1μm in diameter, aerobic diplococcus and shows a large degree of phenotypic variation [Kahler & Stephans, 1998].

The major antigens of the outer membrane of meningococci vary greatly and these variations have been exploited to develop typing systems for epidemiological surveillance. These include capsule, a variety of outer membrane proteins, pili and endotoxin. The main antigens are found anchored to the typical Gram-negative envelope (Figure 1.3).

Figure 1.3 Capsular polysaccharide, outer membrane proteins (OMP) and transmembrane proteins, pili, lipooligosaccharide (LOS) of meningococci



1.2.1 Capsular vaccines

The first major success in development of vaccines against meningococcal disease was through the recognition of the immunogenicity of the capsular polysaccharide of these bacteria.

Originally meningococci were divided into serogroups for epidemiological purposes based on agglutination of capsular antigens. Currently 12 major antigenically distinguishable polysaccharide capsules have been identified: A, B, C, H, I, K, L, X, Y, Z, 29E and W135 which vary in their composition and arrangements of oligosaccharide units [Frasch *et al.*, 1985 a & b]. The most prevalent serogroup structures are presented in Table 1.2. The majority of meningococcal disease is caused by serogroup A, B and C. Groups B and C are responsible for most disease in Europe and the Americas while group A is more prevalent in Africa, Russia and causes periodic epidemics in Romania [Pinner *et al.*, 1989; Mihalcu *et al.*, 1994].

Table 1.2 Oligosaccharide structures of the major pathogenic meningococcal polysaccharide capsules

Serogroup	Capsular Polymer	Reference
A	*N*-acetyl-3-0-acetyl mannosamine phosphate (α1→6), (*O*-acetylated-2-acetamido-2-deoxy-D-mannose-6-phosphate)	Gotschlich *et al.*, 1969
B	Up to 200-residue polysaccharide units of (2→8) linked *N*-acetylneuramic acid	Gotschlich *et al.*, 1969
C	O-acetylated or non acetylated (2→9) linked N-acetylneuramic acid	Gotschlich *et al.*, 1969
X	*N*-acetyl glucosamine phosphate (α1→4), or (2-acetamido-2dedoxy-D-glucose-4-phosphate)	Evans *et al.*, 1968
Y	*N*-acetyl neuraminic acid:glucose, partially *O*-acetylated alternating sequences of D-glucose and *N*-acetylneuramic acid	Evans *et al.*, 1968
W-135	4-*O*-α-D-galactopyranosyl-β-D-N-acetyl-neuraminic acid, alternating sequences of D-galactose and N-acetylneuramic acid	Evans *et al.*, 1968

Polysaccharide capsules are an effective way for pathogens to evade the human immune responses. Compared with non-capsulate meningococci, which are usually eliminated by bactericidal and opsonising antibodies in human serum, heavy

polysaccharide capsulation is thought to reduce the ability of complement to bind and kill meningococci [McKinnon *et al.*, 1993; Vogel *et al.*, 1996; Klein *et al.*, 1996; Taylor, 1983; Hammerschmidt *et al.*, 1994; Kahler *et al.*, 1998]. Group B meningococci express a poorly immunogenic $\alpha 2 \rightarrow 8$ linked poly-sialic capsule similar to some human antigens such as the Neural Cell Adhesion Molecule (N-CAM) [Finne *et al.*, 1983; Hayrinen *et al.*, 1995].

1.2.1.1 Purified capsular antigen vaccines

Capsular polysaccharide vaccines against the serogroups A, C, Y, and W-135 induce protective immunity against these meningococcal serogroups in older children and adults. Immunoprophylaxis with the group C vaccine was effective in studies of army recruits in the United States of America [Artenstein *et al.*, 1970], and vaccination with the group A vaccine was effective in Finnish army recruits [Mäkelä *et al.*, 1975]. While these vaccines appear to be effective in adults, vaccines based on meningococcal polysaccharides are less effective in young children [Gold *et al.*, 1975; Peltola *et al.*, 1977; Reingold *et al.*, 1985]. Group C vaccines are thought to be ineffective in children younger than 2 years of age, and in children under 6 months for group A vaccines [Ceesay *et al.*, 1993]. The duration of protection elicited by capsular antigens is thought to be short lived, varying between two to four years after administration of the vaccine in adults and children [Wahdan *et al.*, 1977].

1.2.1.2 Conjugated capsular antigens

This lack of wide-scale protection within the young age group (6 months to 5 years) that is most susceptible to meningococcal disease led to the development of conjugated group C vaccine. The principal is that used for the successful development of a vaccine for *H. influenzae* type b (Hib) in which the polysaccharide was conjugated to a carrier protein. Conjugation of polysaccharides to protein carriers induces a T-cell dependent response compared to polysaccharide alone which induces a T-cell independent response. Large molecular weight polysaccharide antigens like the meningococcal capsule bind to several receptors on B cells followed by cross-linking of these receptors. This triggers the production of

immunoglobulin IgM and the transformation of the stimulated B cell into plasma cells. This T-cell independent immunity is short lived and does not generate memory.

Conjugation of polysaccharide antigens to protein carriers induces a T-cell dependent response. The protein-carbohydrate antigen is ingested by antigen presenting cells (APC) and expressed on their cell surface within the major histocompatible complex type II receptor (MHC II). T-helper (T_H) cells expressing the MHC II receptor (CD4) and CD28 (B7 receptor) are activated by the APC leading to clonal proliferation and T_H cell maturation with some developing into memory T-cells. The activated T_H cells lead to differentiation of B-cells that bind directly to the hapten, *i.e.* a second encounter with the antigen or antigen present on the APC, followed by proliferation of B-cells, their differentiation into antibody producing plasma cells, or memory B-cells. This T-cell dependent immunity is long lasting and able to produce a wide range of classes of immunoglobulin [Weir & Stuart, 1997]. A serogroup C conjugate vaccine was introduced as part of a mass vaccination program in the United Kingdom in the autumn of 1999 [Richmond *et al.*, 1999]. Initial observations on the effectiveness of the conjugated vaccine in Scotland following mass vaccination of children and young adults indicate a reduction in disease caused by group C meningococci, while its impact on carriage of meningococci and disease is currently being investigated [personal communication, S. Clark, SMPRL].

1.3 The remaining problem of serogroup B

While the conjugate C vaccine appears to have reduced disease due to this serogroup, the NeuNAc capsule of group B meningococci is thought to be ineffective due its low immunogenicity and its presence on some human tissues (*i.e.*, neural cell adhesion molecule, N-CAM) [Finne *et al.*, 1983]. Poly-sialylated N-CAM is an antigen found in several tumours associated with the immune evasion of some malignant metastatic cells [Roth *et al.*, 1993]. Conflicting observations have been reported about the ability of NeuNAc capsular antigen to induce autoimmune responses [Wyle *et al.*, 1972; Finne *et al.*, 1983; Bartolonie *et al.*, 1995]. Protein vaccines containing the B capsular antigen did not show such effects in animal models [Lifely & Wang, 1988] or in humans [Zollinger *et al.*, 1979]. Vaccines based on group B capsular polysaccharide are poorly immunogenic, and shortlived. It rarely induces antibodies in patients [Romero & Outschoorn, 1997]. Attempts to increase the immunogenicity by conjugation with protein carriers were not successful. Because of the problems outlined above, other surface antigens of meningococci have been assessed for their use as vaccine candidates for serogroup B meningococci.

1.3.1 Pili

Successful colonisation of epithelial surfaces in the oropharynx depends on overcoming of mucosal flux by binding of the meningococcus to receptors on the epithelial surface. Early studies implicated the importance of pili, protein extensions on the bacterial cell surface [de Voe & Gilchrist, 1975].

Meningococci expressing pili during initial colonisation of epithelial tissues *in vivo* and epithelial cell lines *in vitro* bind in greater number to such cells [Stephens & McGee, 1981]. Although the establishment of meningococcal colonisation is aided by the expression of pili, prolonged carriage appears to reduce their expression. Mechanical stress on the mucosal surface and the presence of proteinases in the mucus might also remove pili from the meningococcus. Pilus expression can be controlled by stimuli from the host leading to a phase variation of this phenotype *in vivo*. This loss of the pilus surface antigen is important in reducing the formation of secreted IgA towards pili epitopes.

There is some evidence that the loss of pili does not alter the virulence of meningococci suggesting that these structures are not necessary for the invasion of meningococci [Stephens & Farley, 1991]. While pili are associated with colonisation but not with invasive disease, the pilus antigens have not been assessed as vaccines because of the hypervariability of their terminal protein sequences.

1.3.2 Trans-membrane and outer membrane proteins (OMP)

Neisseriae species have a typical Gram-negative envelope consisting of a lipid bi-layer around a semi-rigid peptidoglycan sheet. Proteins can be anchored to the outer lipid layer alone, but usually form monomeric or polymeric structures penetrating both lipids and peptidoglycan layers (trans-membrane OMP).

Five classes of outer membrane proteins have been identified in NM. The classification is based on the molecular weight of proteins separated by sodium dodecyl sulphate (SDS)-polyacrylamide electrophoresis (Table 1.3). Monoclonal

antibodies to class 2 and class 3 outer membrane proteins have been used in epidemiological typing of meningococci (serotype), as have monoclonal antibodies to class 1 to determine the subtype [Frasch *et al.*, 1985a; Evans *et al.*, 1994].

Table 1.3 Characteristic of meningococcal OMPs

Class	Mol.wt. (kDa)	Characteristics	Function
1	44 - 47	Trypsin sensitive; deoxycholate insoluble	Cation porin; no known homology with *N. gonorrhoea*
2	40 – 42	Trypsin resistant; deoxycholate insoluble	Porin; homologous to gonococcal protein IB
3	37 – 39	Trypsin resistant; alternate expression to class 2	Porin; homologous to gonococcal protein IA
4	33 – 34	Trypsin resistant; common to all strains	Widely homologous to gonococcal protein III and OmpA of *Escherichia coli;* inhibits bactericidal activity
5	26 – 30	Protease sensitive; deoxycholate soluble; heat modifiable; highly variable in expression and molecular weight; more than one class 5 protein may be expressed	Analogous to gonococcal protein II; associated with invasion and colonisation of epithelial cells
5c	26 – 30	Small amounts ; phase variable	Invasion and colonization

1.3.2.1 Class 1 proteins

Class 1 OMP are porins selective for cations and are expressed on meningococcal isolates obtained from carriers and patients [van der Ley *et al.*, 1991]. There is antigenic variability within the class 1 OMP which has been used to develop monoclonal antibodies used in epidemiological surveillance [Suakkonen *et al.*, 1989]. These antigens induce antibodies in animal models, and strains expressing multiple subtype antigens are being constructed and assessed for their potential as vaccines against serogroup B [van der Ley *et al.*, 1995]. Currently 14 antibodies (P1.1-P1.7, P1.9, P1.10, and P1.12-P1.16) are used for routine subtyping by reference laboratories.

1.3.2.2 Class 2 and 3 proteins

All known strains of meningococci express either OMP class 2 or class 3, but never both at the same time [Hitchcock, 1989]. A serotyping scheme is based on the use of monoclonal antibodies to different epitopes in variable regions of these class 2/3 OMP was developed.

1.3.2.3 Class 4 proteins

Class 4 OMP are trans-membrane proteins common to all known strains of meningococci. There is evidence for structural similarities between meningococcal class 4 OMP and proteins from *N. gonorrhoea* and *Escherichia coli*. Although these OMP induce an antibody response in man, they are not bactericidal. One study indicates that antibodies against class 4 OMP's inhibit the bactericidal activity of antibodies directed against other classes of meningococcal OMP's [Munkley *et al.*, 1991].

1.3.2.4 Class 5 proteins

Up to four different proteins of Class 5 OMP can be expressed at any one time by some meningococci [Achtman, 1991]. These heat variable proteins share some homology with protein II of *N. gonorrhoea*, but no unifying epitope of this class has yet been identified. Included in class 5 is a protein involved in the invasion and colonisation of meningococci [Fernandez de Cossio *et al.*, 1992]. Although bactericidal antibodies could be raised against these class 5c proteins, their expression appeared to undergo phase variation resulting in low or no expression on the meningococcal surface within the bacterial population. Investigations into the ability of OMP to elict antibodies showed that deglycolysation of all investigated classes (1-5) resulted in no significant antibody production *in vivo*. This suggests that antigenicity of OMP depends on post-translational glycolysation, or the presence of other oligosaccharides that must be considered in evaluation of these surface components for their use in vaccines.

1.3.2.5 Lip and Ctr

In addition to the five major classes of outer membrane proteins, several other molecules are present on the meningococcal surface [Frasch & Pepper, 1982; Ferrón *et al.*, 1992; Ala'Alden *et al.*, 1993; Stimson *et al.*, 1995; Mackinnon *et al.*, 1999; Pizza *et al.*, 2000]. The H.8 or Lip antigen, a lipoprotein with porin function, is found exclusively in pathogenic *Neisseriae*, but it is not found in non-pathogenic species [Bhattacharjee *et al.*, 1988]. It failed to induce bactericidal antibodies [Bhattacharjee *et al.*, 1990]. A meningococcal endogenous membrane-associated lipoprotein, CtrA, was described in NM serogroups producing sialyl transferases B, C, W-135 and Y. It is thought to be part of a capsule expression system [Frosch *et al.*, 1992].

1.3.2.6 Iron binding proteins

Several iron binding OMP with variable molecular weights are currently under investigation as potential vaccine candidates for meningococci (Table 1.4). Some of these antigens show some homology with other *Neisseria* species [Lee, 1994; Troncoso *et al.*, 2000].

Table 1.4 Iron regulated OMP

kDa	Characteristics	Function	Reference
55	Partly expressed in iron deficient and sufficient media; cross-reactive with *N. lactamica*	Haemin-binding protein	Troncoso *et al.*, 2000
50	Only expressed in iron deficient media not cross reactive with *N. lactamica*	Haemin binding protein	Lee, 1994
68 - 95	Minor antigen	Transferrin binding proteins TpbA and TbpB	Ferreirós *et al.*, 1994
70	Iron-limition-indicable OMP	Specific for human lactoferrin	Pettersson *et al.*, 1990

1.3.2.7 Outer membrane vesicle (OMV) vaccines

Various vaccines derived from OMV were tested in animal models [Quakyi *et al.*, 1999] and in clinical trials in Norway [Rosenqvist *et al.*, 1995], Brazil [de Moraes *et al.*, 1992], Cuba [Rodriguez *et al.*, 1999] and Chile [Boslego *et al.*, 1995]. These vaccines induced bactericidal antibodies in the immunised group but were protective mainly against strains expressing the serotype/subtype antigens of the strain from

which the vaccine was produced. There was limited protection against strains expressing other OMP antigens. Due to the heterogeneity of antigens on meningococcal strains and the introduction of new strains into the vaccinated population, the use of OMV would only be effective in closed populations (*e.g.,* Cuba). De-glycolysated OMV vaccines showed poor immunogenicity after the removal of its toxic LOS moieties [van der Ley *et al.*, 1998]. LOS appears to be an essential component of anti-meningococcal protein vaccines, perhaps acting as an adjuvant in the human host.

1.4 Lipooligosaccharides (LOS)

All Gram-negative species have a family of glycolipids called endotoxin embedded into the hydrophobic outer membrane lipid layer [Nikaido *et al.*, 1966; Lüderitz *et al.*, 1966; Osborn, 1966]. These macromolecules share a common basic structure consisting of :

1. a basal lipid A region anchored into the outer membrane;
2. a rough (R) core region consisting of a backbone of 2-keto-3-deoxyoctulosonic acid (KDO) and/or heptose (Hep) phosphate;
3. a highly variable region of saccharide domains differing in length and composition bound to the core heptose residue.

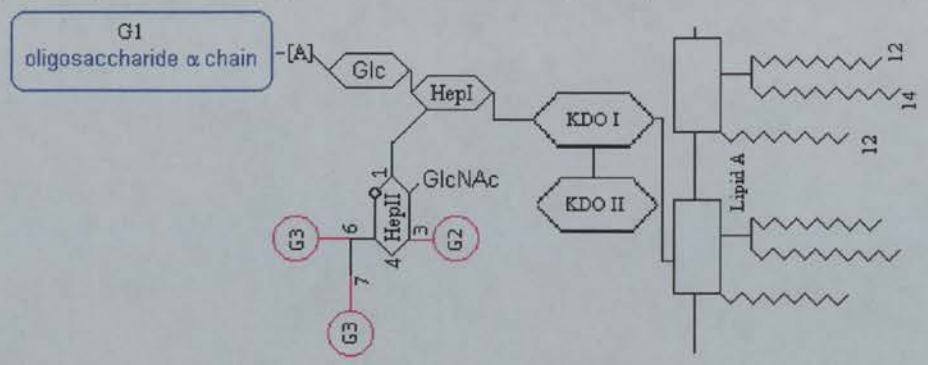
Lipid A is a phosphorylated di-glucosamine disaccharide substituted with fatty acids of variable length, and it is responsible for the biological activity which induces inflammation (endotoxin). Enteric Gram-negative species show a characteristic long linear chain of polysaccharide, called O-antigen, linked to the R-core giving the endotoxin of these species the name lipo**poly**saccharide (LPS). In contrast, the saccharide chains of all *Neisseria* species consist of very few residues, giving its endotoxin the name lipo**oligo**saccharide (LOS) [Kulshin *et al.*, 1992].

1.4.1 Variation in meningococcal LOS and immunotyping

Thirteen major LOS types were identified for *N. meningitidis* using polyclonal and monoclonal antibodies by passive haemagglutination inhibition techniques [Zollinger & Mandrell, 1977; Achtman *et al.*, 1991] and whole cell ELISA [Scholten *et al.*, 1994]. The majority of meningococcal isolates express one or more of the immunotypes L1-L12, while non-typable and L13 immunotypes are rare. The twelve major LOS types have a relative molecular weight ranging from 3.15 to 7.1 kDa [Kim *et al.*, 1988]. The oligosaccharide chain, also referred to as the α-chain or variable LOS region one (R1), is composed of the saccharides glucose (Glc), galactose (Gal), N-acetylglucosamine (GlcNAc), N-acetylgalactosamine (GalNAc). Sialylated forms contain the terminal saccharide N-acetylneuramic acid (NeuNAc) which is added to terminal galactose residues by endogenous or exogenous sialyl transferases [Kahler & Stephans, 1998; Kahler *et al.*, 1998]. The LOS saccharide and core structures are shown in Table 1.5 and Figure 1.4. The following abbreviations for core moieties were used throughout this study: glycero-D-manno-heptopyranoside (Hep or heptose); phospho-ethanolamine (PEA); 2-keto-3-deoxyoctulosonic (KDO).

The complete structures of immunotypes L10 – L13 are not elucidated, but there is evidence that L10 contains the paragloboside residue [Scholten *et al.*, 1994] and L11 shows some homology with L1 [Griffiss *et al.*, 1994]. The PEA residue of immunotype L2 can be expressed in two forms that undergo phase variation: The PEA on the G3 region can be linked in (1→6) or (1→7) conformation; and the PEA can be replaced by a hydrogen (H) atom. The PEA residue of immunotypes L4 and L6 express both PEA (1→6) and (1→7) linkages. The expression of meningococcal immunotypes is associated with serogroups [Tsai *et al.*, 1991; Vedros, 1987; Verheul *et al.*, 1993; Kim *et al.*, 1988]. Immunotypes L8, L9, L10, L11 and L12 are found on group A strains [Salih *et al.*, 1990], while serogroup B and C meningococci express immunotypes L1 – L8.

Figure 1.4 Schematic structure of meningococcal LOS immunotypes
[Jennings *et al.*, 1983 and 1984; Romero & Outschoorn, 1994; Scholten *et al.*, 1994]



G3: PEA (1→6) L2, L4, L6; **G2:** PEA (1→3) L1, L8, L(3,7,9); H (→3) L4, L6; αGal (1→3) L2

1.4.2 Immunotypes and pathogenicity

LOS immunotype expression is thought to be linked to the pathogenicity of the organism. Immunotypes L(3,7,9) are isolated predominantly from patients with invasive meningococcal disease [Scholten *et al.*, 1994; Dunn *et al.*, 1995]. Other immunotypes are found predominantly among carrier strains [Jones *et al.*, 1992]. Immunotypes L3, L7 and L9 are thought to be similar in their immunochemical structures [Zollinger & Mandrell, 1977; Poolman *et al.*, 1982] with immunotype L3 being sialylated by endogenous sialyl transferases. Immunotypes L3 and L7 are found on serogroup B and C meningococci and they have similar G2 core components, PEA (1→3) HepII. Immunotype L9 is expressed on group A strains.

The presence of the sialylated phenotype on invasive meningococci is associated with resistance to complement-mediated killing by masking the terminal galactose with NeuNAc. This mechanism is thought to reduce the recognition of the epitope by anti-LOS antibodies directed against the non-sialylated epitopes [Estabrook *et al.*, 1997; Vogel *et al.*, 1997] (Figure 1.5). Free or membrane bound sialyl-L(3,7,9) also upregulates neutrophil activation markers and results in increased injury of epithelial cell lines [Klein *et al.*, 1996]. Sialyl L(3,7,9) phenotypes can evade the complement mediated bacteriolysis cascade [Mackinnon *et al.*, 1993]. This phenotype also reduces complement and anti-LOS antibody mediated phagocytosis by professional phagocytes [Kim *et al.*, 1992; Lehmann *et al.*, 1997].

1.4.3 Expression of major and minor immunotypes by *N. meningitidis*

Meningococci are able to express more than one immunotype. Isolates from patients with meningococcal disease in the Netherlands (1989-1990) showed different immunotype combinations [Scholten *et al.*, 1994]:

1. Group A meningococci L**9** (54%), L**9,8** (8%), L**10** (24%), L**10,11** (8%) and non-typable (**NT**) (8%).
2. Group B meningococci L**1** (1%), L**1,8** (11%), L**2** (10%), L**3,7** (36%), L**3,7,1** (4%), L**3,7,1,8** (2%), L**3,7,8** (28%), L**4** (4%), and L**8** (5%).
3. Group C meningococci L**1,8** (2%), L**2** (30%), L**3,7** (37%), L**3,7,1** (1%), L**3,7,1,8** (3%), L**3,7,8** (7%), L**4** (15%), L**8** (3%), and **NT** (3%).

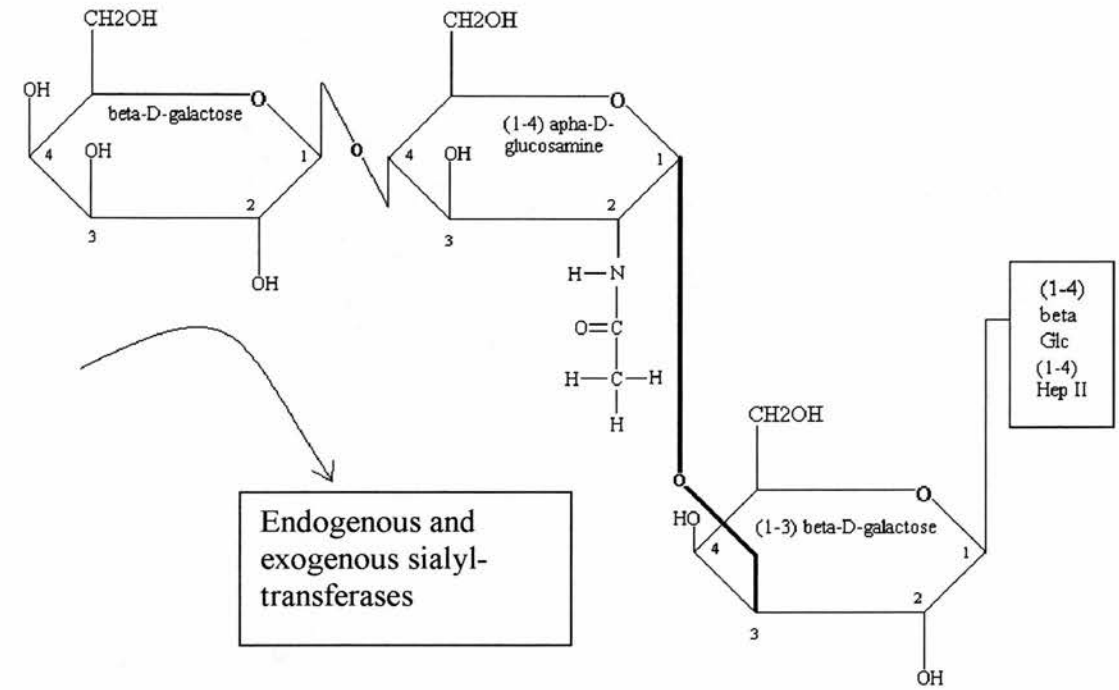
The expression of multiple immunotypes within a meningococcal population allows the organism to diversify its antigenic structure. Selective pressure due to the presence of antibodies in the host to one LOS immunotype allows the strain to express other immunotypes increasing their chance of survival. This ability of meningococci to alter its LOS structure has to be taken into account in understanding the development of natural immunity, and in the choice of immunotypes as potential vaccine candidates. Sialylation [Poolman *et al.*, 1985] and the expression of paragloboside gene cluster IgtABE [Jennings *et al.*, 1999] are the main phase variable phenotypes known.

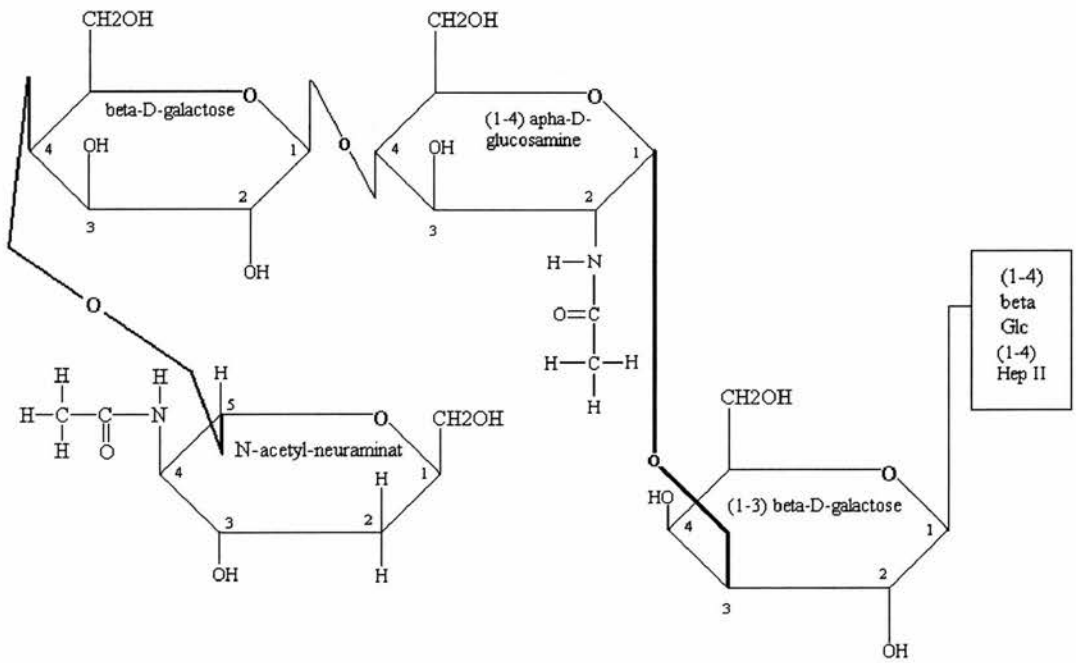
Table 1.5 Oligosaccharide and core structures of LOS immunotypes

LOS type	Terminal oligosaccharide α chain oligomer of the G1 region	Core			Reference
		[A]	G2	G3	
L1	Galα (1→4) Galβ (1→4) Glcβ	-	PEA (1→3)	H	Di Fabio *et al.*, 1990; Wakarchuk *et al.*, 1998
L2	Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) Glcβ	-	Glcα (1→3)	PEA H, (1→6), (1→7)	Rahman *et al.*, 1998; Gamain *et al.*, 1992
L3	NeuNAcα (2→3) Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) Glcβ	-	PEA (1→3)	H	Pavliak *et al.*, 1993
L4	Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) Glcβ	-	H (→3)	PEA (1→6), (1→7)	Dell *et al.*, 1990
L5	Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) Glcβ	Glcβ (1→4)	Glcα (1→3)	H	Michon *et al.*, 1990 Kogan *et al.*, 1997
L6	GalNAcβ (1→3) Galα (1→4) Glcβ	-	H (→3)	PEA (1→6), (1→7)	Di Fabio *et al.*, 1990
L7	Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) Glcβ	-	PEA (1→3)	H	Kogan *et al.*, 1997
L8	Galβ (1→4) Glcβ	-	PEA (1→3)	H	Griffiss *et al.*, 1994 Gu *et al.*, 1992
L9	Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) Glcβ	-	PEA (n.e.)	n.e.	Jennings *et al.*, 1983
L10	Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) Glcβ	n.e.	PEA (n.e.)	n.e.	Scholten *et al.*, 1994
L11	Galα (1→4) Galβ (1→4) Glcβ	n.e.	PEA (n.e.)	n.e.	Griffiss *et al.*, 1994
L12	n.e.	n.e.	PEA (n.e.)	n.e.	Griffiss *et al.*, 1987 Gu *et al.*, 1992
L13	n.e.	n.e.	n.e.	n.e.	Achtman et al., 1992

Abbreviations: Gal, galactose; GlcNAc, N-acetylglucosamine; Glc, glucose; NeuNAc, sialyc acid; Hep, heptose (glycero-D-manno-heptopyranoside); PEA, phospho-ethanolamine; H, hydrogen; n.e., not elucidated

Figure 1.5 Schematic structure of *N. meningitidis* immunotype L(3,7,9) (LOS) in its un-sialylated (L7, L9) and its sialylated form (L3)
[modified from Kahler & Stephans,1998]





1.4.4 Phase variability of LOS expression

The expression of meningococcal immunotypes undergoes phase variation due to *in vitro* growth conditions [Poolman *et al.*, 1985], and they can be modified in the presence of exogenous sialyl transferases *in vivo* [Tsai *et al.*, 1991, Mandrell *et al.*, 1991].

The expression rate and molecular size of meningococcal LOS was shown to depend on the *in vitro* growth conditions. Poolman and co-workers [Poolman *et al.*, 1985] showed an increase in LOS molecular weight, OMP profile and the macroscopic appearance of colonies of meningococci grown in stationary phase compared with those grown in exponential growth conditions. It was suggested that these 'larger' LOS molecules reflect more accurately the *in vivo* phenotypic characteristics of meningococci. It is not clear if the length of meningococcal LOS observed was due to structural modification of the LOS R-core, sialylation, or linkage of several LOS molecules via the lipid A base forming dimers or larger cross-linked LOS polymers. Other studies have confirmed the variability of meningococcal phenotypes and LOS expression depending on growth rate and phase, as well as the presence of exogenous sialyl transferases [Poolman *et al.*, 1985; Tsai *et al.*, 1991, Mandrell *et al.*, 1991].

1.4.5 Structural homology between LOS and human blood group antigens

Some LOS residues mimic human blood group antigens [Mandrell *et al.*, 1988, 1993] (Table 1.6).

Table 1.6 Homology of human blood group antigens with meningococcal LOS residues.

oligosaccharide	**α chain moiety**
P1 blood group	Galα (1→4) Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) Glcβ
p^k, CD77	Galα (1→4) Galβ (1→4) Glcβ
P globoside	GalNAcβ (1→3) Galα (1→4) Galβ (1→4) Glcβ
Paragloboside	Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) Glcβ
i a determinant	Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) GlcNAcβ(1→3) Galβ (1→4) Glcβ
i b determinant	Sialyl-Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) GlcNAcβ(1→3) Galβ (1→4) Glcβ
Cer- dihexocide	Galβ (1→4) Glcβ

Gal, galactose; GlcNAc, N-acetylglucosamine; Glc, glucose; Sialyl, sialyc acid; cer, ceramide; the Glcβ on the reduced end is linked to (1→1) ceramide

The G1 regions of L1 and L11 LOS show identical terminal oligosaccharide residues of ceramide trihexocide, the p^k blood group antigen (CD77, globoside) [Mandrell & Apicella, 1993]. The lacto-N-neotetranose of immunotypes L2, L(3,7,9) and L5 are identical to paragloboside [Tsai & Civin, 1991], a precursor of P1 blood group antigen found in 75% of Caucasians, and the 3 terminal sugars are present in the Ii antigen. Immunotype L6 shares its two terminal sugars with the P blood group antigen, and L8 shares its terminal disaccharide with the common precursor of the P blood group system and steroid receptors. Both blood group antigens and meningococcal LOS with a terminal galactose residue can exist as sialylated and non-sialylated forms [Mandrell *et al.*, 1991; Wakarchuk *et al.*, 1998].

1.4.6 LOS vaccines

The use of meningococcal LOS as a vaccine would have several advantages over other vaccines. The most common LOS immunotype associated with disease is L(3,7,9) found in both group B and C outbreak strains of meningococci in Europe and America. An anti-L(3,7,9) vaccine would be effective against more than 90% of outbreak strains. Meningococcal LOS is highly immunogenic in all age groups, including young children, leading to long lasting protective immunity.

Meningococcal LOS is closely associated with the severity and fatality of disease. This is mainly due to its involvement in inducing large amounts of pro-inflammatory cytokines in a CD14 dependent mechanism (Table 1.1). Anti-meningococcal LOS antibodies are not only bactericidal, but also opsonising in nature [Sjursen *et al.*, 1990], resulting in the phagocytosis of invading bacteria and LOS containing blebs by human monocytes. Normal human serum of adults usually contains antibodies against meningococcal LOS [Goldschneider *et al.*, 1969], suggesting its important role in development of natural immunity to meningococcal disease.

Meningococcal LOS vaccine would induce a strong and potentially fatal inflammatory response due to the toxicity of its lipid A component. The oligosaccharide from which lipid A has been removed is not immunogenic [Verheul *et al.*, 1993].

1.5 Development of natural immunity to meningococci

The majority of people who encounter a pathogenic strain of NM do not develop disease, nor do they necessarily become carriers of this organism. The development of invasive disease in susceptible individuals is associated with the lack of antibodies to the strain of *N. meningitidis* encountered, referred to as an "immunologically virgin population" [Goldschneider *et al.*, 1969].

The protection induced by asymptomatic carriage of meningococci was considered to be an important factor in the development of natural immunity to meningococcal outbreak strains [Goldschneider *et al.*, 1969]. Newborn children have high levels of bactericidal antibodies to meningococci, passive immunity derived from the mother. This protection rapidly declines within the first 6 months of life and coincides with the beginning of greater vulnerability to meningococcal disease at about 6 months to 5 years of age.

1.5.1 Acquisition of antibodies to meningococci by non-groupable meningococci

Goldschneider and colleagues [1969] suggested that there was a steady increase in protective antibodies during early childhood and adult life induced by naso-pharyngal carriage of serogroup B or C meningococci. Later studies found an association between carriage of non-serogroupable meningococci and increased protection against serogroupable meningococci [Reller *et al.*, 1973; Turner *et al.*, 1982; Ross *et al.*, 1985]. Long term carriage of meningococci was reported to induce serum antibodies of the IgG class [Craven & Frasch, 1982; Rosenqvist *et al.*, 1988]. These IgG antibodies were usually directed against outer membrane proteins. Due to antigenic variability and regional variations of these OMP antigens, development of protection would involve the carriage of several strains with different antigenic phenotypes. Sequential carriage of different strains of NM is unlikely to occur in 95% of adults. The involvement of other species in the development of natural immunity is, therefore, more likely to explain "natural" protection against meningococcal disease.

1.5.2 Epitopes on bacterial species cross-reactive with meningococci

Other species were reported to share common antigens with some meningococci. The *N. meningitidis* group A polysaccharide capsule shares cross-reactive epitopes with *Bacillus pumilis* [Robbins *et al.*, 1972]. The K1 antigen of *Escherichia coli*, an exopolysaccharide, is immunologically identical to the poly-α-NeuNAc capsule of group B meningococci [Grados & Ewing, 1970]. There is also evidence that pyocines produced by *Pseudomonas* species recognise different meningococcal and gonococcal LOS epitopes [Blackwell *et al.*, 1979; Blackwell & Law, 1981]. Other mucosal pathogens also share LOS epitopes with meningococci. These include common childhood pathogens such as *Campylobacter* [Aspinall *et al.*, 1992], *Bordetella pertussis* [Maskell *et al.*, 1995], and some *Haemophilus* species [Schweda *et al.*, 1995].

1.5.3 *Neisseria* species

There is a growing body of evidence that epitopes other than proteins or capsular polysaccharides are involved in the development of natural immunity to meningococci. Early studies to phenotypic similarities between meningococci and gonococci showed common LOS epitopes identical to the paragloboside precursor of the P1 blood group [Apicella *et al.*, 1987, Mandrell *et al.*, 1988]. These studies suggest a high degree of genetic and phenotypic similarity between members of the genus *Neisseria* in relation to LOS biosynthesis and structure.

N. lactamica (NL) is a non-pathogenic commensal [Rio *et al.*, 1983], rarely reported to cause disease in humans [Herbert & Rusken, 1981]. Although it is carried with a frequency between 5-14 % among young children [Cartwright *et al.*, 1987], other studies observed a much higher carrier rate of up to 59% within the first four years of life, which was linked to an increase in bactericidal activity against meningococci [Gold *et al.*, 1978].

This development of protective immunity coincides with incidence of meningococcal disease with children under the age of 5 making up the largest group affected (Figure

1.1). There is evidence that carriage of *N. lactamica* coincides with the progressive increase in the level of natural immunity [Goldschneider *et al.*, 1969; Coen *et al.*, 2000].

1.5.3.1 OMP

There is no evidence that carriage of NL induced antibodies to outer membrane protein obtained from meningococcal isolates causing disease in Greece [Kremastinou *et al.*, 1999b]. Other OMP, including the the H.8 and some iron regulatory proteins were reported to share some structural homologous epitopes found in several *Neisseria* species [Cannon *et al.*, 1984; Mietzner *et al.*, 1986, Troncoso *et al*, 2000].

1.5.3.2 Capsule

Several authors reported that a minority of NL isolates were able to produce small amounts of exo-polysaccharide that resulted in agglutination by serogroup B anti-sera [Saez-Nieto *et al.*, 1985]. Another study revealed, that a maximum of 6 % of isolates from children cross-reacted with group B, and 3 % with group 29E capsular serum [Gold *et al.*, 1978]. Other studies suggested the absence of capsules on NL, because NL isolates did not react with commercially available anti-capsular antibodies routinely used for serogrouping [Kim *et al.*, 1989; Zorgani *et al.*, 1996; Kremastinou *et al.,* 1999a].

1.5.3.3 LOS

The structures and expression of LOS of NL appear to be as diverse as those of meningococci. Kim and colleagues [1989] identified epitopes common to NL which were recognised by two monoclonal antibodies produced against NM. The antibody D6A bound to meningococcal immunotypes L1, L8, L(3,7,9), L10 and L11. The antibody 06B4 bound to L2, L4, L8 and L(3,7,9) immunotypes.

None of these immunotypes share any of the P-blood group related saccharides of the

G1 terminal α-chain of meningococcal LOS. Later analysis of the LOS structure revealed that these immunotypes share a common core structure, the G2 and G3 region of the second core heptose (HepII) (Table 1.4). The third carbon shares an hydroxyl (-OH) group, and the fourth carbon contains a PEA residue [Plested *et al.*, 1999]. These findings might partially explain the ability of some NL strains to absorb cross-reactive bactericidal antibodies against meningococcal outbreak strains [Zorgani *et al.*, 1996]. The binding of the 6DA and 06B4 monoclonal antibodies to NL suggests that these two antibodies recognise the common HepII/H/PEA core domain present in some NL strains.

1.5.4 Moraxella catarrhalis

M. catarrhalis (MC) is a commensal Gram-negative diplococcus previously classified within the genera *Branhamella* and *Neisseria* [Murphy, 1996; Catlin *et al.*, 1982, 1990]. Recent genetic studies resulted in the re-classification of MC into the genus *Moraxella* [Enright & McKenzie, 1997].

Although MC is associated with some childhood diseases [Hager *et al.*, 1987], it is frequently isolated as a commensal from the respiratory tract in healthy young children. Children were reported to be colonised with 3-4 different strains of MC within the first two years of life [Faden *et al.*, 1994; Ejlersten *et al.*, 1994]. It is isolated more frequently than *Neisseria* species during the first 6 months of life when infants are developing antibodies to the bacteria in their environment [Harrison *et al.*, 1999]. One member of the genus, *Moraxella nonliquefaciens* expresses a capsular antigen similar to group B meningococci and *E. coli* K1, and it is isolated from about 20% of healthy carriers [Devi *et al.*, 1991].

Several surface antigens are thought to be involved in the development of immunity to MC. Carriage and infections with MC are associated with the development of protective IgG from an early age [Goldblatt *et al.*, 1990]. These include protein antigens and glycoconjugates. Two OMP are associated with protective immunity, UspA1 and UspA2 [Aebi *et al.*, 1997; Cope *et al.*, 1999] possibly due to structural homology and cross-reactivity detected with monoclonal antibodies [Klingman &

Murphy, 1994].

Although LOS from MC differs structurally from meningococcal LOS [Schneider *et al.*, 1984], both species share some homology in their oligosaccharide chain moieties. Terminal oligosaccharide residues found on the non-reducing end of MC LOS share some homology with human blood group antigens [Vaneechoutte *et al.*, 1990; Rahman & Holme, 1996]. Combination of five different saccharide residues of the α or β chains determine the immunotype of MC LOS (Gal, galactose; Glc, glucose; GlcNAc, N-acetylglucosamine; KDO, 2-keto-3-deoxyoctulosonic):

1. Galα (1→4) Galβ (1→4) GlcNAcα (1→2) Glcβ;
2. Galα (1→4) Galβ (1→4) Glcα (1→2) Glcβ;
3. GlcNAcα (1→2) Glcβ;
4. Glcα (1→2) Glcβ;
5. Glcβ.

1.6 Aims

The aims of this study were to assess the role of cross-reactive antigens found on commensal bacteria (NL and MC) in the development of natural immunity to meningococci, and to identify potential vaccine candidates that would be safe and effective in children and adults against meningococcal disease caused by group B meningococci.

The objectives of this study were to address the following questions:

1. Are there antigens on NL that induce antibodies bactericidal for meningococci of different LOS immunotypes?
2. Are there antigens on MC that induce antibodies bactericidal for meningococci of different LOS immunotypes?
3. Are there oligosaccharide antigens common to NL, MC and meningococcal immunotype reference strains?
4. Are the antibodies to NL and MC cross-reactive with meningococci capable of neutralising the bioactivity of LOS?
5. Do the antibodies to NL and MC cross-reactive with meningococci have opsonising activities?

Chapter 2 General Material and Methods

2.1 Procedure for handling infectious specimens and hazardous substances

All biological specimen were treated as potentially infectious material and marked with appropriate safety labels and hazard signs. Laboratory coats and gloves were worn throughout when handling specimen, and eye, face or respiratory protection used when appropriate. All carcinogenic and toxic substances were prepared in a fume hood. Once the substance had been diluted to the appropriate concentration it was handled in accordance with COSHH risk assessment procedures. Sharps were disposed for incineration in safety boxes, and biological material autoclaved. Risk assessments for each procedure were completed, approved by the supervisor and filed before the work began.

2.2 Phosphate buffered saline

Phosphate buffered saline (PBS) contained sodium chloride (6.8 g) (Sigma), di-sodium-phosphate (1.43 g) (Na_2HPO_4 $2H_20$) (BDH) and potassium-di-hydro-phosphate (0.43 g) (KH_2PO_4 $2H_2O$) (Fisons) in 1 litre of distilled water and adjusted to pH 7.2 with hydrochloric acid (0.1 N) (HCl) (BDH) or sodium hydroxide (0.1 N) (NaOH) (Sigma).

2.3 Formalin

Bacteria were fixed with formalin buffered PBS (0.5, % v/v) containing formalin (6.25 ml, 40 % v/v) (BDH) in PBS (494 ml).

2.3 Paraformaldehyde

Eukaryotic cells were fixed in paraformaldehyde (2%, w/v) which is used as a crosslinking reagent for membrane proteins and cell markers, allowing fixed cells to be stored for extended periods with little degradation. Paraformaldehyde (10 g) and FA Bacto buffer (5 g) (Difco) were dissolved in distilled water in the dark overnight.

Once the solution cleared, the pH was adjusted to 7.3-7.4 with HCl (0.1 N) or NaOH (0.1 N). Distilled water was added to a final volume of 500 ml. The solution was stored in the dark for up to one month [Crissan & Steinkamp, 1999].

2.4 Vindelov's Propidium Iodide (VPI)

Tris buffer (1 N) was prepared by dissolving Tris HCl (8.88 g) (Sigma) and Tris base (5.3 g) (Sigma) in 100 ml distilled water and the pH adjusted to pH 8. Tris (1N pH 8 (10 ml), RNAse (700 mg) (Sigma), Nonidet P-40 (1 ml) (Sigma), NaCl (58.4 g), and propidium iodide (PI) (50 mg) (Sigma) were dissolved in 1 litre distilled water. The VPI solution was stored in the dark at 4°C for up to three months. The solution was sterilised by filtration before use [Vindelov, 1977].

2.5 Growth of *Neisseria* species

2.5.1 Human Blood agar (HBA)

Whole blood (100 ml) obtained from the Scottish National Blood Transfusion Service (SNBTS) was frozen at -20°C to lyse red blood cells. The lysed blood was allowed to thaw at 37°C. Special peptone (23 g) (Difco), corn starch (1g) (Sigma), NaCl (4.5 g) (Sigma), D-glucose (1 g) (Sigma), technical grade agar (10 g) (OXOID), K_2HPO_4 (4 g) and KH_2PO_4 (1 g) were added to 900 ml of distilled water and autoclaved at 121°C for 15 min. After allowing the agar to cool to approximately 50°C, lysed human blood (100 ml) was added aseptically. The human blood agar (15 ml) was poured into sterile plastic plates (Greiner) and allowed to cool at room temperature overnight. The HBA plates were kept sealed in plastic bags for up to two weeks at 4°C.

2.5.2 Commercially obtained media

Other growth media were purchased from OXOID: GC selective agar plates containing lincomycin (1 $\mu g\ ml^{-1}$), trimethoprim (6 $\mu g\ ml^{-1}$), colistin (6 $\mu g\ ml^{-1}$),

amphoteracin B (1 μg ml^{-1}) and trimethoprim lactate (6.5 μg ml^{-1}); horse blood agar plates (Oxoid).

2.6 Growth conditions:

Bacterial strains were inoculated onto plates pre-warmed to room temperature (RT) and were incubated at 37°C for 18 h in a humidified atmosphere with 5 % (v/v) carbon dioxide (CO_2).

2.7 Complement source

Human serum was co-incubated with 18 h cultures of the test strains (10^{10}- 10^{12} ml^{-1}) and incubated at 4°C overnight. The serum was centrifuged at 1000 x *g* for 30 min, the supernatant filtered through a 0.22 μm filter (Nu-flow OXOID) and tested for sterility by culturing 100 μl on HBA medium. Aliquots were stored at –70°C for up to 6 months. Fresh sheep rbc obtained from the Scottish Antibody Production Unit (SAPU) were washed with PBS at 200 x *g* for 5 min and re-suspended in PBS to a final concentration of 4% (v/v). Equal volumes of 4% sheep rbc and donkey anti-sheep rbc serum (SAPU) diluted 1 in 100 were incubated at 37°C for 30 min. Doubling dilutions of the absorbed complement source (25μl) were prepared in PBS. Sensitised rbc (25 μl) diluted 1 in 2 were added to the diluted serum and incubated at 37°C for 30 min. Lysis of the cells was analysed macroscopically. The working titre was the one preceding the highest serum dilution showing full haemolysis.

2.8 Bacterial strains

The serogroup, serotype and subtype (*e.g.*, B:2a:P1.5,2) of meningococcal or NL strains were assessed by commercially available typing reagents (Wellcome Diagnostics) or by the standard whole cell ELISA used by reference laboratories from which the isolates were obtained. All Scottish *M. catarrhalis* isolates (Chapter 5) were obtained from our culture collection (Dr. O. El-Ahmer). Standard immunotype strains of meningococci L1-L12 were obtained from Dr. W. D. Zollinger, Walter Reed Army Institute of Research, Washington D.C., USA (Table

2.1.a). Additional strains of NM (Table 2.1.b) and NL (Table 2.2) were obtained from our culture collection or from: Dr. P. Krizova, National Reference Laboratory for Meningococcal Disease, Prague, Czech Republic; Dr. K. Jónsdóttir, University Hospital, Reykjavik, Iceland; Dr. G. Tzanakaki, National Meningococcal Reference Laboratory, National School of Public Health, Athens, Greece; and Dr. S. Clarke, Scottish Meningococcal and Pneumococcal Reference Laboratory (SMPRL), Glasgow, Scotland.

Table 2.1.a *N. meningitidis* immunotypes reference strains

Strain	Code	Major LOS	Minor LOS	Origin
C:NT:P1.2	126E	L1	8	Washington
C:2c:P1.1	35E	L2	3,7	Washington
B:2a:P1.5, 2	6275	L3	8	Washington
C:11:P1.16	89I	L4		Washington
B:4:P1.NT	M981	L5	3,7	Washington
B:5:1.7, 1	M992	L6		Washington
B:9:P1.7, 1	6155	L7	3, 8	Washington
B:8:P1.7, 1	M978	L8	3, 4, 7	Washington
A:21:P1.10	120M	L9	6, 8	Washington
A:21:P1?	7882, A39	L10	8	Washington
A:21:P1.10	7889, A44	L11		Washington
A:21:P1.NT	7897, A42	L12		Washington

Table 2.1.b *N. meningitidis* strains

Strain	Code	Origin
B:NT:NT	I&I, 99-760	Scotland
B:15:NT	I&I B15	Scotland
B:14:P1.16	B14, ICE99	Iceland
B;15:P1.16	I&I, B08, A11	England
NG:4:NT	NG4	Greece
B:2a:P1.2, 1.5	STB	SMPRL
C:2a:P1.2	STC	SMPRL

Table 2.2 *N. lactamica* strains

Phenotype	CODE	Geographical source
NG:NT:NT	CZ1	Czech Republic
NG:NT:NT	CZ2	Czech Republic
NG:NT:NT	CZ3	Czech Republic
NG:NT:NT	CZ4, NL7	Czech Republic
NG:NT:NT	GRE162, NL6	Greece
NG:NT:NT	GRE179	Greece
NG:NT:NT	GRE184ns	Greece
NG:NT:NT	GRE184SW	Greece
NG:NT:NT	GRE211	Greece
NG:NT:NT	GRE213	Greece
NG:NT:NT	GRE213	Greece
NG:NT:NT	GRE227ns	Greece
NG:NT:NT	GRE227sw	Greece
NG:NT:NT	GRE228NS	Greece
NG:NT:NT	GRE228SW, NL4	Greece
NG:NT:NT	GRE268ns	Greece
NG:NT:NT	GRE268sw	Greece
NG:NT:NT	GRE309	Greece
NG:NT:NT	GRE334	Greece
NG:NT:NT	GRE359NS, NL5	Greece
NG:NT:NT	GRE359SW	Greece
NG:NT:NT	GRE409	Greece
NG:NT:NT	GRE534	Greece
NG:NT:NT	GRE538	Greece
NG:NT:NT	GRE619ns	Greece
NG:NT:NT	GRE619sw	Greece
NG:NT:NT	GRE634ns	Greece
NG:NT:NT	GRE634sw	Greece
NG:NT:NT	GRE806	Greece
NG:NT:NT	GRE854ns	Greece
NG:NT:NT	GRE854SW	Greece
NG:NT:NT	ICE, NL3	Iceland
NG:NT:NT	I&I SCO 1751	Scotland
NG:NT:NT	I&I NL1	Scotland
NG:NT:NT	I&I SCO1567	Scotland
NG:NT:NT	I&I SCO1568L	Scotland
NG:NT:NT	I&I SCO1568S	Scotland
NG:NT:NT	I&I SCO318	Scotland
NG:NT:NT	I&I SCO390	Scotland
NG:NT:NT	I&I SCO393	Scotland
NG:NT:NT	I&I SCO395NS	Scotland
NG:NT:NT	I&I SCO395SW	Scotland
NG:NT:NT	I&I Sco99/248F, NL2	Scotland
NG:NT:NT	I&I Sco99/141, NL8	Scotland

2.9 Procedure for handling adherent cell lines and cell lines in suspension

Products of human and animal origin are potential biohazards. Growth media were prepared by adding aseptically appropriate amounts of supplements to basal media. Sterility was checked by suspending 2 ml of medium in 50 ml nutrient broth in glass bottles (SNBTS), followed by incubation overnight aerobically at 37°C and plating 1 ml of the broth onto nutrient agar to detect contamination. If there was concern that sterility was compromised during cell culture procedures, media were sterilised by filtration using a 0.22 μm filter.

2.9.1 Thawing frozen cultures

Cell lines were obtained from the European Collection of Animal Cell Cultures (ECACC, UK) (Table 2.3). Gloves were worn while handling frozen ampoules stored in the gaseous phase of liquid nitrogen. Eye and face protection was used throughout to avoid injury due to the risk of ampoules exploding during thawing.

Table 2.3 Cell lines

Name	Description	Source Number
L929	Mouse C3H/An areolar and adipose tissue, fibroblasts	85103115 CB2400
THP 1	Human monocytic leukaemia, monocyte	88081201 Lot 98/K/018
Bristol 8	Human B lymphocyte	85011436 CB2452

Ampoules were kept at room temperature briefly and transferred to a 37°C water bath for 2 min until fully thawed. The outside of the ampoule was disinfected with a tissue soaked in ethanol (70 %, v/v) and the contents of the vials were slowly pipetted into 30 ml pre-warmed (37°C) medium supplemented with foetal calf serum (FCS) (10%, v/v), penicillin (100 U ml^{-1}), streptomycin (100 μg ml^{-1}), and L-glutamine (1mM) (PSG, Sigma). The suspension was split into halves and each part transferred into a sterile cell culture flasks (Nunc) with an effective growth area of 75 cm^2 and incubated in a humidified atmosphere with 5 % (v/v) CO_2. Every 2-3 days

medium was partly exchanged by replacing 10 ml with fresh pre-warmed medium until approximately 70% confluent growth was achieved.

2.9.2 Culture of adherent cells

To split cells that had reached confluence of 70-80 %, the medium was decanted, the cell sheet washed twice in pre-warmed (37°C) sterile PBS, and cells were incubated with 2 ml of pre-warmed porcine trypsin (0.5 g l^{-1})/ EDTA (0.2 g l^{-1}) (Sigma) or EDTA (0.2 g l^{-1} EDTA 4Na in HBSS) alone (Sigma) at 37°C for a maximum of 10 min until all cells detached. Alternatively, cells were carefully scraped with a sterile cell scraper (Greiner). Cells were resuspended in pre-warmed medium at the appropriate cell densities and cultured.

2.9.3 Culture of non-adherent cells

Once the cell culture reached a density of 5 x 10^5 cells ml^{-1}, the cell suspension was decanted carefully into 25 ml sterile pyrogen free universal tubes (Greiner) and centriguged at 100 x *g* for 5 min. The cells were suspended in one part current medium and 2 parts of fresh medium to a final recommended cell density (THP-1 cells, $2x10^5$ - $9x10^5$) and grown in tissue culture flasks as described above.

2.9.4 Freezing cells

Cells were harvested, centrifuged at 100 x *g* for 5 min and the pellet washed once in medium omitting antibiotics. The pellet was re-suspended in heat inactivated foetal calf serum (FCS) and transferred into an equal volume of 80 % (v/v) FCS and 20 % (v/v) cryopreservant dimethyl sulphoxide (DMSO, Sigma) in cryovials (Greiner). Vials were placed into an iso-propanol freezing box (Nalgene) and frozen at a rate of 1-3°C min^{-1} in a -70°C freezer overnight. Vials were transferred into the gaseous phase of liquid nitrogen.

2.10 Extraction of LOS

LOS was extracted by the hot phenol water method of Westphal & Luderitz [1954] as described by Hancock & Poxton [1988].

2.10.1 Hot phenol water method

Bacteria grown on HBA were harvested, washed in sterile pyrogen free PBS, centrifuged at 1000 x *g*, re-suspended in pyrogen-free distilled water and aliquots were kept frozen (-70°C). The bacteria were freeze-dried and re-suspended in pyrogen free water (5%, w/v). The cell suspension was heated to 67° C in a water bath, and an equal amount of pre-warmed (67° C) phenol (90%, w/v) (Sigma) dissolved in pyrogen free water was added in a fume cabinet. The cells were incubated for 30 min at 67° C and transferred to an ice bath. The phenol-soluble protein and cell fragments were removed from the water-soluble LOS by ultra-centrifugation at 10,000 x *g*. The top aqueous solution was carefully removed and dialysed against tap water for 18 h in a 2 kDa permeable membrane (Spectra Por) (Fisher). The contents of the dialysis membrane were centrifuged for 4 h at 100,000 x *g*. The pellet was recovered, frozen at –70° C and freeze-dried. The purified LOS contained protein contamination of <1% (w/w) as assessed (2.10.2) against a standard of bovine serum albumin (BSA) (Sigma). The LOS was re-suspended in RPMI-1640 medium (Sigma), and filtered through a 0.22 μm membrane filter. Aliquots were stored at –70° C and two samples from each batch were incubated in normal nutrient broth (1 ml) (SNBTS) at 37° C for 18 h to test for sterility.

2.10.2 Protein assay

The Bradford reagent contained Coomassie blue G250 (0.01%, w/v) (Sigma), ethanol (4.7% v/v) and phosphoric acid (8.5%, w/v) (BDH) in pyrogen-free distilled water. The solution was filtered through Whatman No. 1 filter paper prior to use.

Serial dilutions of BSA (0.01 μg ml^{-1} to 1 mg ml^{-1}) (Sigma) and freeze-dried LOS stock were dissolved in sterile distilled water. Each solution was mixed with

Bradford reagent at a ratio of 1 to 5 and incubated at room temperature for 5 min. The absorbance of the solution was measured at OD_{595} using distilled water as a blank. The amount of the protein contamination ($\mu g\ ml^{-1}$) was calculated by comparing the absorbance value of the LOS samples with the absorbance values of the BSA protein standard [modified from Bradford, 1976].

2.11 Immune mouse sera

N. lactamica strain NL1 or *N. meningitidis* immunotype strain L3 (B:2a:P1.5,2) were killed by heating for 60 min at 100°C. Individual strains (10^9 bacteria , 100 µl) were injected in adjuvant free and pyrogen free saline (SIGMA) into the tail vein of three six week old male BALB/c mice. Eight consecutive injections were given allowing the mice to rest for three weeks between each injection. The final injection was LOS (10 ng ml^{-1}) obtained from the corresponding strain (2.10) dissolved in adjuvant free and pyrogen free saline [Yount *et al.*, 1968; Harlow & Lane, 1988].

Antibodies to the bacterial strains in samples collected prior to immunisation and samples collected 24 h prior to the final injection were assessed by whole cell ELISA as detailed in chapter 6. Three days after the final injection, blood was collected aseptically by cardiac puncture, allowed to clot, centrifuged at 500 x *g* for 15 min at 4°C. The supernatant was collected and diluted in pyrogen free saline (1 in 100). Complement was inactivated by heat treatment (56°C for 30 min) and the sera were stored in aliquots (1 ml) at –70°C. Antibodies (IgG) to the bacteria were assessed by whole cell ELISA (5.2.4). The production of antibodies was covered by an animal licence obtained from the British Home Office.

2.12 Analysis of surface antigens and ingested bacteria by confocal microscopy

Cell suspensions (50 µl) of test and control samples were added to clean microscope slides (Menzel), and a glass cover slide was carefully placed to avoid air bubbles. The sides of the coverslip were sealed with clear nail polish (Boots) and allowed to air dry in the dark at RT. The samples were kept in the dark for not more than 24 hours before being analysed by confocal microscopy (Dr. J. Bard, Department of

Anatomy, University of Edinburgh Medical School). Magnifications used were 1,000 x using water immersion fluorescence and dark field lenses. Sections of 0.9-1.2 μm were used to build up a three dimensional image of the stained cell. Compensation was used to account for wavelength interference for samples in which two different colour stains were used. Images were analysed by Imaging for MS-Windows and Adobe Photoshop 3.0 for Apple Macintosh computers. Slides and photographic hard copies were produced by the Department for Medical Illustrations, University of Edinburgh Medical School.

2.13 General flow cytometric method for cell surface and intracellular staining analysis

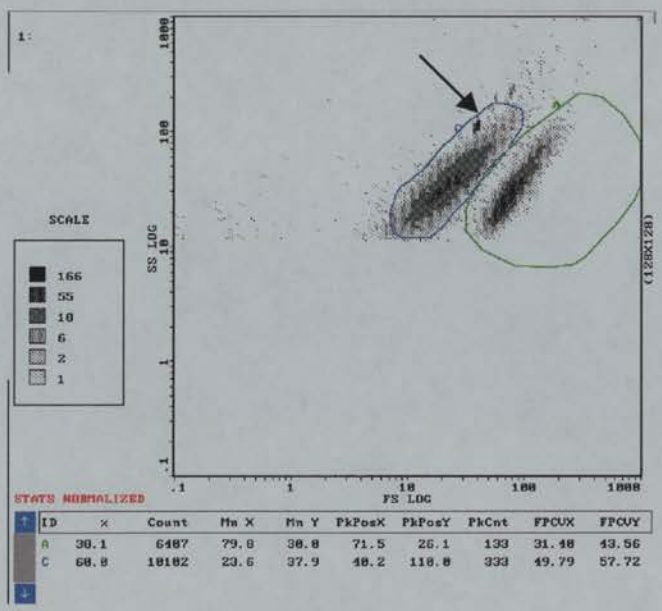
2.13.1 Optimising flow cytometric procedures

Initial optimisation of the assay procedures required the assessment optimal working dilutions. Although the manufacturer's recommended dilution or previously published dilutions for the reagents were used as a guideline, each batch of antibodies was assessed for optimal working dilutions [Robinson, 1993; Lenkei *et al.*, 1998; Serke *et al.*, 1998; Smith & Ellis, 1999; Horsburgh *et al.*, 2000]. Two-fold dilutions of the antibody were prepared in PBS above and below the recommended dilution and were used in a standard single colour flow cytometry assay using a known positive cell line or bacterial strains reported to express a particular antigen. The analysis of fluorescence histograms showed a range of percentages of positive cells in the different populations (two percent of background method) and fluorescence intensities (MnI). The optimal titre was selected by comparing these two parameters against published literature.The optimal assay conditions for each antibody and cell type were used throughout. To avoid release of bound antibody from the cell surface or loss of fluorescence, all cells samples were fixed in paraformaldehyde (2%, w/v) and were kept in the dark on ice for not more that 60 min until analysed with an Epics XL flow cytometer (Coulter, Luton).

Directly conjugated primary antibodies were analysed using the appropriate class of immunoglobulin (*i.e.* non-specific iso-matched fluorochrome-conjugated mouse IgG

when antibodies raised in mice were used) as a negative control. A distinctive cell population with a fluorescence greater than the control (>2%) was considered to be positive. If the fluorescence of the control was ≥5% of the autofluorescence of cells alone, the data were rejected and the experiment repeated due to high non-specific binding of the secondary antibody. A blocking solution containing PBS and bovine serum albumin (BSA) (1%, w/v) (Sigma) was then used throughout the staining procedure. Mean fluorescence intensity (MnXI, MnYI), the mean fluorescence (MnX, MnY), the median (MdX, MdY), the standard deviation of the appropriate cell population, and the percentage of fluorescent cells (F %) were recorded (Figure 2.2).

Figure 2.2 Analysis of THP1 cells on a forward and side scatter histogram: The population on the right (A) contains THP-1 cells, the population on the left (B) cell debris and un-bound bacteria. Fluorescence alignment beads (marked by arrow)



2.13.2 Flow cytometry analysis sample preparation

The cell samples were kept in the dark on ice for not more that 60 min until analysed. The EPICS XL flow cytometer contained an air-cooled 15 MW argon laser operating at 488 nm, and an air-cooled 10 MW helium-neon laser operating at 633 nm. Forward angle light scatter detection was measured via a split photo diode detector,

while right angle light scatter and fluorescence was measured by four photo-multiplier tubes with a spectral range from 200 to 800 nm, and dichroic splitting and bandpass filters. Not less that 5,000 events were sorted (Region A) by size and granularity using graphical logarithmic forward scatter (logFS) and graphical logarithmic side scatter (logSS) respectively.

Prior to analysis, the flow cytometer was aligned to avoid day to day variation in laser strength, gating, or channel settings. Fluorescence alignment beads (10 µl containing 5x 10^6 beads) (ImmunoCheck, Coulter) were added to 1 ml PBS and vortexed. Samples were gated on a forward light scatter (logFS) and side light scatter (logSS) and quantified by exciting the fluorochromes embedded within the beads with an argon laser operating at 488 nm. The emitted green fluorescence of the beads was measured between 505 and 545 nm (logFL1) and for red fluorescence between 595 and 645 (logFL3). The mean fluorescence intensity (MnI) of the beads was adjusted daily to a signal reading of 500. After each reading session the EPICS XL was cleaned using reagents provided by Coulter.

Compensation was used to account for wavelength interference in two colour stains. In two colour assays, fluorescein isothiocyanate (FITC) (green) or R-phycoerythrin (PE, R-PE) (red) wavelength interference can be reduced by subtracting the green fluorescence (FITC) from the red (PE) in a logFL1, logFL2 (logFL3 for PI) histogram. The compensation was set to a red fluorescence of less than 1%. All settings of laser strength and compensation were recorded for future references.

2.13.3 Guidelines for identification of positive and negative samples

Flow cytometry was used to assess the expression of cell surface antigens on bacteria (Chapter 5), on eukaryotic cells (Chapter 6) and the quantitative enumeration of ingested or bound bacteria in phagocytosis assays (Chapter 7).

Several models can be used for the analysis of histograms including: the channel-by-channel subtraction method; cumulative subtraction and maximum positive difference methods; the two-percent-of-background method; and the relative mean

fluorescence intensity analysis [Lampariello & Aiello, 1998]. The most commonly used methods are the "two-percent-of-background" method and the "relative-mean-fluorescence-intensity" methods and were used in this study.

2.13.3.1 Two-percent-of-background method

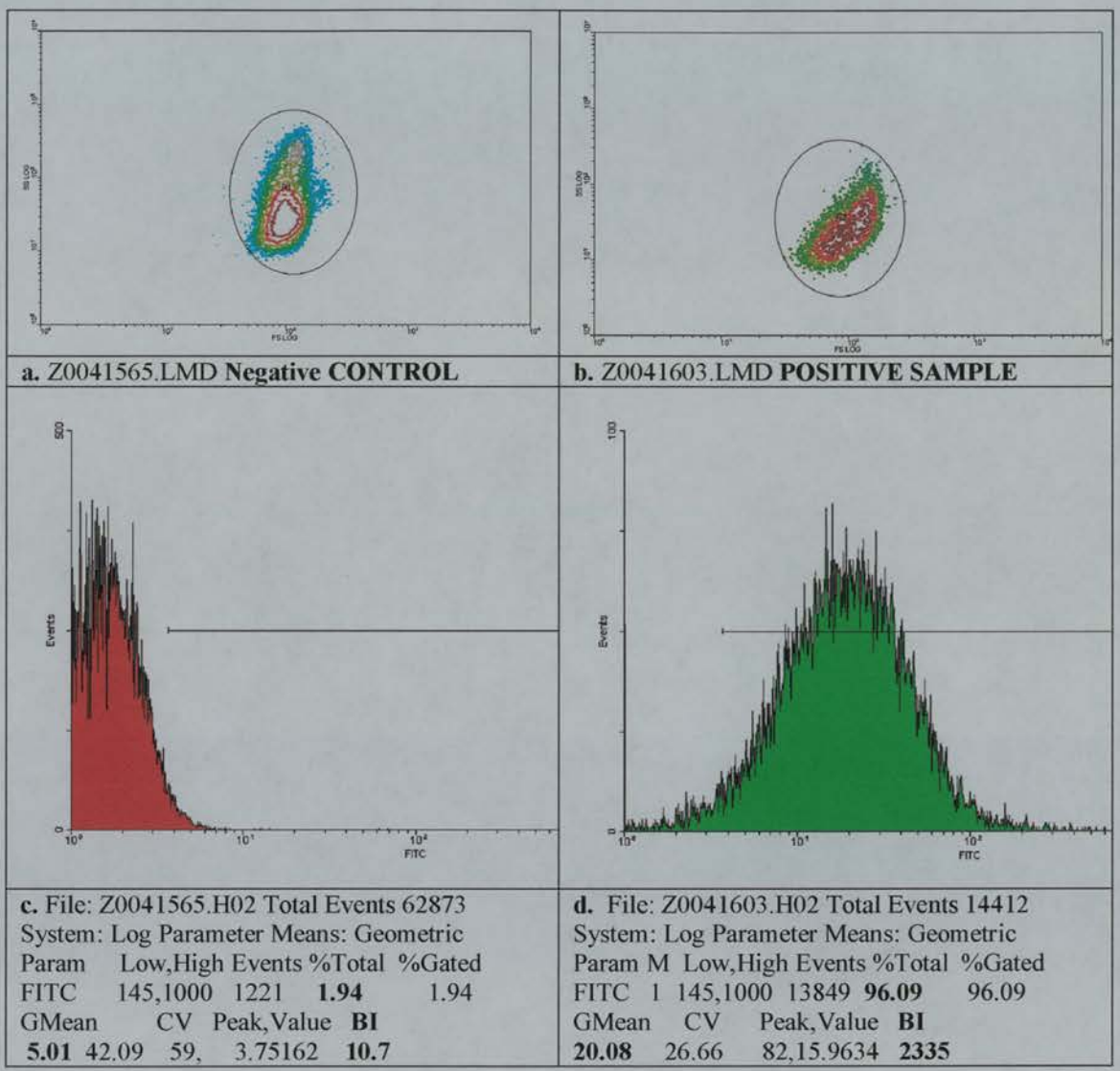
This method is based on the assumption that the primary monoclonal antibodies used are specific for the epitope to be investigated. The use of polyclonal antibodies does not allow the detection of a single epitope, but of an antigen that might possess more than one epitope to be investigated. Secondly, the epitope must be accessible, otherwise pre-treatment with biological (*i.e.*, enzymes) or chemical (*i.e.*, ethanol) reagents is needed to make the cell membrane permeable to the antibodies. This method assumes that each epitope is occupied by an antibody or the true expression of the antigen would be under-estimated. The affinity of the secondary conjugated antibody (sandwich method) must ensure that all primary antibodies are recognised by the secondary antibody. The secondary antibody must not cross-react with non-specific epitopes on the cell surface in greater numbers than the number of primary antibodies bound to specific antibodies.

Each sample requires of a positive and a negative control. The negative control for conjugated-primary antibodies consists of iso-antibodies, *i.e.*, FITC-labelled mouse IgG1 antibodies if the primary antibody is of mouse IgG1 origin. Using a sandwich method, the negative control consists of cells incubated with the conjugated secondary antibody in the absence of the primary antibody. In both cases, this negative control is assessed for fluorescence. This population is gated for a region that contains less than 2% of the total events (Figure 2.5). Measuring the sample incubated with the conjugated primary or un-conjugated primary and conjugated secondary antibody identifies the positive population (>2%) (Figure 2.5). A more stringent definition was applied in Chapter 5, where a population showing less than 5% was considered as borderline and scored as not expressing the epitope or antigen.

2.13.3.2 Relative mean fluorescence intensity method

By converting the geometric mean of the fluorescence signal (logFL1) into units of relative fluorescence, the mean fluorescence intensity (MnI) can be calculated. The relative MnI in relation to fluorescence standard beads (Immunocheck, Coulter) allowed the expression of fluorescence intensity in units of bound fluorochrome [Gratama *et al.*, 1998]. This method is based on the assumption that the affinity of the secondary conjugated antibody, if any, must ensure that all primary antibodies bind an equal number of secondary antibodies. Otherwise multiple binding of secondary antibodies to the primary antibody would result in over-estimation of the antigen present. Calculation of the percentage of positive cells in a population from the two-percent-of-background method with the MnI allows an accurate semi-quantitative enumeration of antigen present. In addition to assessment of cell surface markers, this method could be used to assess quantitative phagocytosis described in Chapter 7.

Figure 2.5 Forward and side scatter histogram of red blood cells stained with (a) FITC-labelled anti-mouse IgM, (b) anti-paragloboside (mouse IgM) and FITC-labelled anti-mouse IgM;
the corresponding FITC-histograms of the two populations are shown in c and d, respectively



a. Z0041565.LMD **Negative CONTROL**

b. Z0041603.LMD **POSITIVE SAMPLE**

c. File: Z0041565.H02 Total Events 62873
System: Log Parameter Means: Geometric

Param	Low,High	Events	%Total	%Gated
FITC	145,1000	1221	**1.94**	1.94

GMean	CV	Peak,Value	**BI**
5.01	42.09	59, 3.75162	**10.7**

d. File: Z0041603.H02 Total Events 14412
System: Log Parameter Means: Geometric

Param	M	Low,High	Events	%Total	%Gated
FITC	1	145,1000	13849	**96.09**	96.09

GMean	CV	Peak,Value	**BI**
20.08	26.66	82,15.9634	**2335**

Chapter 3 Assessment of the antigenic cross-reactivity between *Neisseria lactamica* and *N. meningitidis*

3.1 Introduction

Carriage of both non-serogroupable and groupable strains of meningococci is considered to be an important factor in the development of natural immunity to meningococcal outbreak strains [Goldschneider *et al.*, 1969; Reller *et al.*, 1973; Turner & Hendley, 1982; Ross *et al.*, 1985]. There is evidence that carriage of another member of the genus *Neisseria*, the non-pathogenic commensal *N. lactamica* (NL), coincides with a progressive increase in the level of natural immunity against meningococci [Coen *et al.*, 2000]. Between 5-59% of children under the age of four have been reported to be carriers of NL, and their carrier status was linked to an increase in bactericidal antibodies against meningococci [Gold *et al.*, 1978].

It has not been established which epitopes are responsible for the induction of antibodies that are cross-reactive between NL and other *Neisseria* species. Some OMP of NL were reported to share homology with meningococcal OMPs [Troncoso *et al.*, 2000]. Carriage of NL does not, however, induce significant production of either IgG or IgM to OMP derived from meningococcal strains causing disease in Greece [Kremastinou *et al.*, 1999a]. Very few NL strains react with monoclonal antibodies used for serotyping and subtyping [Kremastinou *et al.*, 1999b; Zorgani *et al.*, 1996], and several studies have reported that NL is not serogroupable using anti-capsular sera [Kim *et al.*, 1989; Zorgani *et al.*, 1996].

The most promising candidate for cross-reactive antigens is the LOS structure of NM and NL. Kim and colleagues [1989] identified common epitopes on meningococcal immunotypes L1, L(3,7,9), L4, L8, L10 and L11 and some strains of NL. The immunotype L(3,7,9) is isolated most frequently from patients with meningococcal disease (>90%) compared with other immunotypes which were found primarily among carriers [Jones *et al.*, 1992; Romero & Outschoorn, 1994]. This homology between immunotype L(3,7,9) LOS from NM and some NL isolates and the

observation that high levels of antibodies to NL strains were associated with the killing of serogroupable strains of meningococci [Zorgani *et al.*, 1996] suggest the endotoxin of these bacteria might be one of the antigens that induces protective antibodies against disease causing strains.

The objective of this part of the study was to investigate NL isolates from different European countries for their ability to absorb bactericidal antibodies to meningococcal strains of different immunotypes.

3.2 Materials and Methods

3.2.1 Bacterial strains

Standard immunotype strains of meningococci L1-L12 were obtained from Dr. W. D. Zollinger, Washington D.C (Table 2.1). NL isolates used in this part of the study were obtained from Scotland (NL1 and NL2), the Czech Republic (NL7), Iceland (NL3) and Greece; (NL4, NL5 and NL6).

3.2.2 Bactericidal Assay

The microtitre plate method described by Zorgani *et al.* [1996] was used to screen for bactericidal activity.

3.2.2.1 Serum source

A pool was prepared with serum from eight healthy adult donors with no known history of meningococcal disease. None of the donors had been vaccinated with the new conjugate meningococcal group C vaccine or had taken antibiotics within the two weeks before collection of the blood sample. One donor had received the A/C polysaccharide vaccine. The pool was inactivated at 56°C for 30 min, divided into aliquots which were absorbed twice at 4°C overnight with viable individual strains of NL (10^{10} bacteria ml^{-1}), centrifuged at 1000 x *g* and filter sterilised using a 0.22 μm membrane (Nu-flow, Oxoid). Aliquots of the absorbed sera were tested for sterility and stored at -70°C.

3.2.2.2 Complement Source

The complement source was prepared from a blood sample from a healthy adult volunteer with no known history of meningococcal disease. Serum was supplemented with 1mM EDTA to stabilise the complement activity and to ensure the bactericidal activity observed reflected the classical antibody-mediated complement killing and not the alternative pathway. The serum was absorbed twice

with a pool of the meningococcal test strains grown overnight at 37°C in a humidified atmosphere with 5% (v/v) CO_2 on human blood agar HBA. The absorbed serum was filter sterilised through a 0.22 μm membrane. The complement source was tested for sterility and stored in aliquots (50 μl) at -70°C. Complement titres were assessed with sensitised sheep red blood cells and used in the assays at a dilution of 1 in 16 (2.7).

3.2.2.3 Bacterial cultures

Cultures were grown overnight at 37°C on HBA, washed twice in PBS by centrifugation at 2000 x *g*. The total count for each strain was determined microscopically with a Thoma counting chamber and adjusted to approximately 10^5 colony forming units (cfu) per ml in sterile D-PBS containing $MgCl_2$ (0.5 mM), $CaCl_2$ (0.9 mM) and glucose (0.1%, w/v) (Sigma) (pH 7.2).

3.2.2.4 Bactericidal assay

Triplicate samples containing equal volumes (40μl) of the test strain (approximately 400 cfu/well) and the heat inactivated serum pool were incubated with 20 μl of the complement source for 30 min in sterile U-bottomed 96 well microtitre plates. Three drops (10 μl) from each sample well were placed on HBA plates which had been dried for 48 hours at room temperature. The plates were incubated overnight at 37°C, the mean cfu recorded and used to calculate the serum bactericidal activity. Each assay included two controls: 1) bacteria + complement source + D-PBS but no serum; 2) bacteria + heat inactivated complement source + absorbed or unabsorbed heat inactivated serum pool.

The absorbed and unabsorbed pools were tested in parallel and the bactericidal activity of the absorbed and unabsorbed pools were compared. Compared with results obtained with the unabsorbed serum, reduction in bactericidal killing ≥ 80% with the absorbed serum was taken as evidence that the NL strain absorbed significant levels of bactericidal activity.

3.3 Results

In three independent experiments, the absorbed and unabsorbed pools were tested for bactericidal activity against 7 isolates of NL from the following countries: Scotland (2); Iceland (1); the Czech Republic (1); and Greece (3) (Table 3.1). The results obtained were consistent in each of the experiments. Eighteen NM isolates, including the twelve immunotype reference strains, were also tested in the bactericidal assays (Table 3.2). The unabsorbed pool killed all the strains tested; the cfu of each strain was reduced by $\geq 80\%$ that of their respective controls.

3.3.1 NL1 (Scotland)

Bactericidal activity against the following NL strains was absorbed by NL1: NL2 from Scotland; NL3 from Iceland; NL4 and NL5 from Greece; NL7 from the Czech Republic. One strain from Greece (NL6) was killed by the absorbed sera. Bactericidal activity against the following NM strains was absorbed by NL1: immunotype reference strains C:NT:P1.2:**L1,8**, B:2a:P1.5,1.2:**L3**, C:11:P1.16:**L4**, B:4:P1.NT:**L5**, B:9:P1.1:**L7**, B:8,19:P1.7:**L8**, and A:21:P1.10:**L9**; B:15:P1.16 from England; B:15:P1.16 from Iceland; B:NT:NT, B:15:NT, from Scotland; B:2a:P1.2 from Greece.

3.3.2 NL7 (Czech Republic)

Bactericidal activity against the following NL strains was absorbed by NL7: NL1 from Scotland; NL3 from Iceland. Bactericidal activity against the following NM strains was absorbed by NL7: immunotype reference strains C:NT:P1.2:**L1,8**, C:2c:P1.1:**L2**, B:5:1.7,1:**L6**, B:9:P1.1:**L7**, and B:8,19:P1.7:**L8**; B:15:NT and B:NT:NT from Scotland; and NG:4:NT from Greece. All other strains were killed by the absorbed serum pool.

3.3.3 NL3 (Iceland)

Bactericidal activity against the following NL strains was absorbed by NL3: NL1 and NL2 from Scotland; NL7 from the Czech Republic. Bactericidal activity against

the following NM strains was absorbed by NL3: immunotype reference strains C:2c:P1.1:**L2**, B:5:P1.7,1:**L6**, and B:9:P1.1:**L7**; B:15:NT and B:NT:NT from Scotland; NG:4:NT from Greece.

3.3.4 NL6 (Greece)

Bactericidal activity against NL4 one of the other Greek isolates, was absorbed by NL6. Bactericidal activity against the following meningococcal isolates was absorbed by NL6: immunotype reference strain B9:P1.1:**L7**; and the Greek carrier isolate NG:4:NT. All other strains were killed by the unabsorbed and the absorbed serum pools.

Table 3.1 Absorption of bactericidal antibodies of adult human pooled serum by *N. lactamica*

Phenotype	Origin		NL1	NL6	NL7	NL3
NG:NT:NT	Scotland	NL1	+	-	+	+
NG:NT:NT	Scotland	NL2	+	-	-	+
NG:NT:NT	Iceland	NL3	+	-	+	+
NG:NT:NT	Greece	NL4	+	+	-	-
NG:NT:NT	Greece	NL5	+	-	-	-
NG:NT:NT	Greece	NL6	-	+	-	-
NG:NT:NT	Czech Republic	NL7	+	-	+	+

Table 3.2 Absorption of bactericidal activity against meningococcal strains by *N. lactamica* isolates from different parts of Europe

Phenotype	Origin	NL1	NL6	NL7	NL3
B:15:P1.7,16	England	+	-	-	-
C:2a:P1.2	Greece	+	-	-	-
NG:4:NT	Greece	-	+	+	+
B:15:P1.7,16	Iceland	+	-	-	-
B:15:NT	Scotland	+	-	+	+
B:NT:NT	Scotland	+	-	+	+
C:NT:P1.2: L1,8	USA	+	-	+	-
C:2c:P1.1: L2	USA	-	-	+	+
B:2a:P1.5,1.2:L3	USA	+	-	-	-
C:11:P1.16:L4	USA	+	-	-	-
B:4:P1.NT:L5	USA	+	-	-	-
B:5:1.7,1:L6	USA	-	-	+	+
B:9:P1.1:L7	USA	+	+	+	+
B:8,19:P1.7:L8	USA	+	-	+	-
A:21:P1.10:L9	USA	+	-	-	-

3.4. Discussion:

3.4.1 Diversity of NL isolates

NL strains from countries of northern, central and southern Europe showed variations in their ability to absorb bactericidal antibodies against different strains of NM. These findings suggest that NL strains in different areas of Europe are phenotypically and genetically diverse, as has been demonstrated for meningococcal strains [Tzanakaki *et al.*, 1993, 1997; Krizova *et al.*, 1996, Caugant *et al.*, 1994].

The Scottish strain NL1 and the Icelandic strain NL3 had similar absorption patterns. The NL7 strain from the Czech Republic showed some homology with these two strains, but the strains from north and central Europe differed markedly from the Greek isolates tested (Table 3.1). NL1 absorbed bactericidal activity against a broad range of meningococcal strains tested. The NL3 strain from Iceland and the NL7 strain from the Czech Republic gave similar results, while the Greek isolate NL6 showed little cross reactivity.

The findings of phenotypic heterogenicity of NL isolated from different geographic regions were consistent with those of Kim *et al.* [1989] who showed antigenic variability and different binding patterns for monoclonal antibodies against meningococcal immunotypes among NL strains isolated from the People's Republic of China, Africa and Romania.

While the pooled human serum was able to kill all of the NL and NM strains tested, the targets of the bactericidal antibodies were unknown. Although the initial hypothesis that NL might play an important role in the acquisition of natural immunity to meningococci was supported, the absorption studies provide evidence that carriage of more than one strain is probably required to develop a broad range of cross-reactive antibodies to meningococci. These findings are consistent with preliminary observations by Bennett *et al.* [2000] who showed that infants carried genetically different strains of NL.

3.4.2 Conditions for the bactericidal assay

Several studies on the bactericidal activity of normal human serum found that the choice of complement source affected bactericidal killing [Zollinger *et al.* 1983]. While baby rabbit complement has been successfully used in several studies [WHO, 1976; Maslanka *et al.*, 1997], Craven *et al.* [1982] showed that the complement source affected the results obtained. Human complement induced killing of only some meningococcal strains, but the use of heat inactivated human serum with baby rabbit complement killed all meningococcal strains tested. A human complement source was used in these studies to try to keep the conditions as similar as possible to those *in vivo*.

Growth conditions were also reported to affect the colony morphology of group B meningococci due to increased capsular expression [Masson & Holbein, 1985] as well as accessibility of OMP and variation in LOS phenotypes [Poolman *et al.*, 1985]. Meningococci grown in stationary phase express longer chain LOS, probably due to its sialylation compared to strains grown in log phase. It has been suggested that outer membrane antigens were more exposed to typing antibodies in strains grown in stationary phase [Poolman *et al.*, 1985]. Most standard bactericidal assays used strains grown in exponential phase [Anderson *et al.*, 1987, 1988; Maslanka *et al.*, 1997]. Although it is impossible to mimic growth conditions found during oro-pharyngal carriage, all strains were harvested in stationary phase to reflect more closely the phenotype of *Neisseria* species during carriage.

3.4.3 Cross reactive antigens

3.4.3.1 Capsule

Capsular antigens are unlikely to be the source of cross reactivity. NL are non-capsulate, and several studies have reported that NL is not serogroupable using anti-capsular typing sera [Kim *et al.*, 1989; Zorgani *et al.*, 1996; Kremastinou *et al.*, 1999a]. None of the NL strains used for absorption reacted with anti-capsular polyclonal typing sera (Gibco) for serogroups A, B or C. The absorption of

bactericidal antibodies by NL was independent of the serogroup of the test strains of meningococci.

3.4.3.2 OMP antigens

Normal human serum usually contains IgG antibodies to OMP antigens used for the serotyping and subtyping of meningococci [Poolman & Zanen, 1980], but none of the NL strains tested bound the monoclonal reagents used for typing. Although it is possible that NL expresses epitopes homologous to meningococcal OMP not used for serotyping, the evidence that successful absorption of bactericidal antibodies was independent of serotype or subtype makes these OMP unlikely sources of cross reactive antigens. Other proteins have been assessed for a role in the development of natural immunity to meningococci: 1) transferrin binding antigens over expressed under iron deficient growth [Ferrón *et al.*, 1992; Ferreiós *et al.*, 1994; Troncoso *et al.* 2000]; 2) a highly conserved surface protein of 18-22 kDa [Martin *et al.*, 1997].

The use of complete genome sequencing to identify genetic homology and structurally similar proteins between NM strains and NL might identify some common OMP able to induce bactericidal antibodies and antigens responsible for the development of natural immunity to meningococci. So far, 570 putative secreted or surface proteins of meningococci have been identified, their corresponding DNA sequences expressed in *E. coli*, and tested as vaccines in mice. Two highly conserved OMPs were identified that were able to elicit bactericidal antibodies, but their homology between different strains of meningococci or NL have not been demonstrated [Pizza *et al.*, 2000]. The data from the current study suggest that more than one NL strain would need to be assessed to identify antigens cross-reactive with different NM phenotypes.

Carriage of meningococci was associated with detection of IgG and IgM antibodies to OMP isolated cases of meningococcal disease in Greece. IgG antibodies to meningococcal OMP were predominant among teenagers and young adults (military recruits) but IgM to the meningococcal OMP were predominant among school children. Carriage of NL was not associated with the detection of either IgG or IgM

antibodies to meningococcal OMP [Kremastinou *et al.*, 1999b]. These findings indicate that *in vivo* carriage of NL does not induce significant levels of IgG and IgM against OMP of NM strains isolated from patients with meningococcal disease.

3.4.3.3 LOS

The most promising candidates are shared LOS epitopes. The basic structure of NM LOS is shown in Figure 1.4. Kim *et al.* [1989] identified two epitopes common to meningococcal immunotypes L1, L(3,7,9), L4, L8, L10 and L11 and some strains of NL. The epitope that bound the D6A monoclonal antibody was shared between some NL isolates, all serogroup A isolates tested, and some group B and C immunotypes. None of these immunotypes had similar oligosaccharides in the G1 α chain moiety, but these immunotypes exclusively shared a common core structure, in the G2 and G3 regions.

So far, four main inner core structures have been identified in meningococcal LOS (Figure 3.1, Table 1.5):

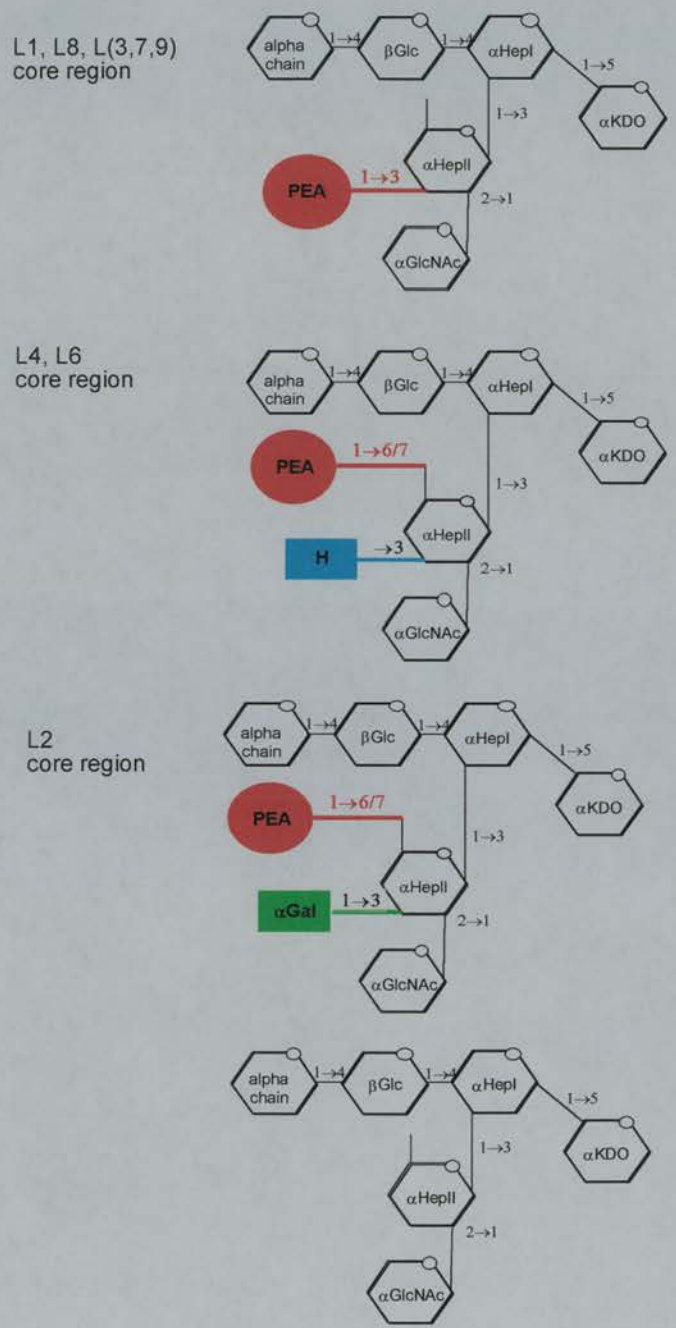
1) α glucose present at the 3rd carbon of HepII;
2) PEA linked to the 3rd carbon of HepII (G2);
3) PEA linked to the 6th or 7th carbon of HepII (G3);
4) PEA and α glucose residues are absent from the inner core.

The immunotypes found on group A meningococci are not fully defined.

The PEA on the carbon 3 of the HepII core structure was also identified as a possible source of cross reactivity among meningococcal strains. This anti-core antibody (Mab B5) was cross reactive with most common immunotypes: L1, L3, L7 to L12 [Plested *et al.*, 1999]. Another antibody (9-2-L3,7,9) eliciting bactericidal activity recognised the saccharide lacto-N-tetranose. Galβ (1→4) GlcNAcβ (1→3) Galβ, contained in the L2, L3, L4, L5, L7, and L9 immunotypes and blood group p^{k} in combination with the core phosphotidyl-ethanolamine at carbon 3 of HepII

[Zollinger *et al.*, 2000]. This combination of G1 α chain oligosaccharide and core G2 structure is found in immunotype L(3,7,9).

Figure 3.1 Main inner core structures of meningococcal LOS [modified from Scholten *et al.*, 1994]



3.4. Conclusions

1) The results indicate that there are epitopes on NL that can absorb bactericidal activity against other NL isolates and a variety of meningococcal phenotypes. There are, however, variations in the ability of individual NL strains to absorb the bactericidal activities.
2) The results highlight the need to assess stains from different geographical regions as illustrated in previous studies on serotype and subtype characteristics of meningococcal isolates from different areas of Europe [Tzanakaki *et al.*, 1993; Krizova *et al.*, 1996].
3) Epitopes on LOS are the most likely source of cross-reactivity between NL and NM eliciting bactericidal activity against meningococci. If carbohydrate domains on LOS or glycoconjugate epitopes are found to be cross-reactive between the two species, examination of the genetic homology of glycosyltransferases of NL and NM and other commensals might be useful.
4) If vaccine candidates were being prepared using antigens from NL to cover the immunotype strains tested, a mixture of antigens from the NL1 strain and the NL3 or NL7 strains would be needed.

Chapter 4 Assessment of the role of *M. catarrhalis* on the induction of natural immunity to meningococcal disease

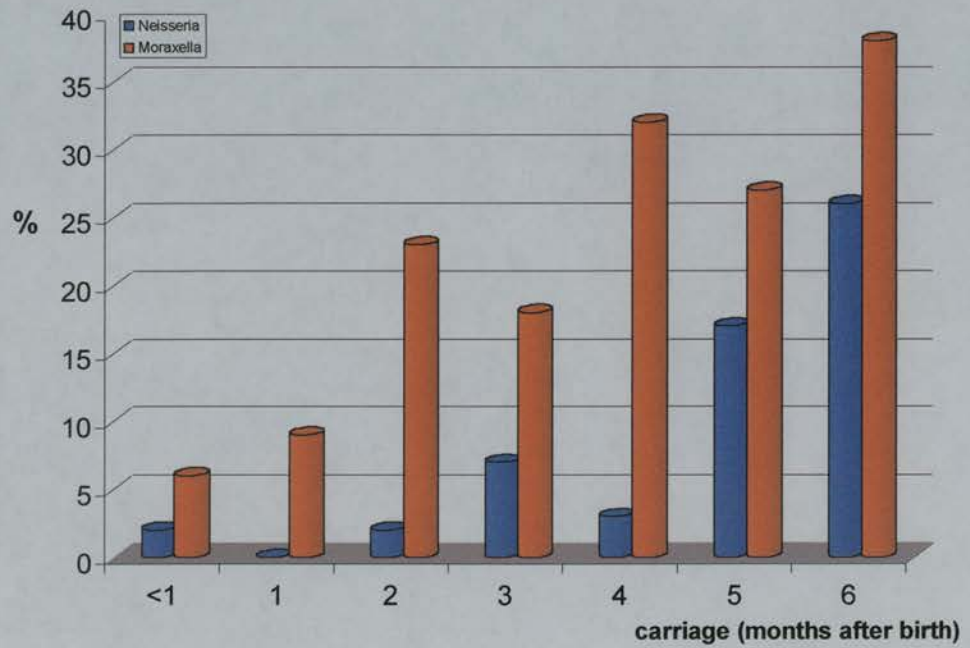
4.1 Introduction

4.1.1 Carriage of MC

In the previous chapter, NL strains from different geographic areas were demonstrated to express a variety of antigens that absorb bactericidal activity to some but not all strains of meningococci [Chapter 3]. Another commensal respiratory species isolated much more frequently from infants and young children is *Moraxella (Branhamella) catarrhalis* (MC) which is increasingly reported to play a role in some childhood diseases [Catlin, 1990; Murphy, 1996].

During the first 6 months of life when infants are developing their immunity to microbial flora, MC is isolated much more frequently than *Neisseria* species from babies [Harrison *et al.*, 1999] (Figure 4.1).

Figure 4.1 Carriage of *M. catarrhalis* and *Neisseria* species during the first 6 months of life [adopted from Harrison *et al.*, 1999]



4.1.2 Epidemiology of MC

4.1.2.1 Classification

MC is a Gram-negative, pilate diplococcus with a diameter of 2-4 μm. It was previously classified within the *Neisseria* or *Branhamella* genera. There is still uncertainty about the appropriate classification of MC. It has little genetic homology with most *Neisseria* species [Catlin & Reyn, 1982], and opinion is divided if MC should be classified within the mostly rod-shaped *Moraxella* [Enright & McKenzie, 1997] or within the *Branhamella* [Murphy, 1996].

4.1.2.2 Association with disease

MC is usually a non-pathogenic respiratory tract commensal, but it is the third most common pathogen isolated from children with otitis media (approximately 20% of all cases) [van Hare *et al.*, 1987]. It is also an important pathogenic agent of lower respiratory tract infections in adults [Hager *et al.*, 1987].

4.1.2.3 Carriage rate

In comparison with NL and meningococci, there is a much higher pharyngal carriage rate of MC in healthy children (average 56%) which decreases with age [Ejlertsen *et al.*, 1994; Faden *et al.*, 1991]. Repeated infections and carriage with a variety of MC strains from an early age results in high serum IgG antibody titres within the normal population [Goldblatt *et al.*, 1990]. Most children were exposed to 3 – 4 different strains of MC [Faden *et al.*, 1994], with colonisation rates varying between 66% of children carrying MC within the first year of life, increasing to 77.5% in the second year. Young adults were found to carry MC rarely [Jousimies-Somer *et al.*, 1989].

4.1.3 Development of immunity to NM and MC

Antigens involved in the development of natural immunity to MC are thought to be glycoconjugates. The main virulence factors are thought to be LOS and OMP including iron-regulated proteins [Storm *et al.*, 1991; Murphy & Bartos, 1989].

4.1.3.1 OMP of MC

Several OMP were recently investigated for their ability to induce protective antibodies between different strains of MC, and one promising candidate for the induction of cross-reactivity between strains of MC is thought to be the ubiquitous surface protein (UspA). This is a high molecular weight OMP complex comprised of two different proteins with molecular masses greater than 250 kDa, UspA1 and UspA2 respectively [Aebi *et al.*, 1997; Cope *et al.*, 1999]. Both proteins share a common antigenic region of 140 identical amino acids recognised by monoclonal antibodies. Molecular analysis identified an oligomeric structure of several monomeric peptide units [Klingman & Murphy, 1994]. While UspA1 is associated with adhesion of MC to human epithelial cell lines, the second high molecular weight complex, UspA2, was found to be involved in resistance to bactericidal action of normal human serum and can function as adhesions to human epithelial cells [Lafontaine *et al.*, 2000].

Both protein complexes show some functional homology with some strains of NM. UspA1 shows a high affinity for human fibronectin and vitronectin, proteins involved in the adherence of meningococci to the epithelial surface [McMichael *et al.*, 1998]. Adherence of meningococci to human epithelial cell lines is partly associated with vitronectin and fibronectin acting as bridges to the human epithelial receptor complexes CD41/CD66 and CD51/CD66, respectively. The close functional homology might reflect cross-reactive epitopes found on MC and NM vitronectin and fibronectin binding proteins, Opa. There might be antigenic similarities between these proteins which could correlate with the development of natural immunity to meningococci in response to childhood carriage or infection with MC.

4.1.3.2 LOS

Serological typing of MC is based upon its LOS. All serotypes of MC have LOS of a similar molecular weight, about 5.5 kDa [Holme *et al.*, 1990]. This differs greatly from immunotypes found in meningococci and *N. gonorrhoeae* which have variable sizes of LOS, 4.1-5.0 kDa and 3-5 kDa, respectively [Schneider *et al.*, 1984].

There are three major LOS types (A, B, and C) found in approximately 95 % of all MC isolates identified in 61%, 29%, and 5% of isolates, respectively [Vaneechoutte *et al.*, 1990; Rahman & Holme, 1996; Edebrink *et al.*, 1994, 1995, 1996; Masoud *et al.*, 1994]]. Lipid A is anchored in the outer membrane of the bacterial envelope linked to KDO-I that is linked to KDO-II (Figure 4.2). A Glcβ (3→1) Glc moiety is linked to (1→5) KDO-I forming the backbone of all known MC immunotypes. The LOS β-chain is linked to the 4th carbon, the α-chain to the 6th carbon of the glucose (1→5) KDO-1 residue. Both chains are variable in length and oligosaccharide composition that determines the MC immunotypes. In contrast to the LOS of meningococci (Figure 4.3), heptose is not present in the LOS of MC (Figure 4.4) (Table 4.1) [Holme *et al.*, 1990]. The major difference between the immunotypes of MC is that group A and C contain GlcNAcα within its β-chain, while group B contains Glcα in its place.

Figure 4.3 Schematic structure of *N. meningitidis* LOS

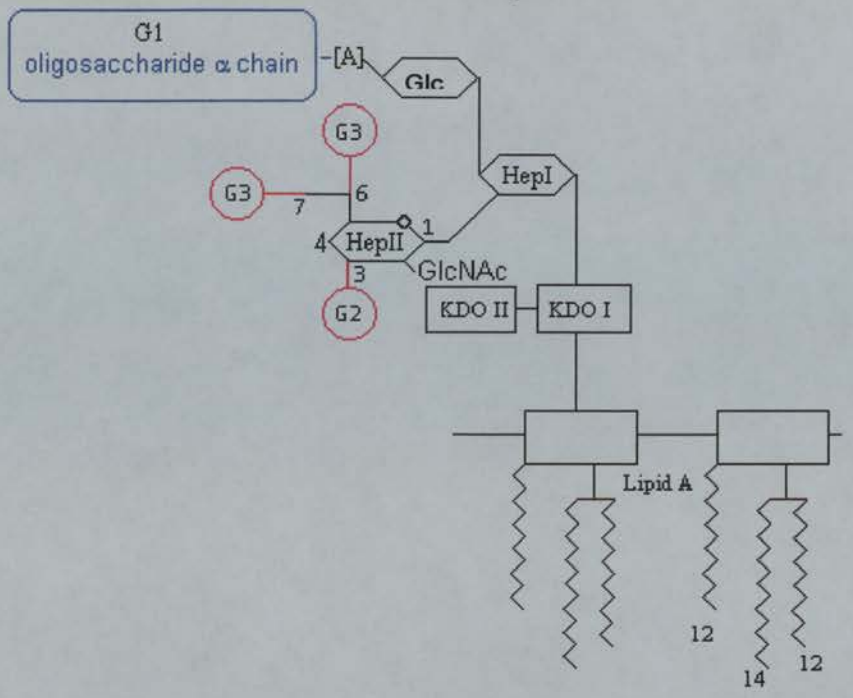


Figure 4.4 Schematic structure of *M. catarrhalis* LOS

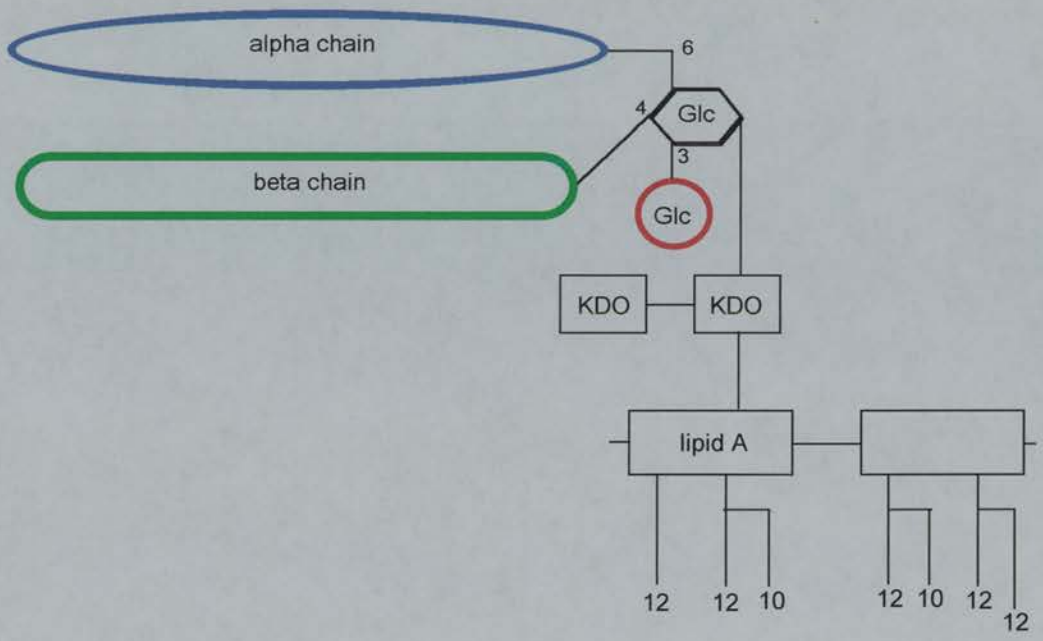


Table 4.1 Primary structure of α and β chains of MC LOS of MC immunotypes A, B and C

LOS type	Chain	Terminal oligosaccharides (variable regions)	Homology with human oligosaccharides	Homology with NM immunotypes
A	α	Galα (1→4) Galβ (1→4) Glcα (1→2) Glcβ	p^K blood group	L1, L11
	β	GlcNAcα(1→2) Glcβ		
B6	α	Glcα (1→2) Glcβ		
	β	Glcβ		
B7	α	Glcα (1→2) Glcβ		
	β	Glcα (1→2) Glcβ		
B8	α	Glcα (1→2) Glcβ		
	β	Galβ(1→4) Glcα (1→2) Glcβ	Ceramide dihexoceramide	L8
B9	α	Galβ(1→4) Glcα (1→2) Glcβ	Ceramide dihexoceramide	L8
	β	Galβ(1→4) Glcα (1→2) Glcβ	Ceramide dihexoceramide	L8
B10	α	Galα (1→4) Galβ(1→4) Glcα (1→2) Glcβ	p^K blood group	L1, L11
	β	Galβ(1→4) Glcα (1→2) Glcβ	Ceramide dihexoceramide	L8
B11	α	Galα (1→4) Galβ(1→4) Glcα (1→2) Glcβ	p^K blood group	L1, L11
	β	Galα (1→4) Galβ(1→4) Glcα (1→2) Glcβ	p^K blood group	L1, L11
C8	α	Glcα (1→2) Glcβ		
	β	Galβ(1→4) GlcNAcα(1→2) Glcβ	Paragloboside	L2, L(3,7,9), L5
C10	α	Galα (1→4) Galβ(1→4) Glcα (1→2) Glcβ	p^K blood group	L1, L11
	β	Galβ(1→4) GlcNAcα(1→2) Glcβ	Paragloboside	L2, L(3,7,9), L5
C11	α	Galα (1→4) Galβ(1→4) Glcα (1→2) Glcβ	p^K blood group	L1, L11
	β	Galα (1→4) Galβ(1→4) GlcNAcα(1→2) Glcβ	P1 blood group	

4.1.3.3 Phenotypic characterisation of MC and potential virulence factors

Previous studies of MC indicate there are at least two groups of these bacteria with different characteristics that could contribute to virulence. These were represented by strains MC1 and MC2. MC1 grew on modified New York City (MNYC) medium, was serum resistant and bound in greater numbers to the Hep-2 cell line infected with respiratory syncytial virus (RSV). MC2 was sensitive to the selective antibiotics in MNYC (colistin, lincomycin, trimethoprim), serum sensitive and bound in significantly lower numbers to RSV-infected cells [El Ahmer *et al.*, 1996, 2000].

4.1.4 Serum resistance as a virulence factor of NM and MC

Protective immunity against meningococcal disease is associated with the presence of serum antibodies against the invading strain [Goldschneider *et al.*, 1969]. The importance of functional complement mediated and opsonising activity is thought to be crucial. Evasion of bactericidal killing by NM includes expression of capsular antigen [Kahler *et al.*, 1998], and sialylation of LOS [Vogel *et al.*, 1996; Klein *et al.*, 1996; Estabrook *et al.*, 1997]. Both mechanisms were associated with reduced binding of antibodies, opsonins, and complement [McNeil *et al.*, 1994; Read *et al.*, 1996] and are major virulence factors of NM [Jones *et al.*, 1992]. MC does not express a polysaccharide capsule; therefore, other factors must contribute to the ability of some MC to resist complement mediated killing. The UspA2 and another protein the 81 kDa CopB are associated with serum resistance [Helminen *et al.*, 1993]. As has been noted for meningococcal LOS, sialylation of terminal galactose moieties in MC LOS by exogenous sialyl-transferases might contribute to increased virulence.

The objective of this part of the study was to determine if MC of the two types represented by strain MC1 and MC2 expressed antigens cross-reactive with those on meningococci as reflected in their abilities to absorb bactericidal activity against NM isolates, immunotype reference strains and NL strains from different regions of Europe.

4.2 Materials and methods

4.2.1 Bacterial strains

In addition to the meningococcal strains tested in Chapter 3, additional isolates were included from our culture collection. The strains of MC and NL used in Chapter 3 were also examined in these experiments.

4.2.2 Bactericidal assay

The microtitre plate method described in Chapter 3 was used. A human complement source absorbed with a pool of the strains to be tested in the bactericidal assay was prepared as outlined in 3.2.

4.2.3 Bacterial cultures

Cultures were grown overnight at 37°C on HBA, washed twice in PBS by centrifugation at 2000 x *g* and adjusted to 10^4 colony forming units (cfu) per ml in sterile D-PBS, containing $MgCl_2$ (0.5 mM), $CaCl_2$ (0.9 mM) and glucose (0.1% w/v) (Sigma) (pH 7.2).

4.2.3 Assessment of bactericidal activity

The unabsorbed pool of human serum and aliquots of the pool absorbed with MC1 or MC2 were tested in parallel and the bactericidal activity of the absorbed and unabsorbed pools were compared. Compared with results obtained with the unabsorbed control serum, reduction in bactericidal killing $\geq$ 80% by the absorbed serum was taken as evidence that the MC strain bound significant levels of antibodies with bactericidal activity.

4.2.4 Statistical analyses:

The chi square test with Yates correction was used to compare the numbers of strains for which bactericidal activity was absorbed by MC1 and MC2 [Gardiner, 1997].

4.3 Results

The unabsorbed serum pool killed all strains tested (>80 % killing).

4.3.1 MC1

In three independent experiments, MC1 absorbed bactericidal activity against MC2 and MC3 but not the other two MC isolates tested. It absorbed bactericidal activity against NL3 from Iceland and NL4 and NL5 from Greece (Table 4.2). MC1 absorbed bactericidal activity against 13/30 (43%) meningococcal isolates tested: immunotype reference strains L1, L4, L5, and L9 (Table 4.3); B:15:P1.7,16 from England; B:15:P1.7,16 and C:4:P1.15 from Iceland; B:2a:P1.2, B:15:NT and B:NT:NT from Scotland; B:2a:1.2, B:NT:P1.9 and B:4:P1.15 from Greece (Table 4.4).

4.3.2 MC2

In three independent experiments, MC2 absorbed bactericidal activity against MC1, the Greek NL4 and NL8 from Scotland (Table 4.2). It absorbed bactericidal activity against 5/30 (17%) meningococcal strains tested: B:2a:P1.2, and B:NT:NT from Scotland; B:2a:P1.2, B:NT:P1.9 and B:4:P1.15 from Greece. All the immunotype reference strains were killed by the sera absorbed with MC2 (Tables 4.3 and 4.4).

Table 4.2 Absorption of bactericidal activity against MC and NL isolates by MC1 and MC2 (results of 3 independent experiments)

Code	Source	MC1	MC2
MC1	Scotland	+	+
MC2	Scotland	+	+
MC3	Scotland	+	-
MC4	Scotland	-	-
MC5	Scotland	-	-
NL1	Scotland	-	-
NL2	Scotland	-	-
NL8	Scotland	-	+
NL3	Iceland	+	-
NL4	Greece	+	+
NL5	Greece	+	-
NL6	Greece	-	-
NL7	Czech Republic	-	-

+ Reduction in bactericidal activity ≥80% compared with the unabsorbed pool
- Reduction in bactericidal activity < 80% compared with the unabsorbed pool

Table 4.3 Absorption of bactericidal activity against meningococcal immunotype reference strains by MC1 or MC2 (results of 3 independent experiments)

Phenotype	LOS oligosaccharide α chain	MC1	MC2
C:NT:P1.2: L1	NeuNAcα (2→3) Galα (1→4) Galβ (1→4) Glcβ	+	-
C:2c:P1.1:L2	(2→3) Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) Glcβ	-	-
B:2a:P1.5,2:L3	NeuNAcα (2→3) Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) Glcβ	-	-
C:11:P1.16:L4	(2→3) Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) Glcβ	+	-
B:4:P1.NT:L5	(2→3) Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) Glcβ	+	-
B:5:P1.7,1:L6	NeuNAcα (2→3) GalNAcβ (1→3) Galα (1→4) Glcβ	-	-
B:9:P1.7,1:L7	Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) Glcβ	-	-
B:8,19:P1.7,1:L8	NeuNAcα (2→3) Galβ (1→4) Glcβ	-	-
A:21:P1.1.10:L9	Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) Glcβ	+	-
A:21:P1.10:L10	Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) Glcβ	-	-
A:21:P1.10:L11	Galβ (1→4) Galβ (1→4) Glcβ	-	-
A:21:P1.NT:L12		-	-

\+ Reduction in bactericidal activity ≥80% compared with the unabsorbed pool
\- Reduction in bactericidal activity < 80% compared with the unabsorbed pool
Meningococcal immunotypes are highlighted in red.

Table 4.4 Absorption of bactericidal activity against meningococcal isolates from different geographic regions by MC1 or MC2 (results of 3 independent experiments)

Phenotype	No.	Origin	MC1	MC2
B:15:P1.7,16	A11	England	+	-
B:NT:P1.9	1766	Greece	+	+
B:NT:P1.13	PE255	Greece	-	-
NG:NT:NT	ST776	Greece	-	-
NG:NT:NT	P481	Greece	-	-
B:2a:P1.2	TH39	Greece	-	-
B:2a:P1.2	TH44	Greece	+	+
B:4:P1.15	A43	Greece	+	+
C:2a:P1.2	A14	Greece	-	-
C:4:NT	A26	Greece	-	-
NG:4:NT	A48	Greece	-	-
B:15:P1.7,16	B14	Iceland	+	-
C:4:P1.15	Ice155	Iceland	+	-
B:15:NT	99-1787	Scotland	+	-
B:2a:P1.2,5	Sto B	Scotland	-	-
B:NT:NT	99/760	Scotland	+	+
C:2a:NT	A25	Scotland	-	-
C:2a:P1.2	StoC	Scotland	-	-
B:2a:P1.2	SNMP	Scotland	+	+

\+ Reduction in bactericidal activity ≥80% compared with the unabsorbed pool
\- Reduction in bactericidal activity < 80% compared with the unabsorbed pool

4.4 Discussion

The aim of this part of the study was to examine the ability of MC to absorb bactericidal activity against meningococci and NL from the pool of normal human serum, and to determine if this species might, like NL and NM, play a role in the development of natural immunity to meningococcal disease.

4.4.1 Absorption of bactericidal antibodies by MC1 and MC2

The results provide evidence for antigenic cross reactivity between MC and some, but not all strains of NM and NL. There were variations in the spectrum of absorption patterns. MC1 absorbed bactericidal activity against a significantly higher proportion of MC strains tested (43%) compared with 17% for MC2 (X^2=6.18, df=1, P=0.013). MC1 absorbed bactericidal activity against immunotype reference strains L1, L4, L5, and L9, some meningococcal strains from the UK, Greece and Iceland. MC2 was able to absorb bactericidal antibodies only against meningococcal strains from Scotland and Greece.

Antigens involved in the development of natural immunity between different strains of MC are thought to be glycoconjugates, and the main virulence factors LOS, OMP and iron-regulated proteins [Murphy & Bartos, 1989]. As there is no evidence of cross-reactivity between meningococcal capsular, serotype or subtype antigens and MC, the published structures for LOS of the two species suggest that these might be the cross-reactive antigens (Table 4.1).

MC1 absorbed bactericidal activity against L4, L5, and L9 which express the paragloboside moiety, a precursor of the P1 blood group antigen (Table 4.1) [Naiki & Kato, 1979; Hakmori & Kannagi, 1986]; however, immunotypes L2, L3, and L7 which also express paragloboside were killed by the absorbed serum.

Carriage of or infection with MC strains might contribute to the development of natural immunity to meningococci by eliciting bactericidal antibodies cross-reactive with meningococcal LOS. Proposed vaccination with anti-MC LOS vaccines to

prevent otitis media [Gu *et al.*, 1998] might induce cross-reactive protection against meningococcal disease. Alternatively, vaccine candidates that eliminated carriage of MC might interfere with development of natural immunity against meningococci. Further studies investigating common antigens on MC, NM and NL LOS were carried out and are summarised in Chapter 5.

4.4.4 Conclusions:

1. This study provided evidence that MC strains express antigens that absorbed bactericidal antibodies against NM.
2. MC1 absorbed bactericidal activity against a significantly higher proportion of meningococcal isolates (43%) than MC2 (17%) (P=0.013).
3. The absorption studies indicated some of the cross-reactivity between MC and NM could be due to expression of carbohydrate moieties similar to epitopes found on human blood group antigens and meningococcal LOS oligosaccharides.
4. These antigens on MC might be involved in the development of natural immunity to some meningococcal phenotypes; their toxicity, therefore, reflected in their ability to induce pro-inflammatory cytokines needs to be investigated.

Chapter 5 Assessment of meningococci and commensal species for cross-reactive surface antigens

5.1 Introduction

The previous two chapters provided evidence that there are antigens present on commensal species that can absorb antibodies bactericidal for meningococci. Evidence for oligosaccharide structures common for meningococci and NL or meningococci and MC has been reported [Mandrell *et al.* 1988]. Meningococci and MC also appear to share oligosaccharide antigens with some found on human tissues including paragloboside, P, P1, p^K, and Ii blood group antigens (Tables 3.4 and 4.1). The expression of blood group related LOS on meningococcal carrier strains and outbreak strains differs greatly. While disease is mainly associated with meningococcal immunotype L(3,7,9) showing homology with the paragloboside antigen (a precursor of the P1 blood group antigen), carrier strains isolated in Britain were found to express LOS immunotypes similar to the p^K (L1) and ceramide-dihexocide (L8) blood group antigens [Jones *et al.*, 1992]. There has been no systematic screening of commensal NL or MC isolates from different regions of Europe with the immunotype antibodies used to classify NM immunotypes or antibodies to human blood group antigens.

5.1.1 P-related blood group system

Carbohydrate antigens are widely distributed on human blood cells and tissues. Their expression is facilitated through glycosyl-transferases during the post-translational modification of proteins (glycoproteins) or linkage to ceramide (N-linked fatty acyl sphingosine). The ABO blood group antigens can be expressed either as glycoproteins or as ceramide glycolipids. The P-blood group system is thought to be expressed in glycolipid form on red blood cells and other tissues [Prokop & Uhlenbruck, 1965; Race & Sanger, 1975; Hakomori & Kannagi, 1986; Bailly *et al.*, 1992; Brown *et al.*, 1993]. It consists of a single oligosaccharide chain linked to a membrane anchored ceramide (Table 5.1) (Figure 5.1). One member of the P-system, the globotriaosylceramide (p^K or CD77) is associated with the differentiation and

maturation of human B cells [Butch & Nahm, 1992] and B-cell Burkitt lymphomas [Wiels *et al.*, 1981]. While other members of the P-system are readily expressed on human red blood cells, the expression of p^K is relatively rare [Marcus *et al.*, 1976]. It is thought that oligosaccharides with a terminal galactose residue can be found in sialylated or non-sialylated forms due to the sialyl-transferases found in human serum, an enzyme that is also associated with the sialylation of meningococcal LOS [Mandrell *et al.*, 1993; Wakarchuk *et al.*, 1998].

5.1.2 Ii-blood group system

Similar to the p^K antigen, Ii determinants are associated with developmental maturation in humans. Although, p^K might be expressed in children and adults, i-antigens are found in foetal tissue but rarely in children or adults. I-antigen expression coincides with the loss of i-blood group moieties [Marsh & Jenkins, 1960; Marsh, 1961; Wiener *et al.*, 1973]. The i-determinant has a single chain oligosaccharide structure (Table 5.1) linked to a membrane anchored ceramide (Figure 5.1), while the I-blood group antigens consist of a branched structure at the third terminal saccharide. Ia and Ib are glycolipids linked to ceramide, while the carbohydrate antigens Ic and Id form the glycosyl structure of glycoproteins linked to the amino acids serine and threonine.

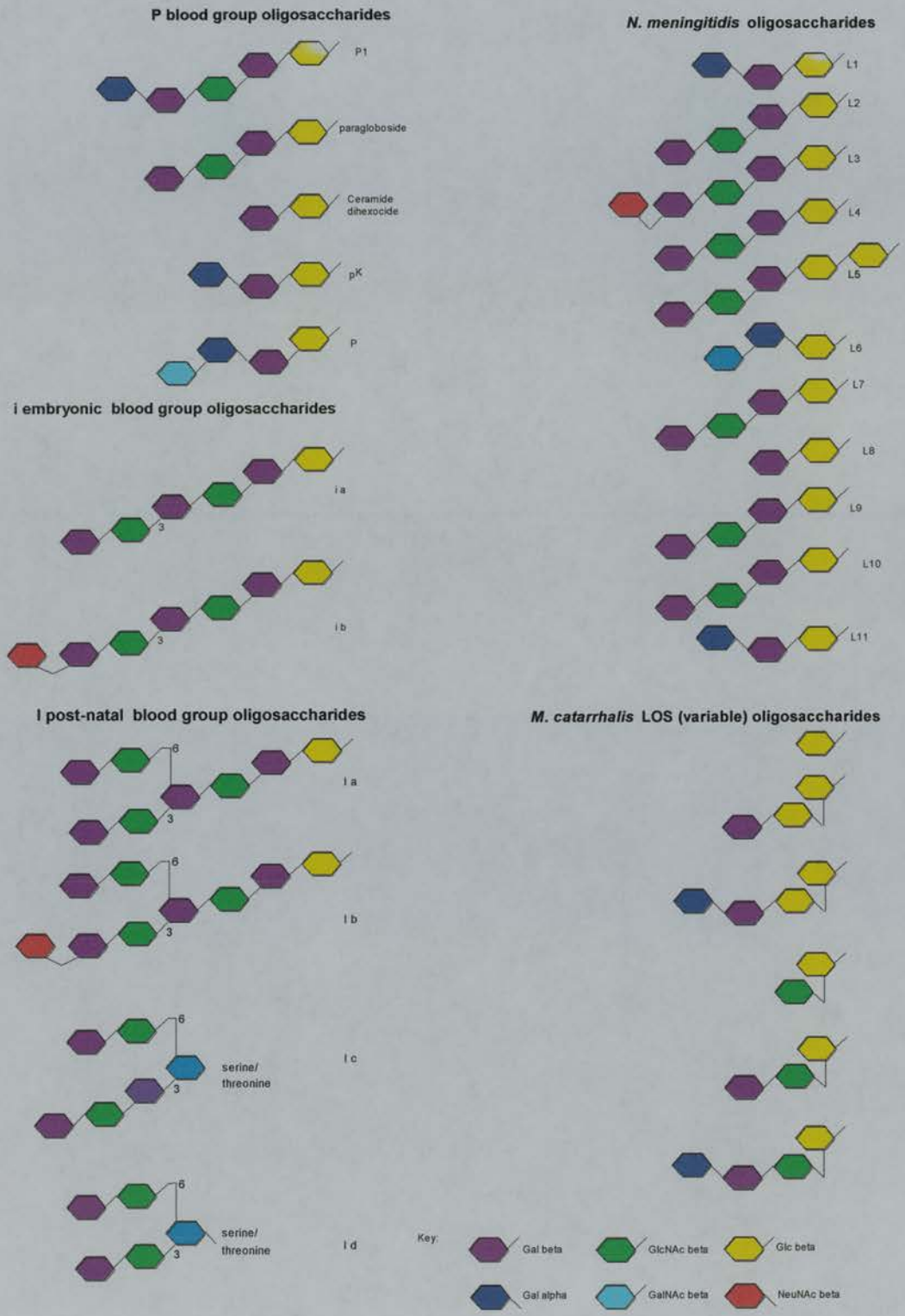
Table 5.1 Blood group antigens of the P- system.

The glucose is linked to the membrane anchored ceramide (Glcβ (1→1) Ceramide)

	α chain moiety
P1	Galα (1→4) Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) Glcβ
p^K, CD77	Galα (1→4) Galβ (1→4) Glcβ
P globoside	GalNAcβ (1→3) Galα (1→4) Galβ (1→4) Glcβ
Paragloboside	Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) Glcβ
i a determinant	Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) Glcβ
i b determinant	**S**-Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) Glcβ
CDH	Galβ (1→4) Glcβ

CDH, ceramide-di-hexocide; S, sialyl

Figure 5.1 Oligosaccharide structures of human P and Ii blood group antigens, and oligosaccharide moieties of meningococcal and MC LOS



5.1.3 Structural homology of *N. meningitidis* LOS with blood group antigens

The oligosaccharide moiety of the α-chain of NM LOS shares structural homology with some human blood group antigens (Figure 5.1), and these structures have been identified in the LOS of several isolates of NM and *N. gonorrhoea* [Mandrell *et al.*, 1988; Kim *et al.*, 1989].

The G1 region of L1 and L11 meningococcal LOS immunotypes show identical terminal oligosaccharide residues of ceramide trihexocide, Galα (1→4) Galβ (1→4) Glcβ, identical to the human p^k blood group antigen (CD77) [Griffiss *et al.*, 1994].

The lacto-N-neotetranose residue, Galβ (1→4) GlcNAcβ (1→3) Galβ, a 4.5 kilodalton LOS component of immunotypes L2, L(3,7,9), L4 and L5 is identical to paragloboside with different distributions in NM, *N. gonorrhoea* and NL [Kim *et al.*, 1989]. Additionally, paragloboside, a precursor of P1 blood group antigen found in 75% of Caucasians, is the terminal structure of the human I-erythrocyte antigen and the embryonic i-antigen (Figure 5.1) [Mandrell *et al.*, 1988, Hakomori & Kannagi, 1986; Tsai & Civin, 1991]. It is also an epitope homologous to type XIV pneumococcal polysaccharide capsules [Siddiqui & Hakomori, 1973].

Immunotype L6 shares its two terminal sugars, Galβ (1→4) GlcNAcβ, with the P blood group antigen, and L8 shares its terminal disaccharide with ceramide dihexocide, Galβ (1→4) Glcβ, the common precursor of the P blood group system and steroid receptors.

5.1.4 Objectives

The objectives of this part of the study were:

1. to compare ELISA and flow cytometry methods for detection of blood group related antigens and immunotype antigens on bacterial cells;
2. to assess NL and MC isolates for binding of antibodies to blood group antigens and NM immunotype antigens;
3. to compare binding of these antibodies by commensal isolates from different geographical regions of Europe.

5.2 Material and Methods

5.2.1 Bacterial strains

Standard immunotype strains of meningococci L1-L12, strains of NM and NL examined in Chapters 3 and 4 were used in these experiments. Meningococci, NL and MC isolates were used from our culture collection (Tables 2.1 and 2.2).

5.2.3 Flow cytometry method for detection of binding of antibodies to bacterial isolates

The primary and secondary antibodies used for flow cytometry are listed in Tables 5.2 and 5.3. Positive controls for blood group expression were red blood cells (rbc) from healthy donors with different blood group phenotypes. The human B lymphocyte Bristol 8 cell line (CB 2452) was used as a positive control for the anti-CD77 antibody. It was obtained from the European Collection of Animal Cell Culture (ECACC), grown in RPMI 1640 supplemented with 2 mM glutamine and 10% (v/v) foetal bovine serum.

Table 5.2 Primary antibodies

Antigen	Species/ isotype	dilution	Code	Source
Anti-meningococcal L1	Mouse IgG	1 in 1000	L1(17-1-L1)	Zollinger
Anti-meningococcal L(3,7,9)	Mouse IgG	1 in 1000	12C10	Zollinger
Anti-meningococcal L8	Mouse IgG	1 in 1000	6E7-10	Zollinger
Anti-meningococcal L10	Mouse IgG	1 in 1000	14-1-L10	Zollinger
Anti-paragloboside	Mouse IgM	1 in 400	1B12-1B7	Zollinger
Anti-human P1	Mouse IgM	1 in 10	Z202	Diagnostic Scotland
Anti-human P	rabbit	1 in 10	A0302 118	DAKO
Anti-human p^K	Rat IgM	1 in 10	MCA579	Serotec
Anti-Ii	human	1 in 100	Z248	Diagnostic Scotland

Table 5.3 Secondary antibodies used to detect binding of primary antibodies in flow cytometry assays

Primary antibody (Table 5.2)	Secondary antibody	Species	dilution	Source
Mouse IgG to immunotypes	FITC-anti-mouse IgG (whole molecule) F(ab') fragment	Sheep	1 in 400	Sigma
Mouse IgM to paragloboside	FITC-anti-mouse IgM μ-chain specific	Goat	1 in 400	Sigma
Rabbit anti-human P	FITC-anti-rabbit IgG	Goat	1 in 400	Sigma
Rat anti-human p^K	FITC-anti-rat IgM μ chain specific	Mouse	1 in 400	Serotec
Human anti-Ii IgG	FITC anti human Ig	Goat	1 in 400	Sigma

A flow cytometric method was developed for the detection of binding of antibodies to blood group antigens to bacterial cells based on the method of Apicella *et al.* [1987]. Overnight cultures grown on HBA were killed by incubation for 30 min in formalin (1%, v/v) and washed twice in PBS (2 ml) by centrifugation at 2000 x *g* for 5 min. The pellet was resuspended in 100 μl PBS. Primary antibody to the blood group antigens (5μl) was added to the cell suspension (100 μl 10^8 bacteria ml^{-1}), vortexed, incubated for 30 min at RT and washed twice in 2 ml PBS. The FITC-labelled secondary antibody (100 μl) was added to the pellet, vortexed, incubated for 30 min at RT, washed twice and re-suspended in buffered paraformaldehyde (Sigma) (0.5%, w/v). The specimens were stored in the dark until assessed by flow cytometry.

Positive controls for the assessment of binding of the monoclonal immunotype antibodies were meningococcal reference strains for which immunotype data had been published: L1 expressing L1 and L8; L3 expressing L(3,7,9) and L8; L10 expressing L10 and L8. Negative controls included strains L4 and L6 (Table 2.1).

The positive population (cells with fluorescence greater than the control cells treated with the FITC-labelled secondary antibody alone) were assessed for percentage of positive cells and mean fluorescence intensity of the positive cell population. A population of bacteria was scored as expressing the antigen when the test population was positive (≥ 5%) compared with the negative control (< 2%) incubated with the secondary fluorochrome-conjugated antibody. This was a more stringent application of the two-percent-of-background method (Chapter 2.13).

5.2.4 Whole cell ELISA (WCE) for the detection of antibodies bound meningococcal surface antigens

WCE was developed to screen for binding of antibodies to blood group and immunotype antigens on bacteria based on previously published methods for the detection of antigens on meningococci [Abdillahi & Poolman, 1987; Scholten *et al.*, 1994].

5.2.4.1 Reagents

5.2.4.1.1 Washing solution

Washing solution was prepared by adding Tween 80 (0.25 ml) (Sigma) to 1 litre of tap water.

5.2.4.1.2 Sodium acetate buffer

Sodium acetate buffer (1.1 mM) was prepared by dissolving of sodium acetate (0.18 g) (Sigma) in 2 litres of distilled water. The pH was adjusted to 5.5 with saturated citric acid (Sigma) containing citric acid (130 g) in 100 ml distilled water.

5.2.4.1.3 Casein Tween (CT) buffer

For the casein Tween buffer, casein Hammarsten (3 g) (BDH) was dissolved in 1 litre of distilled water containing Tween 80 (0.11 ml).

5.2.4.1.4 Antibodies

The HRP-conjugated reagents used to detect antibodies bound to bacteria in the WCE are listed in Table 5.4. One vial of protein A peroxidase conjugate (Sigma P-8651) was diluted 1 in 500 in CT buffer, and 100 µl aliquots were stored at -20°C. Protein A was used to detect mouse and human IgG antibodies.

Table 5.4 Antibodies used in WCE

Primary antibody (Table 5.2)	Secondary antibody	Species	dilution	Source
Anti- L1	HRP-anti-mouse IgG (whole molecule) F(ab') fragment	Sheep	1 in 1000	Sigma
Anti- L(3,7,9)	HRP-anti-mouse IgG (whole molecule) F(ab') fragment	Sheep	1 in 1000	Sigma
Anti- L8	HRP-anti-mouse IgG (whole molecule) F(ab') fragment	Sheep	1 in 1000	Sigma
Anti- L10	HRP-anti-mouse IgG (whole molecule) F(ab') fragment	Sheep	1 in 1000	Sigma
Anti-paragloboside	HRP-anti-mouse IgM μ-chain	Goat	1 in 400	Sigma
Anti-human P1	HRP-anti-mouse IgM μ-chain	Goat	1 in 400	Sigma
Anti-human P	HRP-anti-rabbit IgG γ chain	Rat	1 in 400	Biosource
Anti-human p^K	HRP-anti-rat IgM μ chain	Mouse	1 in 400	Biosource
Anti-li IgG	HRP-anti human IgG γ chain	Goat	1 in 400	Sigma
Anti-li IgM	HRP-anti human IgM μ chain	Goat	1 in 400	Sigma
Anti-li Ig	HRP-protein A	*S. aureus*	1 in 500	Sigma

5.2.4.1.5 Substrate

An aqueous stock solution of the substrate 3,3',5,5' tetramethyl-benzidine (TMB) was purchased from Sigma and kept in the dark at 4°C. Immediately before use in the assay, TMB was diluted 1 in 5 in distilled water.

5.2.4.1.6 Stopping solution

Sulphuric acid (2N) was prepared by adding 10 ml of concentrated sulphuric acid to 170 ml of distilled water. Concentrated sulphuric acid and its vapours are extremely toxic, and the 2 N solution was prepared inside a fume cupboard wearing appropriate protective clothing (safety goggles, and gloves). For neutralisation of spillage, a solution of 0.5 N NaOH was available. Colour change resulting from peroxidase activity was terminated by adding stopping solution (25 μl) to each well.

5.2.4.2 Assay procedure

The method for WCE determination of meningococcal subtypes, serotypes and immunotypes was adapted for use in the present study [Abdillahi & Poolman, 1987; Scholten *et al.*, 1994].

5.2.4.2.1 Coating of plates with bacteria

Bacteria were grown overnight on HBA, harvested in sterile filtered 0.5% (v/v) buffered formalin and washed twice in sterile filtered PBS. The cell suspension was heat inactivated (56°C, 60 min) and adjusted to a final concentration of 10^{10} bacteria ml^{-1} which correlated to an absorption of OD_{546}=0.600 (or OD_{620}=0.1). The cell suspension (100 µl) was distributed into sterile flat bottom 96 well PVC microtitre plates (Greiner) and allowed to dry overnight at 37°C. The coated plates were kept at room temperature for up to three months.

5.2.4.2.2 Assay

The coated plates were washed 3 times with washing solution, aspirated, and blocked for 15 min with 50 µl CT buffer at 37°C. The blocking buffer was removed and 50 µl of primary antibodies were added to the appropriate wells. The plates were incubated at 37°C for 30 min in a moist chamber. The samples were washed three times in washing solution. The peroxidase-conjugated secondary antibody (100 µl) was added to each appropriate well and incubated for 30 min at 37°C in a moist chamber.

The wells were washed three times with washing solution (100 µl), and 100 µl of freshly prepared substrate diluted 1 in 5 in distilled water was added to the appropriate wells. The plates were incubated at room temperature in the dark for 15 min. The peroxidase activity was stopped by adding 50 µl of stopping solution to each well. The absorption of each well was measured at an optical density of 450 nm with a reference filter at 630 nm using a 96 well plate reader (Dynex MRX II) and analysed with the Dynex Revelation software for PCs .

Two separate batches of bacteria were assessed in duplicate in three independent experiments. Each ELISA plate contained a negative control for non-specific binding of the HRP-conjugated secondary antibody tested for each individual strain. This sample was used to set the negative value against which the samples incubated with the primary and secondary antibody was judged as negative or positive. An increase in the absorbance of more than 0.5 in the reading for the test compared to the negative control was considered to be positive for binding of the antibody (+). Values below 0.5 were scored as negative (-) [Scholten *et al.*, 1994].

Every ELISA plate contained a positive control, reported to express the immunotype antigens, immunotype reference strains L1, L3, L7, L8 and L10. Quality control between plates was assessed by adding 5 µl of the HRP-conjugated secondary antibody and TMB substrate (100µl) to an empty well. Variability in absorbance between plates was less than 0.100. Table 5.5 shows the layout for a typical ELISA plate used.

Table 5.5 Layout of a representative 96-well plate for the assessment of cross-reactive antigens using whole cell ELISA

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12
B	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12
C	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12
D	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22	S23	S24
E	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22	S23	S24
F	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22	S23	S24
G	NL1	NL3	NL7	MC1	MC2	L1	L3	L7	L8	L10	-	QC
H	NL1	NL3	NL7	MC1	MC2	L1	L3	L7	L8	L10	-	B

S, bacteria sample; -, QC, quality control containing 5µl secondary antibody and substrate; B, blank; Rows A1-A12, B1-B12, D1-D12, E1-E12, and G1-G11 were incubated with primary, secondary antibody, and TMB substrate to assess the binding of the primary antibody. Rows C1-C12, F1-F12, and H1-H11 were incubated with the secondary antibody and TMB substrate to assess non specific binding (background or negative control).

5.2.5 Assessment of total IgG and IgM in anti-human Ii blood group reagent

5.2.5.1 Coating buffer

Coating buffer contained Na_2CO_3 (1.59g) (BDH), $NaHCO_3$ (2.93g) (BDH) and NaN_3 (0.20g) (Sigma) dissolved in 1 litre of distilled water and adjusted to pH 9.6.

5.2.5.2 Washing buffer

The buffer was prepared by dissolving of NaCl (8 g) (Sigma), KH_2PO_4 (0.2 g) (Fisons), Na_2HPO_4 (1.15g) (BDH) and KCl (0.2 g) (BDH) in 1 litre of distilled water. Washing buffer was prepared prior to use by addition of Tween-20 (0.05%, v/v) (Sigma).

5.2.5.3 Blocking buffer

Blocking buffer contained bovine serum albumin (1%, w/v) (BSA) (Sigma) in sterile PBS.

5.2.5.4 Substrate

TBA (Sigma) was diluted 1 in 5 in distilled water.

5.2.5.5 Stopping solution

The stopping solution contained H_2SO_4 (12.5%, v/v).

5.2.5.6 Assay procedure

To determine the concentration of human IgG and IgM antibodies in the anti-Ii blood group typing serum (Diagnostic Scotland), 50 µl of the serum were diluted in an equal volume of coating buffer and two-fold duplicate dilutions were prepared in a sterile 96 well ELISA plate. Two-fold serial dilutions of reagent grade human IgG (Sigma) (I-2511, Lot 69H4839) or human IgM (I-8260, Lot 87H4832) were made in

coating buffer and used as standards. The plates were incubated at 37°C for 2 h, washed three times in washing buffer, and peroxidase-conjugated anti-human IgM (diluted 1 in 400 in blocking buffer) or anti-human IgG (diluted 1 in 400 in blocking buffer) was added to the appropriate wells and incubated at 37°C for 1 h in a moist chamber. The plates were washed three times in washing buffer, and TMB substrate (100 µl) diluted 1 in 3 in distilled water was added to all wells except the blank and incubated for 15 min at RT in the dark. The reaction was terminated by adding 25 µl of stopping solution and the absorbance was read at an optical density of 450 nm with a reference filter of 650 nm. The concentration of human antibodies in the Ii-reagent was calculated by plotting the IgG and IgM standards (µg ml^{-1}) against their corresponding absorbance values.

5.3 Results

5.3.1 Assessment of IgG and IgM antibodies in the Ii reagent

The mean concentration of anti-Ii antibodies were 12.5 μg ml^{-1} human IgG, but no IgM was detected (< 0.01 μg ml^{-1}).

5.3.2 WCE assays for binding of antibodies to blood group and immunotype antibodies by meningococcal immunotype strains

5.3.2.1 Blood group antibodies

The immunotype reference strains bound the following blood group antibodies: L1 reacted with P and p^K; L2 with P, paragloboside and Ii; L3 with P, p^K, paragloboside and Ii; L4 with paragloboside; L5 with paragloboside and Ii; L6 with P1; L7 and L8 reacted with all blood group antibodies used; L9 with P1, p^K, paragloboside and Ii; L10 with p^K, paragloboside and Ii; L11 P, p^K and Ii; L12 reacted only with paragloboside (Table 5.6). The detection of anti-I antibodies to meningococcal reference strains using either HRP-conjugated protein A or HRP-conjugated anti-human IgG were identical.

5.3.2.2 Meningococcal immunotype antibodies

The immunotype reference strains bound the following anti-LOS antibodies: L1 reacted with L1 and L8; L2 with L(3,7,9); L3 with L(3,7,9) and L8; L4 with none; L5 with L(3,7,9); L6 did not bind any of the antibodies used; L7 reacted with L(3,7,9) and L8; L8 with L8 and L(3,7,9); L9 with L(3,7,9) and L8; L10 with L10 and L8; L11 with L8; and L12 reacted with L10 (Table 5.6).

Table 5.6 WCE to detect binding of antibodies to blood group antigens by meningococcal immunotype strains

Reference strain	Blood group					Immunotype			
	P	P1	p^K	Para-globoside	I	L1	L379	L8	L10
L1	+	-	+	-	-	+	-	+	-
L2	+	-	-	+	+	-	+	-	-
L3	+	-	+	+	+	-	+	+	-
L4	-	-	-	+	-	-	-	-	-
L5	-	-	-	+	+	-	+	-	-
L6	+	+	-	-	-	-	-	-	-
L7	+	+	+	+	+	-	+	+	-
L8	+	+	+	+	+	-	+	+	-
L9	-	-	+	+	+	-	+	+	-
L10	-	-	+	+	+	-	-	+	+
L11	+	-	+	-	+	-	-	+	-
L12	-	-	-	+	-	-	-	-	+

5.3.3 Flow cytometry assays for binding of antibodies to blood group and immunotype antigens to meningococcal immunotype strains

5.3.3.1 Blood group antibodies

The following meningococcal immunotype strains bound anti-P antibody: L1, L2, L3, L6, L7, and L11. The anti-P1 typing serum bound: immunotype reference strains L7, L8 and L9; the antibody bound weakly to immunotype L9 (6.5%, MnI 27.7), but did not bind to L3 (4.5%, MnI 2.17).

Binding of the anti-pK monoclonal antibody by 5% or more of the bacterial population was observed for the meningococcal immunotype reference strains L1, L3, L7, L8, L9, L10 and L11.

For the Ii reagent, high percentages of the bacterial population with high MnI were observed in strains L1, L3, L6, L10, L11 and L12. A high percentage with small MnI were observed in L2, L4, L5, and L9. Less than 5% of cells of immunotypes L7 and L8 bound the Ii reagent (Table 5.7).

Table 5.7 Flow cytometry assay to detect binding of antibodies to blood group antigens by meningococcal immunotype strains

Reference strain	P		P1		p^K		I	
	%	MnI	%	MnI	%	MnI	%	MnI
L1	**25.3**	**44.8**	1.3	8.33	**33.3**	**17.0**	**32.4**	**14.4**
L2	**10.8**	**104.0**	3.5	2.0	2.1	3.3	**21.8**	3.44
L3	1.4	**80.7**	4.5	2.2	**15.2**	**16.1**	**23.8**	**14.7**
L4	3.5	2.1	1.9	4.0	1.9	3.2	**38.8**	4.54
L5	3.2	2.5	2.1	5.1	2.8	5.7	**29.3**	5.7
L6	**8.7**	**93.8**	3.1	3.1	2.1	3.1	**32.4**	**11.7**
L7	**13.3**	**186.0**	**8.1**	**135.1**	**12.1**	**13.6**	2.5	7.33
L8	**26.2**	**60.4**	**39.9**	**24.2**	**26.3**	**17.9**	2.75	39.3
L9	4.6	7.8	**6.5**	**27.7**	**11.1**	**15.2**	**20.1**	4.72
L10	2.6	1.5	3.1	2.6	**7.5**	**12.44**	**54.4**	**39.8**
L11	**11.1**	**87.8**	2.8	1.8	**27.4**	**17.0**	**17.4**	**21.1**
L12	**5.2**	1.9	1.5	6.2	1.7	2.4	**13.9**	**35.8**

%, percentage positive bacteria; MnI, mean fluorescence intensity in relation to fluorescence standard beads (MnI = 500)

5.3.3.2 Immunotype antigens

The meningococcal reference strains bound the immunotye monoclonal reagents as predicted from the published results. Strain L1 bound monoclonals for L1 and L8 epitopes. Strains L2 and L5 bound the monoclonal antibody for L(3,7,9). Strains L3, L7, L8 andL9 bound monoclonal antibodies for the L(3,7,9) and L8 epitopes. Strain L10 bound antibodies for the L8 and L10 epitopes. Strain L11 bound only the L8 antibody. Strain L12 bound the L10 antibody. L4 and L6 did not bind any of the monoclonals used for immunotyping (Table 5.8).

Table 5.8 Flow cytometry assay to detect binding of immunotype antibodies by meningococcal immunotype reference strains

Reference strain	L1		L(3,7,9)		L8		L10	
	%	MnI	%	MnI	%	MnI	%	MnI
L1	**37.2**	**16.2**	1.9	3.2	**36.1**	**19.8**	1.6	2.2
L2	1.4	2.1	**21.8**	**17.1**	1.1	2.7	2.2	3.1
L3	1.7	2.5	**62.4**	**21.2**	**31.5**	**21.3**	3.4	2.0
L4	0.9	3.1	1.2	2.0	2.4	3.0	2.1	1.8
L5	2.1	2.7	**23.4**	**21.6**	2.9	3.5	1.3	2.2
L6	3.4	3.1	2.3	2.2	1.9	3.7	4.1	1.8
L7	1.1	4.3	**73.8**	**19.2**	**14.2**	**16.5**	1.6	5.3
L8	4.7	1.3	**42.2**	**16.5**	**27.8**	**21.8**	2.3	3.3
L9	1.0	3.2	**55.9**	**24.1**	**13.2**	**16.7**	1.2	3.7
L10	2.2	1.2	3.6	2.0	**9.1**	**15.3**	**17.3**	**8.9**
L11	1.1	4.6	2.4	2.4	**31.8**	**21.6**	2.8	2.2
L12	3.5	2.1	3.3	1.9	1.6	2.9	**14.6**	**7.9**

%, percentage positive bacteria; MnI, mean fluorescence intensity in relation to fluorescence standard beads (MnI = 500)

5.3.4 Comparison of results obtained by the two method

The binding of blood group antibodies to meningococcal immunotype reference strains using WCE and flow cytometry (FC) methods were compared (Table 5.9).

5.3.4.1 Blood group antigens

The results for binding of the antibodies to P1 and p^K were the same for both methods for all 12 immunotype strains (Table 5.9) . For the antibody to P, strain L3 was positive by WCE but negative by flow cytometry; however, all the other results were in agreement. The percentage of cells positive for binding the anti-P antibody was small (1.4%); however, the MnI was 80.7 (Table 5.7). This might account for the difference in the two tests.

The results were less comparable for binding of the Ii reagent: Strains L1, L4, L6 and L12 were negative by WCE but positive in flow cytometry. Strains L7 and L8 were positive by WCE but negative by flow cytometry.

Table 5.9 Comparison of methods to detect binding of antibodies to blood group antigens by meningococcal immunotype strains

Strain	P		P1		p^K		I	
	WCE	FC	WCE	FC	WCE	FC	WCE	FC
L1	+	+	-	-	+	+	-	+
L2	+	+	-	-	-	-	+	+
L3	+	-	-	-	+	+	+	+
L4	-	-	-	-	-	-	-	+
L5	-	-	-	-	-	-	+	+
L6	+	+	-	-	-	-	-	+
L7	+	+	+	+	+	+	+	-
L8	+	+	+	+	+	+	+	-
L9	-	-	+	+	+	+	+	+
L10	-	-	-	-	+	+	+	+
L11	+	+	-	-	+	+	+	+
L12	-	-	-	-	-	-	-	+

WCE, whole cell ELISA; FC, flow cytometry

5.3.4.2 Immunotype antigens

The WCE and flow cytometry methods agreed for each of the immunotype antigens tested (Table 5.10).

Table 5.10 Comparison of methods to detect binding of immunotyping antibodies by meningococcal immunotype strains

Strain	L1		L(3,7,9)		L8		L10	
	WCE	FC	WCE	FC	WCE	FC	WCE	FC
L1	+	+	-	-	+	+	-	-
L2	-	-	+	+	-	-	-	-
L3	-	-	+	+	+	+	-	-
L4	-	-	-	-	-	-	-	-
L5	-	-	+	+	-	-	-	-
L6	-	-	-	-	-	-	-	-
L7	-	-	+	+	+	+	-	-
L8	-	-	+	+	+	+	-	-
L9	-	-	+	+	+	+	-	-
L10	-	-	-	-	+	+	+	+
L11	-	-	-	-	+	+	-	-
L12	-	-	-	-	-	-	+	+

WCE, whole cell ELISA; FC, flow cytometry

5.3.5 Binding of antibodies to blood group and immunotype antigens by NL

5.3.5.1 Binding of blood group and immunotype antibodies to immunotype strains detected by WCE

The following NL strains bound both anti-P and anti-P1 antibodies (Table 5.11), NL3, NL4, NL5, NL6 and NL7; NL1 and NL2 bound anti-p^K antibodies. Antibodies to paragloboside bound to NL2, NL3, NL4, NL7 and NL8; NL1, NL3, NL4, NL5, and NL7 bound anti-human Ii blood group antibodies.

NL1 bound L(3,7,9) and L8. Strains NL2, NL3, NL4 and NL8 bound L(3,7,9). NL5 and NL6 did not bind any of the immunotyping antibodies. NL7 bound L1 and L(3,7,9) (Table 5.11).

Table 5.11 WCE to detect binding of antibodies to blood group antigens and meningococcal immunotype antigens to *N. lactamica* strains

	Blood group antibodies					Immunotype antibodies			
Strain	P	P1	p^K	Paragloboside	I	L1	L379	L8	L10
NL1	-	-	+	-	+	-	+	+	-
NL2	-	-	+	+	-	-	+	-	-
NL3	+	+	-	+	+	-	+	-	-
NL4	+	+	-	+	+	-	+	-	-
NL5	+	+	-	-	+	-	-	-	-
NL6	+	+	-	-	-	-	-	-	-
NL7	+	+	-	+	+	+	+	-	-
NL8	-	-	-	+	-	-	+	-	-

5.3.5.2 Binding of blood group and immunotype antibodies to NL isolates detected by flow cytometry

Polyclonal anti-P bound to the following NL isolates: NL3 from Iceland, NL4, NL5 and NL6 from Greece, and NL7 from the Czech Republic: strain NL5 reacted only weakly with anti-P using the two-percent-of background analysis, but were considered to be negative after assessment with the relative-mean-fluorescence-intensity analysis (Chapter 2.13).

The anti-P1 serum bound the following NL strains: NL3 from Iceland; NL4, NL5, and NL6 from Greece; NL7 from the Czech Republic. None of the three Scottish

isolates reacted with anti-P1. Binding of the anti-pK monoclonal antibody by 5% or more of the bacterial population (two-percent-of background analysis) was observed for the Scottish strains NL1 and NL2. Polyclonal human anti-I antibodies bound to strains NL1, NL3, NL4, NL5 and NL7 (Table 5.12).

Table 5.12 Flow cytometry assay to detect binding of antibodies to blood group antigens by *N. lactamica* strains

Strain	P		P1		p^K		I	
Strain	%	MnI	%	MnI	%	MnI	%	MnI
NL1	1.56	11.5	2.7	1.11	**55.5**	**22.4**	**36.2**	**14.3**
NL2	2.1	2.16	1.3	2.73	**42.5**	**36.7**	2.7	3.8
NL3	**21.7**	**74.0**	**28.4**	**17.4**	2.5	8.9	**17.1**	**13.2**
NL4	**15.6**	**236.1**	**27.6**	**54.1**	1.1	3.6	**29.4**	**16.8**
NL5	**5.6**	3.75	**42.9**	**15.7**	1.2	2.5	**31.4**	**14.6**
NL6	**32.2**	**184.3**	**18.2**	**77.1**	1.8	8.3	3.1	2.9
NL7	**34.9**	**103.2**	**41.1**	**19.3**	3.5	9.61	**28.5**	**15.3**
NL8	1.2	1.7	2.2	3.5	2.6	6.2	1.2	4.6

%, percentage positive bacteria; MnI, mean fluorescence intensity in relation to fluorescence standard beads (MnI = 500)

The NL isolates bound the following immunotype antibodies: NL1, L(3,7,9) and L8; NL2, NL3, NL4 and NL8 bound L(3,7,9); NL7 bound L1 and L(3,7,9); NL5 and NL6 did not bind any of immunotype antibodies tested (Table 5.13).

Table 5.13 Flow cytometry assay to detect binding of antibodies to immunotype antigens by *N. lactamica*

Strain	L1		L(3,7,9)		L8		L10	
	%	MnI	%	MnI	%	MnI	%	MnI
NL1	3.2	4.8	**32.9**	**17.5**	**19.1**	**9.5**	2.2	4.1
NL2	2.5	3.9	**28.3**	**15.4**	2.6	1.3	1.9	2.7
NL3	1.0	2.2	**19.8**	**19.3**	3.1	2.2	4.1	1.5
NL4	2.9	4.1	**21.1**	**17.9**	2.9	4.7	1.1	2.7
NL5	3.7	2.4	2.7	3.1	4.2	1.8	2.1	3.4
NL6	3.3	3.1	2.9	1.9	2.8	2.4	2.9	2.8
NL7	**24.8**	**8.9**	**22.9**	**14.7**	2.3	4.1	1.2	3.4
NL8	3.9	2.1	**14.9**	**17.8**	1.2	2.2	3.1	1.9

%, percentage positive bacteria; MnI, mean fluorescence intensity in relation to fluorescence standard beads (MnI = 500)

5.3.5.3 Comparison of results obtained by the two methods

The results for the flow cytometry method and the whole cell ELISA agreed for all strains for the detection of P, P1, p^K and I (Table 5.14). There was no difference in the detection of binding of anti-I antibodies using HRP-conjugated protein A (pA) or HRP-conjugated anti-human IgG.

Table 5.14 Comparison of methods for detection of binding of antibodies to blood group antigens by *N. lactamica* strains

Strain	P		P1		p^K		I	
	WCE	FC	WCE	FC	WCE	FC	WCE	FC
NL1	-	-	-	-	+	+	+	+
NL2	-	-	-	-	+	+	-	-
NL3	+	+	+	+	-	-	+	+
NL4	+	+	+	+	-	-	+	+
NL5	+	+	+	+	-	-	+	+
NL6	+	+	+	+	-	-	-	-
NL7	+	+	+	+	-	-	+	+
NL8	-	-	-	-	-	-	-	-

WCE, whole cell ELISA; FC, flow cytometry

The results for binding of antibodies to meningococcal LOS antigens L1, L(3,7,9), L8 and L10 to NL strains using WCE and flow cytometry methods agreed for all strains (Table 5.15).

Table 5.15 Comparison of methods for detection of binding of immunotyping antibodies by *N. lactamica* strains

Strain	L1		L(3,7,9)		L8		L10	
	WCE	FC	WCE	FC	WCE	FC	WCE	FC
NL1	-	-	+	+	+	+	-	-
NL2	-	-	+	+	-	-	-	-
NL3	-	-	+	+	-	-	-	-
NL4	-	-	+	+	-	-	-	-
NL5	-	-	-	-	-	-	-	-
NL6	-	-	-	-	-	-	-	-
NL7	+	+	+	+	-	-	-	-
NL8	-	-	+	+	-	-	-	-

WCE, whole cell ELISA; FC, flow cytometry

5.3.6 Detection of blood group or immunotype antigens on NL from different sources

The binding of antibodies to blood group antigens and LOS immunotypes to NL isolates from the Czech Republic (n=4), Greece (n=27), Iceland (n=1) and Scotland (n=12) were assessed by WCE in three independent experiments. The binding of blood group antibodies (Table 5.16) and meningococcal immunotype antibodies (Table 5.17) are summarised by country. The binding pattern of individual strains are presented in Table 5.18.

5.3.6.1 Comparison of the binding of blood group antibodies of NL isolates

There was no significant difference in the number of isolates from the Czech Republic (n=4) and Scotland (n=12) expressing blood group antigens of the P-system or I. Significantly fewer NL isolates from Greece (n=27) expressed p^K (P=0.039) paragloboside (P<0.001), and I (P=0.042) (Table 5.16). There was no significant difference in the expression of P or P1 antigen between the samples from different countries tested. The Icelandic strain was excluded from the statistical analysis.

Table 5.16 Binding of antibodies to blood group antigens by *N. lactamica* isolates from different European countries detected by WCE

	Scotland n=12 No (%)	Iceland n=1 No (%)	Greece n=27 No (%)	Czech Rep. n=4 No (%)
P	1 (8.3)	1 (100)	10 (37)	1 (25)
P1	2 (16.7)	1 (100)	4 (14.8)	2 (50)
p^K	8 (66.7)	0 (0)	8 (29.6)	2 (50)
paragloboside	8 (66.6)	1 (100)	1 (3.7)	4 (100)
I	7 (58.3)	1 (100)	8 (29.6)	4 (100)

5.3.6.2 Comparison of binding of meningococcal immunotyping antibodies to NL

There was no significant difference in the distribution of LOS immunotype cross-reactivity between NL samples from the Czech Republic (n=4) and Scotland (n=12) (Kruskal-Wallis analysis of variance by ranks). Meningococcal immunotypes L(3,7,9) ($P<0.02$) and L10 ($P<0.01$) were expressed by fewer NL isolates from Greece (n=27) compared to samples from either Scotland or the Czech Republic (Table 5.17). The Icelandic strain (n=1) was excluded from the statistical analysis.

Table 5.17 Binding of immunotyping antibodies by *N. lactamica* isolates from different European countries detected by WCE

	Scotland n=12 No (%)	Iceland n=1 No (%)	Greece n=27 No (%)	Czech Rep. n=4 No (%)
L1	1 (8.3)	0 (0)	4 (14.8)	1 (25)
L(3,7,9)	9 (75)	1 (100)	9 (33.3)	4 (100)
L8	2 (16.7)	0 (0)	4 (14.8)	2 (50)
L10	4 (33.3)	0 (0)	1 (3.7)	1 (25)

Table 5.18 WCE assay for binding of immunotype antibodies and antibodies to blood group antigens by *N. lactamica* isolates from different European countries

Strain	CODE	Blood group antibodies					Immunotype antibodies			
		P	P1	p^K	Para-globoside	I	L1	L379	L8	L10
	CZ1	-	-	+	+	+	-	+	+	-
	CZ2	-	+	-	+	+	-	+	-	+
	CZ3	-	-	+	+	+	-	+	+	-
NL7	CZ4	+	+	-	+	+	+	+	-	-
NL6	GRE162	+	+	-	-	-	-	-	-	-
	GRE179	-	-	-	-	-	-	-	-	-
	GRE184ns	-	-	-	-	+	-	+	-	-
	GRE184SW	-	-	+	-	-	-	-	-	-
	GRE211	-	-	-	-	+	-	+	+	-
	GRE213	-	-	+	-	+	-	-	-	-
	GRE213	+	-	+	-	-	-	-	-	-
	GRE227ns	+	-	-	-	-	-	-	-	-
	GRE227sw	+	-	-	-	-	+	-	-	+
	GRE228NS	+	+	-	-	-	-	+	-	-
NL4	GRE228SW	+	+	-	+	+	-	+	-	-
	GRE268ns	-	-	-	-	-	-	+	-	-
	GRE268sw	-	-	-	-	+	-	-	-	-
	GRE309	-	-	-	-	-	-	-	-	-
	GRE334	-	-	-	-	+	-	-	-	-
NL5	GRE359ns	+	+	-	-	+	-	-	-	-
	GRE359SW	-	-	+	-	-	-	-	-	-
	GRE409	-	-	-	-	-	-	-	-	-
	GRE534	+	-	-	-	-	-	+	-	-
	GRE538	-	-	+	-	-	-	-	-	-
	GRE619ns	-	-	+	-	+	+	+	+	-
	GRE619sw	-	-	+	-	-	+	+	+	-
	GRE634ns	-	-	+	-	-	-	-	-	-
	GRE634sw	+	-	-	-	-	-	-	-	-
	GRE806	+	-	-	-	-	+	+	+	-
	GRE854ns	-	-	-	-	-	-	-	-	-
	GRE854SW	-	-	-	-	-	-	-	-	-
NL3	ICE	+	+	-	+	+	-	+	-	-
	SCO 1751	-	-	+	-	+	-	+	+	-
NL1	SCONL1	-	-	+	-	+	-	+	+	-
	SCO1567	+	-	+	+	-	-	+	-	+
	SCO1568L	-	-	+	+	-	-	+	-	+
	SCO1568S	-	-	+	+	-	-	+	-	+
	SCO318	-	+	+	+	+	-	-	-	-
	SCO390	-	+	-	+	+	-	-	-	+
	SCO393	-	-	+	+	+	+	+	-	-
	SCO395NS	-	-	-	-	+	-	+	-	-
	SCO395SW	-	-	-	-	+	-	-	-	-
NL2	SCO99/248F	-	-	+	+	-	-	+	-	-
NL8	SCO99/141	-	-	-	+	-	-	+	-	-

CZ, Czech Republic; GRE, Greece; ICE, Iceland; SCO, Scotland; All NL isolates from Greece are AUT:NT:NT

5.3.7 Binding of antibodies to blood group and L(3,7,9) antigens by MC isolates from Scotland

Clinical isolates of MC (n=126) from our culture collection were kindly provided by Dr. El-Ahmer. The binding of blood group antibodies against P, P1, p^K, paragloboside, I and meningococcal immunotype L(3,7,9) were measured by WCE. Because of the large number of strains to be tested and the limited amount of reagents, WCE was used and only the L(3,7,9) monoclonal tested as this epitope is most likely to be the one involved in induction of protective antibodies against disease causing strains.

5.3.7.1 WCE for binding of antibodies to blood group antigens and L(3,7,9) by *M. catarrhalis*

Antibodies to blood group antigens bound to the MC isolates used in the previous chapters (Table 5.19): MC1 bound antibodies to P, P1, p^K and I; MC2 bound only antibodies to p^K; MC3 bound antibodies to P, p^K, and I; MC4 bound antibodies to P1 and p^K; MC5 bound antibodies to p^K and paragloboside. None of the strains bound the L(3,7,9) monoclonal antibody.

Table 5.19 WCE assay to detect antibodies to blood group antigens and anti-L(3,7,9) by *M. catarrhalis* isolates

Strain	P	P1	p^K	Para-globoside	I	L(3,7,9)
MC1	+	+	+	-	+	-
MC2	-	-	+	-	-	-
MC3	+	-	+	-	+	-
MC4	-	+	+	-	-	-
MC5	-	-	+	+	-	-

5.3.7.2 Flow cytometry assay for binding of antibodies to blood group antigens by *M. catarrhalis*

The binding of antibodies to human blood antigens by MC isolates in the flow cytometry assay were as follow (Table 5.20): MC1 and MC2 bound antibodies to p^K and P1; MC3 bound antibodies to P, P1 and p^K; MC4 did not bind any of the antibodies tested; MC5 bound only the antibody to paragloboside. Binding of the L(3,7,9) antibody was not assessed.

Table 5.20 Flow cytometry assay for binding of antibodies to blood group antigens by *M. catarrhalis* (3 independent experiments)

Strain	P		P1		p^K		Paragloboside	
	%	MnI	%	MnI	%	MnI	%	MnI
MC1	3.7	8.4	**27.3**	**14.7**	**41.7**	**15.6**	2.0	2.3
MC2	2.0	5.2	**18.2**	**17.5**	**38.1**	**16.1**	1.8	5.2
MC3	**26.5**	**17.2**	**31.1**	**12.5**	**22.7**	**14.3**	**29.2**	**13.3**
MC4	1.9	2.1	3.7	3.5	2.0	2.77	3.8	2.6
MC5	1.3	4.1	2.7	3.7	2.7	4.1	**31.2**	**17.3**

%, percentage positive bacteria; MnI, mean fluorescence intensity in relation to fluorescence standard beads (MnI = 500)

5.3.7.3 Comparison of WCE and flow cytometry methods

Detection of binding of antibodies to blood group antigens by MC by WCE and flow cytometry agreed except for the following strains (Table 5.21). MC1 bound the antibody to P in WCE but was negative in flow cytometry (3.7%, MnI 8.4). MC2 was negative for P1 in WCE but positive in flow cytometry (18.2%, MnI 17.5). MC3 was negative for P1 in WCE but positive in flow cytometry (31.1%, MnI 12.5), and a similar pattern was observed to binding of the activity to paragloboside (29.2%, MnI 13.3). MC4 was positive for P1 in WCE but was negative in flow cytometry (3.7%, MnI 3.5) as was binding of the antibody to p^K (2.0%, MnI 2.77). MC5 was positive for p^K in WCE but was negative in flow cytometry (2.7%, MnI 4.1).

Table 5.21 Comparison of methods for detection of binding of antibodies to blood group antigens by *M. catarrhalis* strains

Strain	P		P1		p^K		Paragloboside	
	WCE	FC	WCE	FC	WCE	FC	WCE	FC
MC1	+	-	+	+	+	+	-	-
MC2	-	-	-	+	+	+	-	-
MC3	+	+	-	+	+	+	-	+
MC4	-	-	+	-	+	-	-	-
MC5	-	-	-	-	+	-	+	+

5.3.7.4 Screening of MC isolates for binding of antibodies to blood group antigens and immunotype L(3,7,9) by WCE

Most clinical isolates of MC bound one or more antibody to the following antigens (Table 5.22): P (12.7%); P1 (23.8%); p^K (63.5%); paragloboside (17.5%); and I (19.0%); L(3,7,9) (31%).

Table 5.22 WCE for binding of antibodies to blood group antigens and L(3,7,9) by *M. catarrhalis* strains

Antigen	Positive MC strains n=126, No (%)
P	16 (12.7)
P1	30 (23.8)
p^K	80 (63.5)
Paragloboside	22 (17.5)
I	24 (19.0)
No binding of blood group antibodies tested	33 (26.2)
L(3,7,9)	39 (31.0)

Binding pattern of antibodies to blood group and immunotype L(3,7,9) to individual MC isolates are presented in Table 5.23. The most striking observation was the significantly higher proportion of strains from children that bound the L(3,7,9) antibody (16/26, 61%) compared with isolates from adult patients (23/100, 23%) (χ^2=12.59, p<0.001).

Table 5.23 WCE assay for binding of antibodies to blood group antigens and immunotype L(3,7,9) to Scottish *M. catarrhalis* strains

Strain	P	P1	p^K	Paragloboside	I	L(3,7,9)
MC1	+	+	+	-	+	-
MC2	-	-	+	-	-	-
MC3	+	-	+	-	+	-
MC4	-	+	+	-	-	-
MC5	-	-	+	+	-	-
MC6	-	+	-	-	-	-
MC7	-	+	+	-	+	+
MC8	-	+	+	+	+	+
MC9	-	-	-	-	-	-
MC10	-	-	-	-	-	-
MC11	+	+	+	+	-	+
MC12	-	-	-	-	-	-
MC13	+	+	-	-	-	-
MC14	-	+	+	+	-	+
MC15	+	+	+	+	-	-
MC16	-	-	+	-	+	+
MC17	-	-	-	-	-	-
MC18	-	+	-	-	-	-
MC19	-	-	-	-	-	-
MC20	-	+	+	+	-	-
MC21	-	-	+	-	-	-
MC22	-	+	-	-	-	-
MC23	-	-	+	+	-	-
MC24	-	-	+	-	+	+
MC25	+	-	-	-	+	-
MC26	-	-	+	-	-	-
MC27	-	-	-	-	-	-
MC28	-	-	+	-	-	-
MC29	-	-	+	-	-	-
MC30	+	-	-	-	-	-
MC31	-	-	-	-	-	-
MC32	-	+	-	-	-	+
MC33	-	-	+	-	-	+
MC34	-	-	-	-	+	-
MC35	-	-	-	-	-	-
MC36	-	-	-	-	-	-
MC37	-	-	-	-	-	-
MC38	-	+	+	+	-	-
MC39	-	-	-	-	-	-
MC40	-	-	+	+	+	-
MC41	-	-	-	+	-	-
MC42	-	-	-	-	-	-

Table 5.23 (continued)

Strain	P	P1	p^K	Paragloboside	I	L(3,7,9)
MC43	-	-	+	-	-	+
MC44	-	-	-	-	-	-
MC45	-	-	-	-	-	+
MC46	-	-	+	+	-	+
MC47	-	-	-	-	-	-
MC48	-	-	+	+	-	+
MC49	-	-	-	-	-	-
MC50	-	-	+	-	-	+
MC51	-	-	-	-	-	-
MC52	-	-	+	-	-	-
MC53	+	+	+	+	-	+
MC54	-	-	+	-	-	-
MC55	-	-	+	-	+	-
MC56	+	+	+	+	+	-
MC57	-	-	+	-	-	+
MC58	-	-	+	-	-	-
MC59	+	+	+	+	+	+
MC60	-	-	+	-	+	-
MC61	-	-	+	-	-	-
MC62	-	-	+	-	-	+
MC63	-	-	-	-	-	-
MC64	-	-	+	+	+	-
MC65	-	+	+	-	+	-
MC66	-	+	+	-	+	+
MC67	-	+	+	-	-	-
MC68	+	-	-	-	-	-
MC69	-	-	-	-	-	-
MC70	-	-	-	+	+	-
MC71	-	-	+	-	-	-
MC72	-	-	-	-	-	+
MC73	-	-	-	-	-	-
MC74	-	-	-	-	-	+
MC75	-	-	+	-	+	-
MC76	-	+	+	+	-	-
MC77	-	+	+	-	-	-
MC78	-	-	+	-	-	-
MC79	-	-	+	-	-	+
MC80	-	-	+	-	-	-
MC81	-	-	-	-	-	-
MC82	+	+	+	+	-	-
MC83	-	-	-	-	-	-
MC84	-	-	+	-	-	-

Table 5.23 (continued)

Strain	P	P1	p^K	Paragloboside	I	L(3,7,9)
MC85	+	+	+	-	-	+
MC86	-	-	+	-	+	-
MC87	-	-	-	-	+	-
MC88	-	-	-	-	-	-
MC89	-	-	+	-	-	-
MC90	-	-	+	-	+	-
MC91	-	-	+	-	-	-
MC92	-	-	+	-	-	+
MC93	-	+	+	-	-	-
MC94	-	-	-	-	-	-
MC95	+	+	+	+	-	-
MC96	-	-	+	-	-	-
MC97	-	+	-	-	-	-
MC98	-	-	+	-	+	-
MC99	+	+	+	+	+	-
MC100	-	-	+	-	-	+
MC101	-	-	+	-	-	+
MC102	-	+	+	-	-	-
MC103	-	-	+	-	-	-
MC104	-	-	-	-	-	+
MC105	-	-	+	-	-	+
MC106	-	+	+	-	-	+
MC107	-	-	-	-	-	+
MC108	-	-	+	-	-	+
MC109	-	-	-	-	-	+
MC110	-	-	-	-	-	+
MC111	-	-	+	-	-	-
MC112	-	-	+	-	-	-
MC113	-	-	-	-	-	+
MC114	+	-	+	-	-	+
MC115	-	-	-	+	-	-
MC116	-	-	+	-	+	+
MC117	-	-	-	-	-	+
MC118	-	-	+	-	-	+
MC119	-	-	+	-	-	+
MC120	-	-	+	-	-	+
MC121	-	-	+	-	-	-
MC122	-	-	+	-	-	-
MC123	-	-	+	-	-	-
MC124	-	-	+	-	-	-
MC125	-	-	+	-	-	-
MC126	-	-	+	-	-	-

5.4 Discussion

5.4.1 Comparison of results obtained by WCE and flow cytometry

The aim of this part of the study was to determine if the two methods used yielded similar results in assessing the binding of antibodies to meningococci and commensal bacteria. The WCE can accommodate larger numbers of specimens and requires less sophisticated equipment than flow cytometry. This methodology is available in reference laboratories that carry out serotyping and subtyping with monoclonal antibodies against protein antigens of meningococci.

Detection of binding of immunotype monoclonal antibodies and antibodies to blood group antigens by meningococcal reference strains agreed for both methods. The results using WCE and flow cytometry methods agreed except for results obtained with human anti-I and meningococcal immunotypes L1, L4, L6 and L12. Binding of antibodies by these strains was not detected using HRP-conjugated protein A or HRP-conjugated anti human IgG, but binding was measured using FITC-conjugated anti-human immunoglobulin antibodies in the flow cytometric method. The affinity of protein A for immunoglobulins is limited to medium and high affinity IgG of several species, human IgG1, IgG2 and IgG4, but not IgG3 [Harlow & Lane, 1988]. The difference was not related to the FITC-labelled anti-human immunoglobulin binding IgM antibodies in the I reagent as the levels of IgM were below the level of detection by the ELISA (5.3.1).

5.4.2 *N. lactamica*

Detection of binding of immunotype monoclonal antibodies to NL agreed for both methods. Only binding of antibodies to blood group P to NL5 differed in the two methods used. The stringent application of the flow cytometric two-percent-method (Chapter 2.13.3) suggested that binding of anti-P was considered to be negative (5.6%). It could be argued that a less stringent application should have been used, and the binding of anti-P to NL5 should have been considered to be weakly positive (Table 5.12). The reason why this value was considered negative was due to the

observation that all other NL strains binding anti-P had a high proportion of positive cells (15.6-77.1%) and 20 x greater MnI values (Table 5.12).

5.4.3 *M. catarrhalis*

The binding of blood group antibodies agreed except for the following: binding of P to MC1; P1 to MC2 and MC3; p^K to MC4 and MC5; and paragloboside to MC3. This suggests that both methods did not assess accurately the binding of blood group antigens to cross-reactive antigens. The reasons for these differences are not clear as there was no change in methodology, reagents or solutions used and all strains were tested in the same experiments.

5.4.4 Assessment of the two methods

These findings suggest that both methods are effective in assessing the binding of antibodies to carbohydrate antigens on meningococci and NL. While WCE alone could provide accurate information for meningococci and NL, both methods are needed to obtain an accurate picture of binding of these antibodies by MC. Because of the limited quantities of the antibodies, the flow cytometry method was not used to assess binding of immunotype antibodies to the 126 strains of MC.

5.4.5 Binding of antibodies to blood group and meningococcal immunotype antigens by meningococci in relation to previous studies

All immunotype antibodies used bound to strains previously reported to express these antibodies (Chapter 2, Table 2.1) [Scholten *et al.*, 1994]. The anti-paragloboside antibody bound to all immunotype reference strains reported to express this antigen. In addition, immunotype L8 that bound anti-paragloboside IgM co-expresses immunotype L(3,7,9). Immunotype L12 bound anti-paragloboside an antigen found on immunotype L10. The antibody against immunotype L(3,7,9), an antigen that contains the paragloboside moiety, did not bind to immunotypes L4, or L10 that bound anti-paragloboside antibodies. These findings suggest that the L(3,7,9) antibody recognises an epitope that is either not accessible in these

immunotypes, or that the L(3,7,9) antibody is directed against an epitope other than the oligosaccharide moiety. This epitope might be present in the core structure of the L(3,7,9) antigen, or it might include a combination of core and paragloboside structure.

The binding of blood group antibodies corresponds to the presence of these antigens within the published structures of meningococcal LOS. These findings provide evidence that antibodies used for blood group typing can detect similar antigens on meningococci. Antibodies found in human serum directed against blood group antigens might also cross-react with meningococcal oligosaccharide moieties, for example anti-I antibodies found in human serum. Their possible biological functions in relation to complement dependent bactericidal activity (Chapters 3 and 4), ability to neutralise the toxicity of meningococcal LOS (Chapter 6), or ability to opsonise meningococci and commensal species has not been evaluated.

5.4.6 Binding of antibodies to blood group and meningococcal immunotype antigens by NL isolates from different countries in Europe

Significantly fewer NL isolates from Greece bound antibodies to the blood group antigens pK, paragloboside, Ii, and immunotypes L(3,7,9) and L10 compared to isolates from the Czech Republic and Scotland (5.3.6). The Greek isolates were obtained from ethnic Greek school children and children from Russian immigrant families in Athens [Kremastineou *et al.*, 1999a & b], the Czech strains were isolates from children who were carriers, while the Scottish isolates were obtained from adult (student) carriers (n=10) and post mortem isolates from children who died of sudden infant death syndrome (SIDS) (n=2).

Several authors have investigated carriage rates of meningococci and NL within normal populations in the USA [Gold *et al.*, 1978], Norway [Holten *et al.*, 1978], Nigeria [Blakebrought *et al.*, 1982], Spain [Saez-Nieto *et al.*, 1985], England [Cartwright *et al.*, 1987; Coen *et al.*, 2000], Faroe Islands [Olsen *et al.*, 1991], Wales [Davies *et al.*, 1996], Greece [Kremastineou *et al.*, 1999a], and New Zealand [Simmons *et al.*, 2000]. There has, however, been no systematic survey of antigens

on NL cross-reactive with those on meningococci. All of these surveys investigating carriage of NL reported that carriage rates of NL were higher in young children (12-65%) compared to young adults (2-5%). Carriage of NL were found to exceed those for carriage of meningococci within the younger age groups by up to 6 to 1 and the two species are not isolated from the same individual [G. Tzanakaki, personal communications]. This demonstrates that NL is a commensal found world-wide in young children, and its association with the development of natural immunity to meningococcal disease appears to be of great importance in many communities.

The significant differences in the binding antibodies to carbohydrate antigens by strains isolated from Greece, Scotland and the Czech Republic indicate that regional phenotypic differences of NL isolates might contribute to the development of different herd immunities. This could lead to greater susceptibility to meningococcal disease in some populations for example those in which there is a low proportion of commensal strains expressing the L(3,7,9) or L8 epitopes. Little is known about the LOS immunotypes of meningococcal isolates from Eastern Europe, Australia or African countries.

5.4.7 Binding of antibodies to blood group and L(3,7,9) antigens by *M. catarrhalis*

The majority of MC isolates (73.8%) bound one or more antibodies to blood group antigens or L(3,7,9) immunotype (Tables 5.22 and 5.23). Antibodies to the blood group p^K were bound by most of the isolates (63.5%) and the monoclonal antibody to L(3,7,9) was bound by 30.2%. These findings support our hypothesis, that carriage of MC might induce protective immunity against meningococcal disease. Most of these isolates were from adults with respiratory tract infections. Strains isolated from children with otitis media showed a higher proportion of binding of L(3,7,9) antibodies to those MC isolates (n= 26, 61%) compared to isolates causing disease in adults (n=100, 23%) (Table 5.23). Although these findings were highly significant, caution has to be taken due to the unreliability of the WCE method used for MC isolates. Similar studies with MC isolates from children from different geographical regions and ethnic groups with respiratory or ear infections and carrier isolates need

to be carried out. Little is known about natural antibodies induced by MC cross-reactive to meningococci or NL. Naturally occurring IgG2 antibodies that bound to whole cells of MC were detected in children older than 5 years [Goldblatt *et al.*, 1990]. The presence of anti-MC antibodies in older children, its frequent presence in the pharyngeal cavities of children, and apparent high levels of strains with L(3,7,9) epitope isolated from children with otitis media provide evidence that MC might be involved in the development of natural immunity to meningococcal LOS.

5.5 Conclusions

1. Assessment of binding of antibodies by flow cytometry and whole cell ELISA provided evidence that antibodies used for blood group typing could detect similar antigens on meningococci and commensal species.
2. Commensal species express antigens that bound antibodies used for meningococcal immunotyping.
3. Significant differences in the binding of antibodies to carbohydrate antigens were observed among NL strains isolated from Greece, Scotland and the Czech Republic. This indicates that regional phenotypic differences of NL isolates might contribute to the development of different herd immunities that could lead to greater susceptibility to meningococcal disease in some of these populations.

Chapter 6 Pro-inflammatory responses elicited from THP-1 cells by LOS of *N. meningitidis, N. lactamica* and *M. catarrhalis*

6.1 Introduction:

Meningococcal LOS and LOS from related species are being examined as potential vaccine candidates for serogroup B meningococcal disease because they induce antibodies in young children [Verheul *et al.*, 1993]. A major problem is that inflammatory responses to meningococcal LOS contribute to the severity and fatality of disease caused by NM [Brandtzaeg *et al.*, 1989; Westendorp *et al.*, 1995, 1997; Jensen *et al.*, 1996]. Although there are 12 immunotypes of LOS described, L(3,7,9) is associated with rapid progressive meningitis and septicaemia and is isolated from over 90% of patients with disease due to serogroup B or C. Immunotypes L10-L12 are found exclusively on group A meningococci. Other types are obtained primarily from asymptomatic carriers [Romero & Outschoorn, 1994; Jones *et al.*, 1992]. In Chapters 3-5, isolates of NL and MC were screened for antigens cross-reactive with meningococci. Evidence was found indicating that there are common oligosaccharides in the LOS of these species (Chapter 5).

Meningococcal meningitis and septicaemia are exclusively human diseases, and a suitable animal model for safety assessments of LOS vaccines does not exist. Because of the genetically controlled variability of the inflammatory response of individuals to bacterial antigens and toxins [Westendorp *et al.*, 1995, 1997; Nadel *et al.*, 1996; Gordon *et al.*, 1999; Read *et al.*, 2000], an *in vitro* model with a human monocytic cell line was developed for initial screening of cytokine release induced by LOS preparations from meningococci and commensal species.

Although several models were described to investigate the role of MC in the pathogenicity of otitis media [Karalus & Campagnari, 2000], as well as to evaluate effectiveness of MC vaccines [McMichael, 2000], none accurately reflects the events observed on human mucosal membranes, or corresponds to the human immune response *in vivo*.

The objectives of this part of the study were:

1. to develop an *in vitro* model using a human monocytic cell line for initial screening of potential vaccine candidates for their ability to induce inflammatory responses;
2. to use the model system to compare the inflammatory responses to LOS isolated from meningococci and commensal species which absorbed bactericidal activity against different immunotypes of NM;
3. to test the hypothesis that the L(3,7,9) LOS induced higher levels of inflammatory responses than other immunotypes;
4. to use the model system to assess the ability of normal human serum and immune mouse serum to neutralise the effect of meningococcal LOS as reflected in the reduction of pro-inflammatory cytokine responses.

6.2 Materials and Methods

6.2.1 THP-1

THP-1 cells [Tsuchiya *et al.*, 1980] were grown to 10^4 to 10^6 cells ml^{-1} in RPMI-1640 cell culture medium (Sigma) for not more than 18 weeks after establishing the cell line. The calf serum did not contain antibodies against any of the tested bacterial strains as determined by whole cell ELISA (5.2.4).

6.2.2 Analysis of cell surface antigens

THP-1 cells were induced to express the lipopolysaccharide (LPS) receptor CD14 by incubation with 10^{-7} M 1.25-dihydroxyvitamin D3 (VD3) (Calbiochem) for 72 h [Schwende *et al.*, 1996; James *et al*, 1997]. The primary and secondary antibodies used to detect cell surface antigens in the flow cytometry assay are listed in Tables 6.1 and 6.2.

Table 6.1 Primary antibodies and primary fluorochrome-conjugated antibodies used for flow cyometry and confocal microscopy

Anti-human antigen-	Species	Clone	Isotype	Code Lot	Company	Dilution
CD4 (R-PE)/ CD3 (FITC)	Mouse	S4.1/S3.5	IgG2a/ IgG2a	CD3-4 A	Caltac	Neat
CD8 (R-PE) /CD3 (FITC)	Mouse	S4.1/3B5	IgG2a/ IgG2a	CD3-8-A	Caltac	Neat
CD11/18	Mouse	191b FD11	IgG	BC95F 191-1	Seralab	1 in 20
CD11b (PE)	Mouse	2LPM19c	IgG	078(201) R0841	DAKO	1 in 50
CD11c	Mouse	MCA551	IgG	200T	SAPU	1 in 10
CD14 (FITC)	Mouse	TÜK4	IgG	068(201) F0844	DAKO	1 in 10
CD15	Mouse	2-35-14	IgM	hCD15	SAPU	1 in 20
CD16 (R-PE) /CD3 (FITC)	Mouse	3/16/56	IgG	08010102	Caltac	Neat
CD18 (LFA-1 beta chain of complement receptor)	Mouse	MHM23.(1)	IgG1κ	M783 064	DAKO	Neat
CD25 (FITC) (IL-2 receptor)	Mouse	ACT-1	IgGκ	F0801 078	DAKO	Neat
CD29 (FITC) (T cell fibronectin receptor)	Mouse	K20	IgG2aκ	F7068 087	DAKO	1 in 10
CD35 (C3b receptor)	Mouse	To5	IgG1κ	M0710 048(101)	DAKO	1 in 10
CD41 (FITC) α chain (fibronectin receptor)	Mouse	5B12	IgG1κ	F7088 018	DAKO	Neat
CD45 (leukocyte common antigen)	Mouse	T29/33	IgG1κ	M0855 067(201)	DAKO	Neat
CD51 (FITC) α chain integrin	Mouse	51F	IgG	1855	Immuno tech	Neat
CD61 GPIIIa β3 vitronectin receptor	Mouse	Y2/51	IgG1κ	M0753 029	DAKO	Neat
CD64 (FcRI receptor)	Mouse	10.1		MCA756	Serotec	1 in 20
CD77 (anti-p^K)	Rat	38-13	IgM	MCA579 221097	Serotec	Neat
Blood group antigen H	Mouse	92FR-A2	IgMκ	A0583 058A	DAKO	Neat
Mø	Mouse	UCHM1	IgG	5076L	SAPU	1 in 10

κ kappa, mAb monoclonal antibody, pAb polyclonal antibody, FITC, fluorescein isothiocyanate -labelled; R-PE, PE, R-phycoerythrin labelled

Table 6.2 Secondary fluorochrome-conjugated antibodies used for flow cyometry and confocal microscopy

Antigen	Species	Isotype	Code Lot/batch	Company	Dilution
Mouse IgG (whole molecule) F(ab') fragment FITC conjugate	Sheep	IgG	F2883 048H9180	Sigma	1 in 100
Mouse IgG (whole molecule) F(ab') fragment R-PE conjugate	Sheep	IgG	P9287 056H8890	Sigma	1 in 100
Rat IgM FITC conjugate	Mouse	IgG	MCA189F P10802	Serotec	1 in 200
Mouse IgMμ-chain specific FITC conjugate	Goat	IgG	F9259 086H8824	Sigma	1 in 200
Rabbit IgG (whole molecule) FITC conjugate	Goat	IgG	F0382 125H8826	Sigma	1 in 200
Mouse IgG (whole molecule) FITC conjugate	Sheep	IgG	S121-201 8241B	SAPU	1 in 100

6.2.3 Extraction of LOS from meningococcal immunotype strains and commensal isolates

The expression of LOS immunotypes of the NM reference strains are shown in Table 6.3. NL1, MC1 and MC2 examined in Chapters 3-5 were used in this part of the study.

All strains were grown for 18 h in 5% (v/v) CO_2 on HBA. Cells were harvested from plates, washed in sterile pyrogen free PBS, centrifuged at 1000 x *g* and resuspended in pyrogen free distilled water. The purified *Escherischia coli* LPS (strain 026:B6) was purchased from Sigma, UK.

Table 6.3 LOS immunotypes of NM reference strains [Scholten *et al.*, 1994]

Major LOS	Strain	Major L(3,7,9)	Minor LOS	PEA (1→3) HepII	PEA (1→6/7) HepII
L2	35E	-	3,7,9	-	+
L3	6275	+	8	+	-
L4	89I	-		-	+
L5	M981	-	3,7,9	-	-
L6	M992	-		-	+
L7	6155	+	8	+	-
L8	M978	-	3,4,7	+	-
L9	120M	+	6, 8	+	n.e.
L10	7880	-	8	+	n.e.
L11	7889	-		+	n.e.

6.2.3.1 Extraction of LOS

LOS was extracted by the hot phenol water method of Westphal & Luderitz [1954] as described by Hancock & Poxton [1988]. Bacteria grown on HBA were harvested, washed in sterile pyrogen free PBS, centrifuged at 2000 x *g*, re-suspended in pyrogen-free distilled water and frozen (-70°C). The bacteria were freeze-dried and resuspended in pyrogen-free water (5%, w/v). The cell suspension was heated to 67° C in a water bath, and an equal amount of pre-warmed (67° C) 90% (w/v) phenol (Sigma) dissolved in pyrogen free water was added in a fume cupboard. The cells were incubated for 30 min at 67° C and transferred to an ice bath. The phenol-soluble protein and cell fragments were removed from the water-soluble LOS by ultra-centrifugation at 10,000 x *g* for 60 min. The top aqueous solution was carefully removed and dialysed against tap water for 18 h in a 2 kDa permeable membrane (Fisher). The contents of the dialysis membrane were centrifuged for 4 h at 100,000 x *g*. The pellet was recovered, frozen at –70° C and freeze-dried. The purified LOS contained protein contaminants of <1% (w/w) as assessed against a standard of bovine serum albumin (BSA) (Sigma) (6.2.3.2). The LOS was resuspended in RPMI-1640 medium (Sigma), and filtered through a 0.22 μm membrane filter. Aliquots were stored at –70° C and two samples from each batch were incubated at 37° C for 18 h to test for sterility.

6.2.3.2 Protein assay

The Bradford reagent contained Coomassie blue G250 (0.01%, w/v) (Sigma), ethanol (4.7%, v/v) and phosphoric acid (8.5%, w/v) (BDH) in pyrogen-free distilled water. The solution was filtered through Whatman No. 1 filter paper prior to use. Serially diluted amounts (1 mg ml^{-1} to 0.01 μg ml^{-1}) of BSA (Sigma) and freeze-dried LOS stock were dissolved in sterile distilled water. Each solution was mixed with Bradford reagent at a ratio of 1 in 5, and incubated at room temperature for 5 min. The absorbance of the solution was measured at OD_{595} using distilled water as a blank. The amount of the protein contamination (μg ml^{-1}) was calculated by comparing the absorbance value of the LOS samples with the absorbance values of the BSA protein standard [modified from Bradford, 1976].

6.2.4 Immune mouse sera

NL1 and the meningococcal immunotype strain L3 (B:2a:P1.5,2) were killed by heating for 60 min at 100°C (2.11). Individual strains (10^9 bacteria , 100 µl) were injected in adjuvant free and pyrogen free saline (SIGMA) intravenously (*i.v.*) into the tail vein of three six week old male BALB/c mice on three consecutive days. This was followed by repeated *i.v.* inoculations with the same dose and batch of antigen at weeks 4, 8, 12 and 16. In week 20, LOS (100 µl, 100 ng ml^{-1}) obtained by hot phenol water extraction of the immunotype L3 or NL1 strain was injected. Three days after the final injection, blood was collected aseptically by cardiac puncture, allowed to clot, centrifuged at 500 x *g* for 15 min at 4°C. The supernatant was collected and diluted in pyrogen free saline (1 in 100). Complement was inactivated by heat treatment (56°C for 30 min) and the sera were stored in aliquots (1 ml) at –70°C. The production of antibodies was covered by an animal licence obtained from the British Home Office.

Antibodies to NL1 and L3 in samples taken from the mice before immunisation and at the end of the immunisation schedule were detected by WCE against the respective strain (5.2.4).

6.2.5 Induction of pro-inflammatory cytokines

The immature human monocyte cell line THP-1 was incubated for 72 h with 10^{-7} M VD3 to induce expression of the CD14 cell surface antigen [Schwende *et al.*, 1996; James *et al*, 1997]. Triplicate samples of the differentiated cells were challenged for 6 h with tenfold dilutions ranging from 1 pg ml^{-1} to 100 ng ml^{-1} of LOS from the individual immunotypes or *E. coli* endotoxin [Brandtzaeg *et al.*, 1996] in the absence or presence of pooled human serum or immune mouse serum (0.1%, v/v). To determine the concentration to be used in the neutralising assays, human serum or immune mouse serum was serially diluted and tested using WCE (6.2.4) for binding to meningococcal immunotype L3 and NL1. A final dilution of 1 in 1,000 was used in the neutralisation experiments.

6.2.6 ELISA for detection of IL-6

6.2.6.1 Coating buffer

Coating buffer contained Na_2CO_3 (1.59g) (BDH), $NaHCO_3$ (2.93 g) (BDH) and NaN_3 (0.2 g) (Sigma) dissolved in 1 litre of distilled water and adjusted to pH 9.6.

6.2.6.2 Washing buffer

The buffer was prepared by dissolving of NaCl (8 g) (Sigma), KH_2PO_4 (0.2 g) (Fisons), Na_2HPO_4 (1.15 g) (BDH) and KCl (0.2 g) (BDH) in 1 litre of distilled water. Washing buffer was prepared prior to use by addition of Tween-20 (0.05%, v/v).

6.2.6.3 Blocking buffer

Blocking buffer contained BSA (1%, w/v) (Sigma) in sterile PBS.

6.2.6.4 Substrate

TMB (Sigma) was diluted 1 in 5 in distilled water.

6.2.6.5 Stopping solution

The stopping solution contained 12.5% (v/v) H_2SO_4.

6.2.6.6 Detection of cytokine production by ELISA

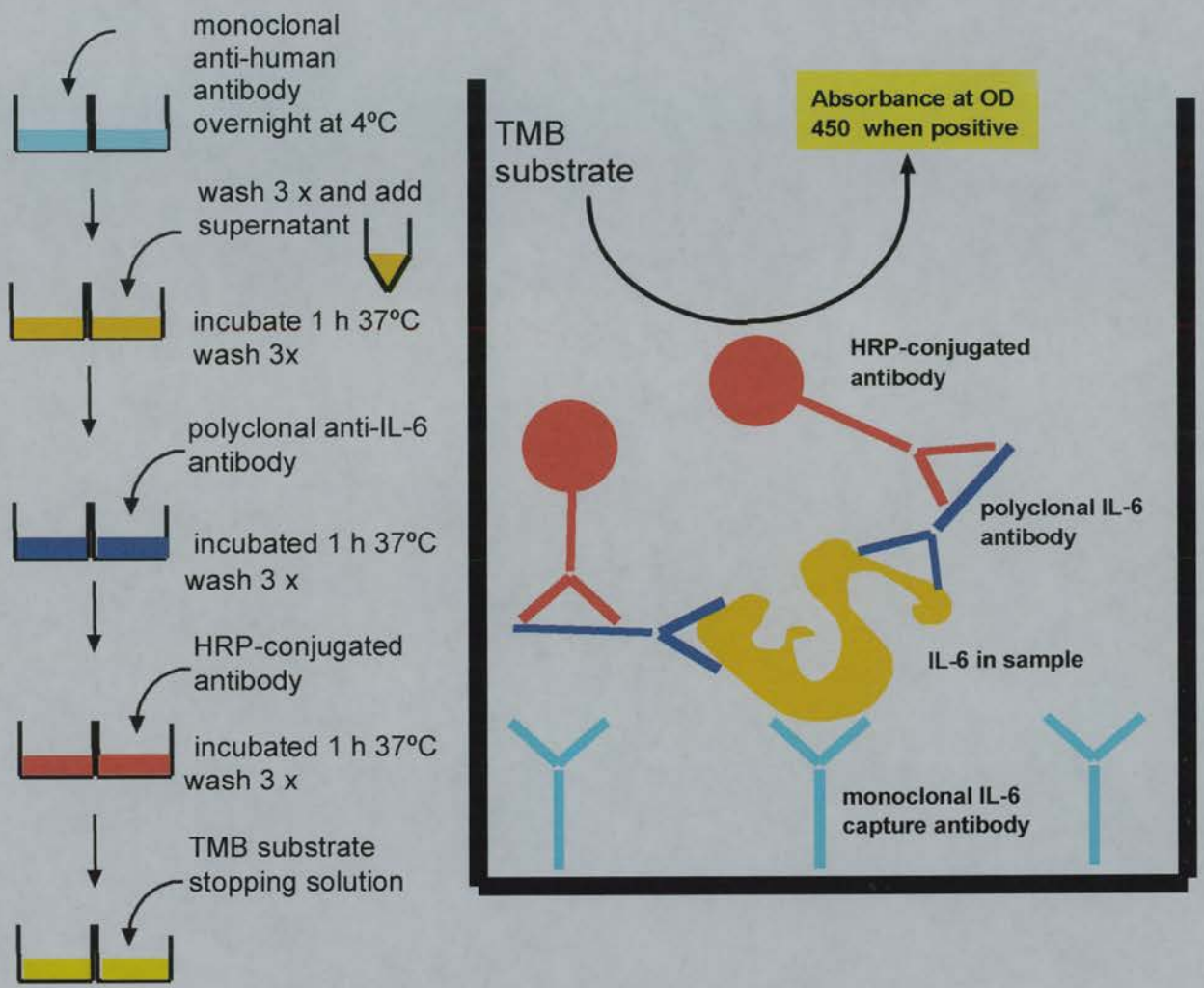
The wells of microtitre plates were incubated overnight at 4°C with 50 µl of mouse monoclonal anti-IL-6 (1 µg ml^{-1}) diluted 1 in 500 (R&D Systems, UK) in coating buffer. The wells were washed 3 times with washing buffer, aspirated and blocked with 50 µl of blocking buffer at room temperature (RT) for 30 min. The wells were washed 3 times and the test samples (neat, 50 µl) were added to duplicate wells. For each plate, dilutions of standard recombinant human IL-6 (R&D) (50 µl) ranging

from 0.19-100 ng ml^{-1} in blocking buffer were added to duplicate wells. The plates were incubated for 2 h at 37°C. The plates were washed 3 times, aspirated and 50 µl of goat polyclonal anti-IL-6 antibody (1 µg ml^{-1}) diluted 1 in 1000 in blocking buffer (R&D) was added to each well except the blank well. The plates were incubated at 37°C for 2 h, aspirated and washed 3 times. The HRP-conjugated donkey anti-goat IgG (50 µl) diluted 1 in 20,000 in blocking buffer (Sigma) was added to each well except the blank well., and the plates were incubated at 37°C for 1 h. The plates were washed 3 times, aspirated and 100 µl of substrate was added. The colour change of the substrate was stopped after 10-15 min by adding 25 µl of stopping solution.

The absorbance was measured at 450 nm and a reference wavelength of 620 nm (Dynex MRX II) and analysed with the Dynex Revelation software for PCs. The concentration of IL-6 (ng ml^{-1}) in each sample was calculated from the standard curve obtained with the recombinant IL-6 samples. The mean IL-6 concentration and standard deviation (SD) were recorded.

Quality control between plates was assessed by adding 5 µl of the HRP-conjugated secondary antibody and TMB substrate (100µl) to an empty well. Variability in absorbance between plates was less than 0.100. The principles of ELISA method are outlined in Figure 6.1.

Figure 6.1 Principle of ELISA



6.2.7 Detection of TNFα by a bioassay

This method was modified from that described by Delahooke *et al.* [1995].

6.2.7.1 L929 cells

The mouse fibroblast cell line L929 was obtained from the ECACC. Cells were grown in 75 cm^3 tissue culture flasks (Greiner) in growth medium containing DMEM medium (Sigma) supplemented with FCS (5%, v/v) (Gibco), L-glutamine (1%, w/v) (Gibco), penicillin (100 IU ml-1) and streptomycin (200 mg ml-1) (Gibco) at 37 °C with 5% CO_2.

6.2.7.2 Seeding of cells into 96-well plates

The semi-confluent monolayer covering approximately 70% of the surface of the flask was washed twice in pre-warmed PBS and dispersed from the plastic surface by adding 1.5 ml trypsin (0.005%, w/v) - EDTA (0.02%, w/v). Excess trypsin-EDTA was discarded after 30 sec and the cells left for 10 - 15 min to detach. The cells were resuspended in growth medium, washed twice by centrifugation for 10 min at 300 x *g*. The cells were counted following a 1 in 10 dilution in trypan blue (0.4%, w/v) using an improved Neubauer counting chamber. The cell count was adjusted to 3 x 10^5 cells ml^{-1} in the growth medium. Cells (100 µl) were added to a sterile 96-well tissue culture plates (Nunc); 6 wells per plate contained medium only to act as negative controls. The plates were incubated at 37 °C in 5% (v/v) CO_2 for 24 h.

6.2.7.3 Sensitising L929 cells for TNFα

The assay medium was prepared by supplementing pre-warmed RPMI 1640 medium (Sigma) with FCS (5%, v/v) (Gibco), L-glutamine (1%, w/v) (Gibco) and actinomycin D (2mg ml^{-1}) (Sigma).

The growth medium was aspirated from all wells and replaced with 100 µl of assay medium and the plates were incubated for 2 h at 37 °C with 5% (v/v) CO_2.

6.2.7.4 Detection of TNFα

The assay medium was aspirated and replaced with duplicate samples (100 µl) diluted 1 in 2 or 1 in 4 in assay medium. Assay medium (100 µl) was added to six wells of cells acting as a negative control. A standard curve was obtained for each plate by adding duplicate samples of human recombinant TNFα (National Institute for Biological Standards and Control) diluted in assay medium ranging from 0 to 100 IU ml^{-1}. The plates were incubated for 20 h at 37 °C in a humidified atmosphere with 5% (v/v) CO_2.

Crystal violet solution contained crystal violet (0.5%, w/v) and methanol (20% v/v) in distilled water. The solution was filtered through a Whatman No. 1 filter paper. Crystal violet (100 µl) was added to each well and the cells were incubated for 2 min followed by gentle washing with running tap water. The plates were allowed to dry at RT. The dye was dissolved in 100 ml of acetic acid (20%, v/v) in distilled water for 10 min. The absorbance was assessed using an ELISA plate reader (Dynex) at OD_{570} blanked on the mean of the negative controls. The results were expressed as IU ml^{-1} of recombinant human TNFα derived from the standard curve.

6.2.8 Confocal microscopy

After flow cytometric analysis, immature and VD3 differentiated THP-1 cells (50µl) stained with FITC-idiotypic antibodies (control), anti-CD14, anti-CD64, or anti-CD11/18 were place on a microscope slide (Greiner) and sealed under a cover slip with clear nail polish (Boots). Images were taken by scanning confocal microscopy (3µm depth) and phase contrast microscopy (670 x magnification) (J. Bard, Department of Anatomy, University of Edinburgh). Images were analysed using imaging software for Windows (Microsoft).

6.2.9 Statistical analysis

The mean, standard deviation (SD) and Student's t-test were calculated using Minitab for the Apple Mackintosh. To determine if the data were normally distributed, normal probability plots were used [Gardiner, 1997]. Regression and analysis of variance showed that cytokine levels were normally distributed. Probability values were calculated with a confidence interval of 5% against the negative control treated with PBS only or the *E.coli* 026:B6 LPS. Two-sided analysis (5% confidence level) was carried out using a paired t-test for different endotoxin samples.

To assess the inflammatory response of known LOS antigens, samples were grouped and a two-sided analysis was performed for:

1) immunotype strains expressing the major L3, L7, and L9 immunotypes were grouped and assessed to determine if the inflammatory response was greater than the response elicited by non-L(3,7,9) immunotypes (L4, L6, L10, and L11) or strains expressing L(3,7,9) as a minor antigen (L2, L5 and L8) (Table 6.1);
2) immunotypes with a PEA (1→3) HepII in their LOS core structure but that did not express L(3,7,9) as a major antigen were grouped (L8, L10, and L11) and compared to meningococcal LOS that did not have PEA moieties in the core of the major immunotype (L5).

6.3 Results

6.3.1 Expression of cell surface antigens

Incubation of THP-1 cells with VD3 for 72 h resulted in expression of most of the cell surface markers tested except H. The greatest increase was observed for CD14 (Table 6.4). Binding of FITC-labelled anti-CD14 antibodies to undifferentiated and VD3 differentiated cells detected by flow cytometry is shown in Figure 6.3. The negative control contained differentiated and undifferentiated cells incubated with FITC-labelled anti-idiotypic mouse antibodies and did not show any detectable staining. Confocal microscopy confirmed that CD14 was expressed in VD3 differentiated cells, but undifferentiated THP1 did not bind any FITC-labelled anti-CD14 antibodies.

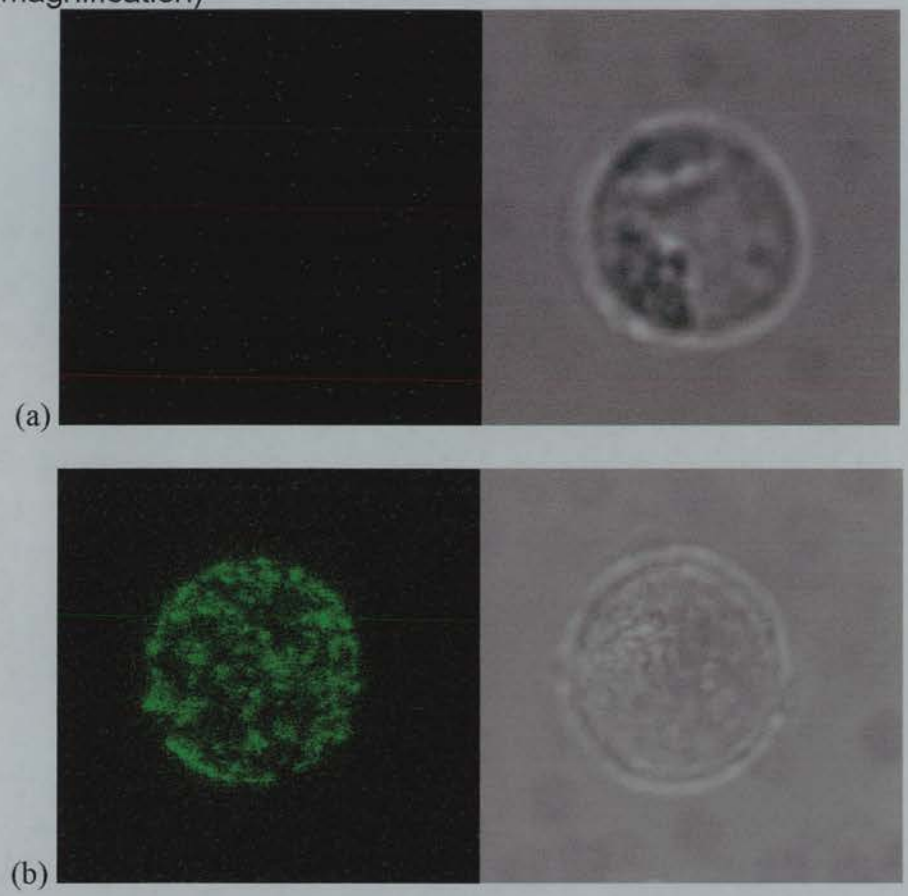
Table 6.4 Expression of cell surface markers following incubation of THP-1 cells with VD3 (% of FL1 positive population pre- or post- differentiation) (mean of 6 experiments, ±SD)

Cell surface marker		Positive population%	
	Description	THP-1	THP-1 + VD3
Control	FITC labelled secondary antibody	1.0	1.0
CD4	MHC class II receptor	4.06 ±1.5	**# 92.7** ±3.2
CD11/18	Complement receptor	99.3 ±0.4	91.2 ±0.8
CD14	Matrix LPS binding receptor	4.3 ±0.8	**# 89.9** ±7.9
CD15	Lewis x	65.6 ±4.8	**# 83.4** ±5.1
CD45	Leukocyte common antigen	87.8 ±6.1	91.9 ±4.5
CD64	IgG3 high affinity phagocytic FcγRI receptor	57.9 ±3.1	53.3 ±2.9
CD77	Pk blood group antigen	47.7 ±4.4	57.8 ±0.8
H	Blood group H	80.0 ±8.1	*** 33.2 ±9.2**
Mø	Monocyte activation antigen	37.1 ±2.3	41.7 ±1.9
CD29	Fibronectin receptor β chain	78.3 ±6.1	86.1 ±9.3
CD41	Common α chain for CD29 and CD51	92.1 ±2.3	91.2 ±5.2
CD51	Vitronectin receptor β chain	87.2 ±6.1	86.1 ±5.2

statistically significant up-regulation of cell surface marker ($p<0.05$) compared to the VD3 undifferentiated THP-1 cells

* statistically significant down-regulation of cell surface marker ($p<0.05$) compared to the VD3 undifferentiated THP-1 cells

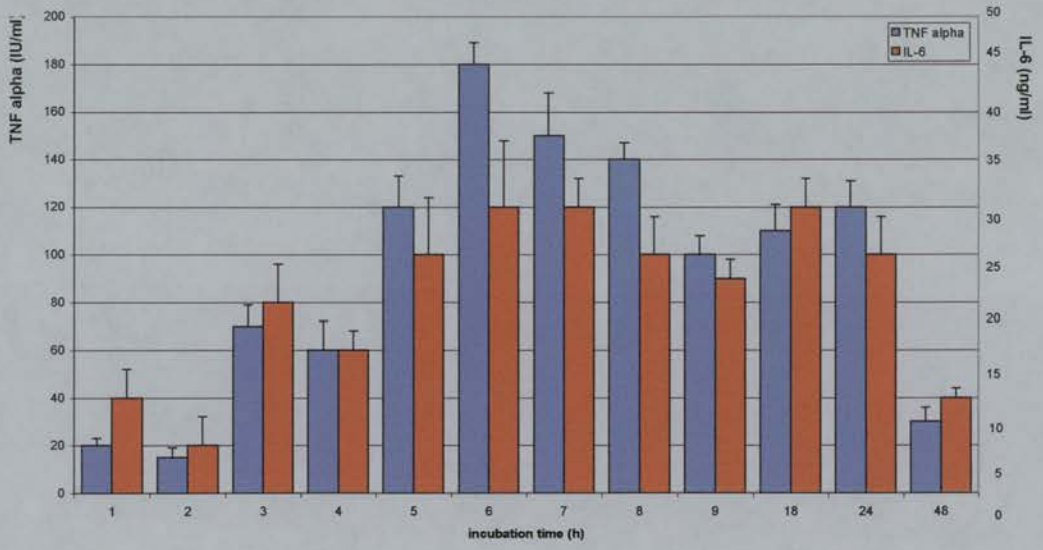
Figure 6.3 Scanning confocal (left) and phase contrast (right) images of (a) undifferentiated THP-1 cells incubated with FITC-anti human CD14 and (b) VD3 differentiated cells incubated with FITC-anti human CD14
(670 x magnification)



6.3.2 Time course for induction of TNFα and IL-6 from differentiated THP-1 cells

In three independent experiments, TNFα was detected from 3 h post exposure to LOS from the L3 immunotype strain ranging from 1 pg ml-1 to 100 ng ml^{-1}. The peak level was observed at 6 h at an optimal concentration of 100 pg ml^{-1}. IL-6 was detected from 3 h and the peak level reached by 6 h (Figure 6.4). In the subsequent experiments, samples were challenged with 100 pg ml^{-1} purified LOS and were taken at 6 h and assessed for TNFα and IL-6.

Figure 6.4 Time course of TNFα (IU ml^{-1}) and IL-6 (ng ml^{-1}) production by CD14 positive THP-1 cells challenged with 100 pg ml^{-1} LOS from immunotype strain L3 (mean of 3 independent experiments)



6.3.3 TNFα responses to LOS of different species in the presence and absence of VD3

In six independent experiments in which each control and test condition was carried out in triplicate, incubation of undifferentiated THP-1 cells with LOS (100 pg ml^{-1}) from immunotypes L3, L6, NL1, MC1, MC2 and *E. coli* resulted in detection of low levels (50-92 IU ml^{-1}) of TNFα compared with cells incubated with PBS (Figure 6.5.a).

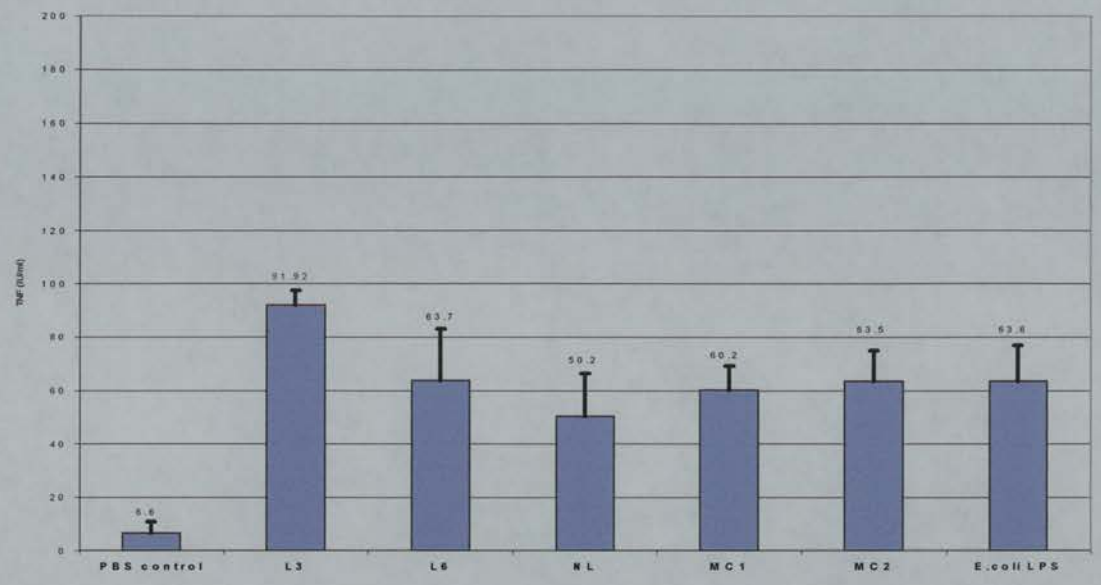
Compared with levels obtained with the undifferentiated THP-1 cells, there was a significant increase in TNFα activity ($p<0.01$) for each of the LOS preparations with the VD3-differentiated cells (Figure 6.5.b). All endotoxin samples except MC2 showed significant increases of TNFα release compared with the *E.coli* LPS. With the VD3 differentiated cells, the highest TNFα levels were obtained with LOS from the L3 immunotype. TNFα levels for NL1, MC1, MC2 and *E.coli* were all significantly lower than those elicited by immunotype L3 LOS.

6.3.4 IL-6 responses to LOS of different species in the presence and absence of VD3

In six independent experiments, a similar pattern was observed for induction of IL-6. Compared with cells incubated with PBS, incubation of undifferentiated THP-1 cells with LOS from the different strains resulted in low levels of IL-6 production (Figure 6.6.a). Compared with IL-6 levels obtained with the undifferentiated THP-1 cells, there was a significant increase in IL-6 levels for each of the LOS preparations for the differentiated cells ($p<0.01$) (Figure 6.6.b). All except LOS from MC2 induced significantly higher levels of IL-6 compared with the *E. coli* LPS (Figures 6.6.a,b). NL1, MC2 LOS preparations and *E. coli* LPS elicited IL-6 levels significantly lower than those obtained with LOS from the L3 strain.

Figure 6.5 TNFα (IU ml^{-1}) responses to LOS from meningococci (L3, L6), commensal species (NL1, MC1, MC2) or *E.coli* endotoxin (100 pg ml^{-1}) by (a) undifferentiated, (b) differentiated THP-1 cells
(n=6, error bars = SD)

(a)



(b)

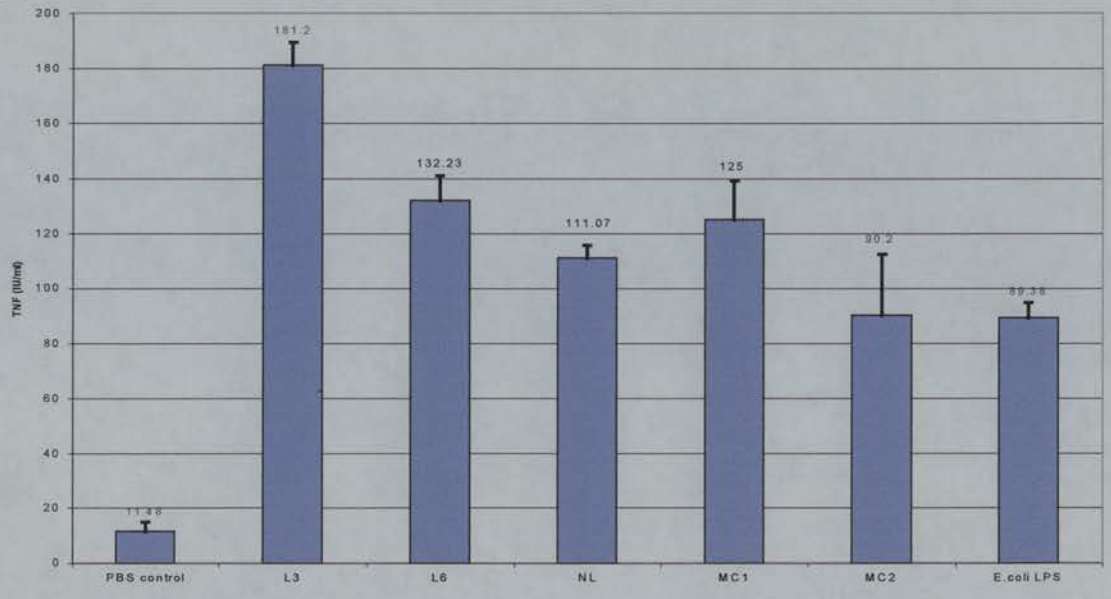
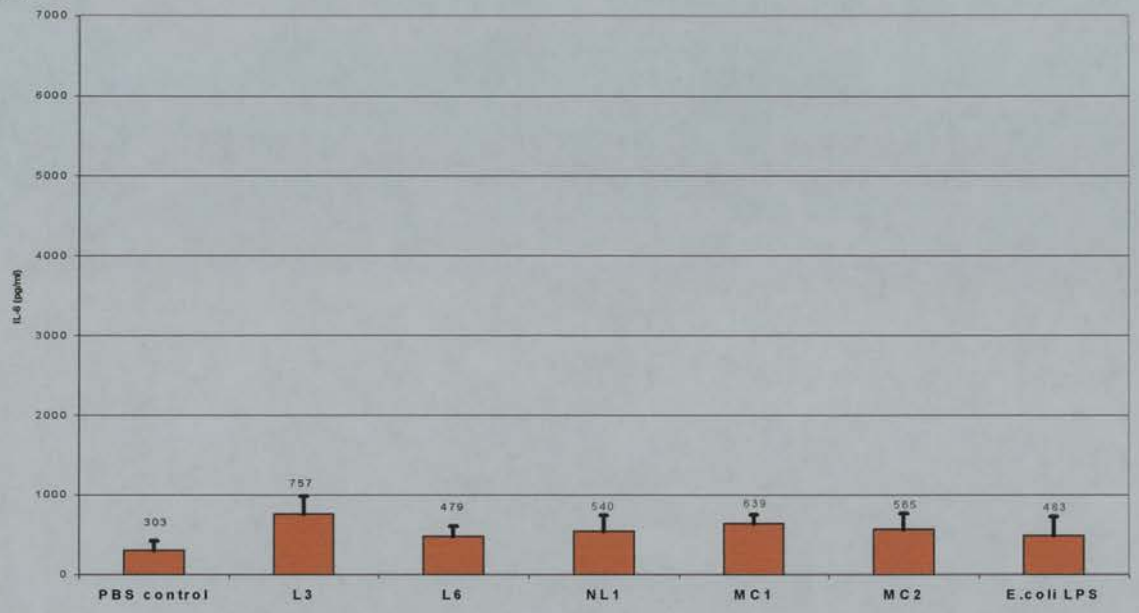
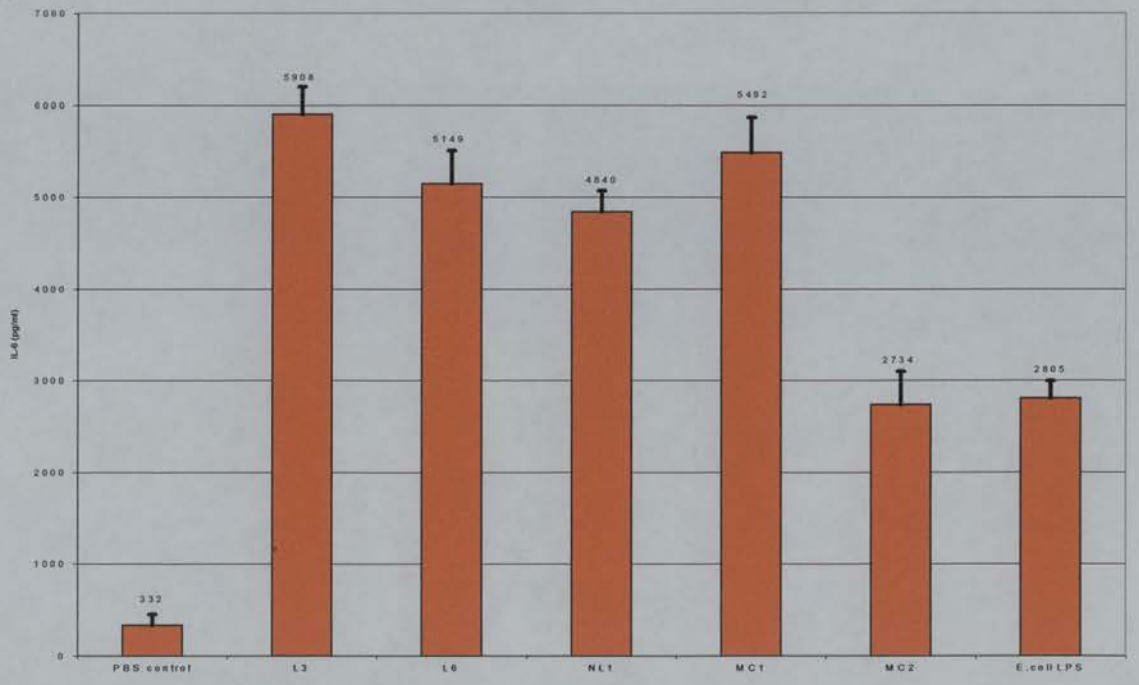


Figure 6.6 IL-6 (pg ml^{-1}) responses to LOS of meningococci (L3, L6), commensal isolates (NL1, MC1, MC2) or *E. coli* endotoxin (100 pg ml^{-1}) by (a) undifferentiated, (b) differentiated THP-1 cells
(n=6, error bars = SD)

(a)



(b)



6.3.5 Effect of LOS immunotype on cytokine levels

In six independent experiments, equivalent amounts of endotoxin (100 pg ml^{-1}) from *E. coli* and NM were assessed for their induction of pro-inflammatory cytokines.

E. coli endotoxin induced significantly lower levels of TNFα and IL-6 than LOS from each meningococcal immunotype tested ($P<0.003$) (Figures 6.7.a and 6.8.a). Immunotypes L3, L7, L8 and L9 induced significantly higher levels of TNFα and IL-6 compared with strains expressing non-L(3,7,9) immunotypes ($p<0.01$) (Figures 6.7 and 6.8).

L7 LOS elicited the highest levels of TNFα compared with all other immunotypes tested including those expressing L(3,7,9) ($P<0.01$) (Figure 6.7.a). L3 LOS elicited TNFα levels significantly higher than those of non-L(3,7,9) strains and L8 (3,7,9) ($P<0.01$), but there were no significant differences observed between results for L3 and L9. L9 LOS elicited TNFα levels significantly higher than those of non-L(3,7,9) strains and L8 (3,7,9) ($P<0.006$), but there were no significant differences observed between results for L3 and L9 ($P>0.19$). LOS of L7 elicited the highest levels of IL-6 compared with all other immunotypes tested including those expressing L(3,7,9) ($P<0.01$). L3 LOS elicited IL-6 levels significantly higher than those all non-L(3,7,9) strains tested ($P<0.006$) but not L8 ($P=0.980$) or L9 ($P=0.864$). Similar patterns were observed for L9.

6.3.6 Cytokine levels in relation to the presence or absence of the major L(3,7,9) structure

To assess the inflammatory response of known LOS antigens, samples were grouped and a two-sided paired analysis was performed as follows: immunotypes L3, L7, and L9 were grouped to assess the inflammatory responses in comparison with immunotypes not expressing L(3,7,9) as their major LOS antigen (Table 2.1) (L2, L4, L5, L6, L8, L10, and L11). The results with immunotypes containing L(3,7,9) were significantly higher for TNFα ($P<0.01$) and IL-6 ($P<0.01$).

6.3.7 Cytokine levels induced by LOS immunotypes in relation to core structure

To assess the role of different core structures, cytokine responses to LOS from immunotypes known to have PEA (1→3) HepII structure in their major LOS immunotype were grouped (L3, L7, L8, L9, L10, and L11) and assessed in relation to responses elicited by LOS from strains that do not have the PEA (1→3) HepII core structure (L2, L4, L5, and L6) (Table 6.3). Levels of TNFα and IL-6 responses induced by LOS structures containing PEA (1→3) HepII were significantly higher. TNFα ($P<0.01$) and IL-6 ($P<0.006$) compared to those without PEA (1→3) HepII. The analysis was repeated excluding immunotypes expressing L(3,7,9) as their major LOS antigen. Cytokine levels induced by LOS of immunotypes possessing the PEA (1→3) core structure (L8, L10, and L11) induced significantly higher responses than LOS from immunotypes with other core structures (L2, L4, L5, L6) although L2 and L5 expressed 3,7,9 as a minor antigens {TNFα ($P<0.01$), IL-6 ($P<0.01$)}.

6.3.8 Effect on cytokine levels following treatment of LOS with pooled human serum

In six experiments, cytokine levels for LOS from the different NM immunotypes incubated with the pooled human serum were significantly reduced compared with the results obtained with the LOS alone. In the presence of serum, the levels of both TNF and IL-6 were reduced to the levels obtained with the PBS control ($P<0.01$) (Figures 6.7.b and 6.8.b).

6.3.9 Effect on cytokine levels following treatment of LOS with immune mouse serum induced by the L3 strain

In six experiments, cytokine levels for LOS from the different immunotypes incubated with the mouse serum induced by the immunoype L3 strain were significantly lower for LOS of immunotype strains L3, L5, L7 and L9 compared with results obtained with the LOS in the absence of serum ($P<0.03$). Treatment of the *E. coli* LPS with the immune mouse serum did not significantly reduce release of either TNFα or IL-6 (Figures 6.7.c and 6.8.c). The pooled non-immune serum sample

obtained from mice prior to immunisation did not significantly reduce cytokine levels for any of the LOS or *E. coli* LPS samples (P>0.65).

Compared with results obtained with LOS alone, TNFα levels induced by LOS co-incubated with immune mouse serum was reduced sixfold for immunotypes L3 and L7 (P<0.01), and fourfold for immunotype L9 (P<0.01). Immunotype L8 co-expressing the L(3,7,9) immunotype showed reduction of TNFα levels by approximately 40% (P<0.01). Release of TNFα by immunotype L5 was also reduced significantly (40%) by treatment with the mouse serum (P<0.01) (Figure 6.7.c).

Compared with results obtained with the LOS alone, IL-6 release was reduced by treatment of the LOS preparations with immune mouse serum by 85-90% for immunotypes L3, L7, and L9 (P<0.01). Immunotypes L5 and L8(3,7,9) were reduced by 50 and 35 %, respectively (P<0.01), L2 by 12% (P<0.02); Ll1 by 5% (P<0.01) (Figure 6.8.c)

6.3.10 Effect on cytokine levels of treatment of LOS with immune mouse serum induced by the NL1 strain

In six experiments, TNFα and IL-6 levels for LOS from different meningococcal immunotypes co-incubated with immune serum of mice vaccinated with strain NL1 were significantly lower compared with cytokine levels obtained with LOS in the absence of serum: L2 (P<0.01); L3 (P<0.01); L7 (P<0.01); L8 (P<0.01); L9 (P<0.01); L11 (P<0.01). TNFα levels were lower for immunotypes L5 (P<0.01) and L10 (P<0.01), but IL-6 levels for these immunotypes were not significantly reduced (P>0.13). IL-6 levels were significantly lower for immunotype L6 (P<0.01), but TNFα levels were not significantly reduced (P=0.34). Cytokine levels for immunotype L4 were not significantly lower (P=0.27). Treatment of *E. coli* LPS with the immune mouse serum induced by NL1 reduced TNFα and IL-6 levels by not more than 15% (Figures 6.7.d and 6.8.d). The non-immune serum did not reduce cytokine levels in any of the endotoxin samples (P=0.89).

Figure 6.7 (a) Release of TNFα (IU ml^{-1}) from VD3 differentiated THP-1 cells challenged with meningococcal LOS or *E. coli* LPS (100 pg ml^{-1}), (b) endotoxins co-incubated with pooled human serum (final dilution 1 in 1000), (c) endotoxins co-incubated with immune mouse serum produced by vaccination with the immunotype L3 (final dilution 1 in 1000), (d) endotoxins co-incubated with immune mouse serum produced by vaccination with NL1 (final dilution 1 in 1000) (n=6; error bars = standard deviation)

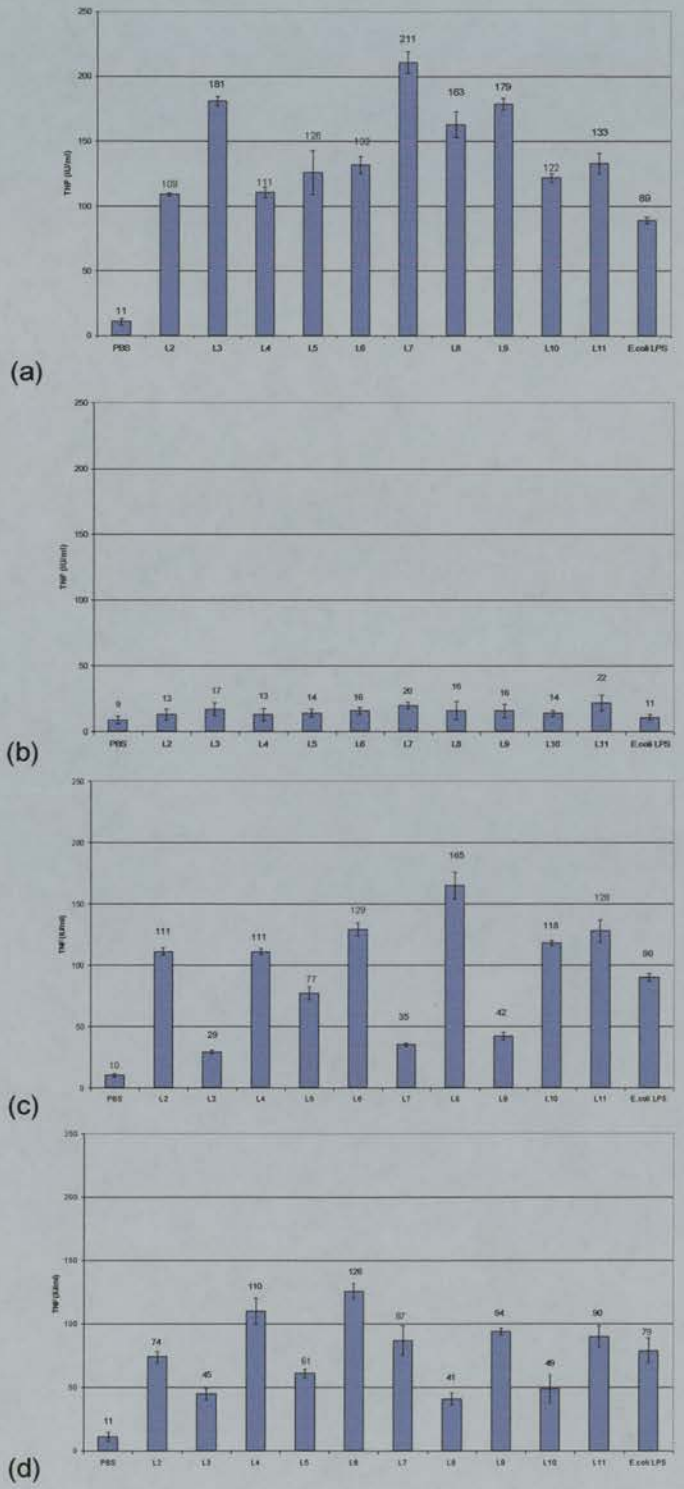
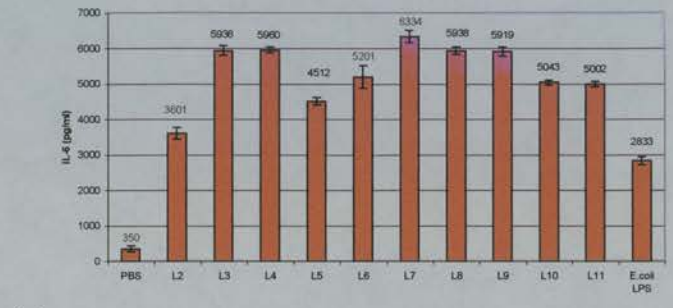
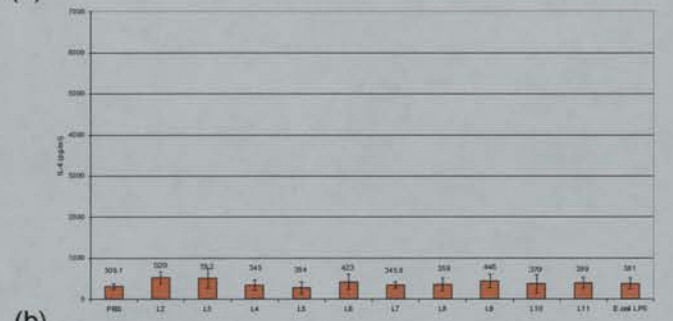


Figure 6.8 (a) Release of IL-6 (pg ml^{-1}) from VD3 differentiated THP-1 cells challenged with meningococcal LOS or *E. coli* LPS (100 pg ml^{-1}), (b) endotoxins co-incubated with pooled human serum (final dilution 1 in 1000), (c) endotoxins co-incubated with immune mouse serum produced by vaccination with the immunotype L3 (final dilution 1 in 1000), (d) endotoxins co-incubated with immune mouse serum produced by vaccination with NL1 (final dilution 1 in 1000) (n=6; error bars = standard deviation)



(a)



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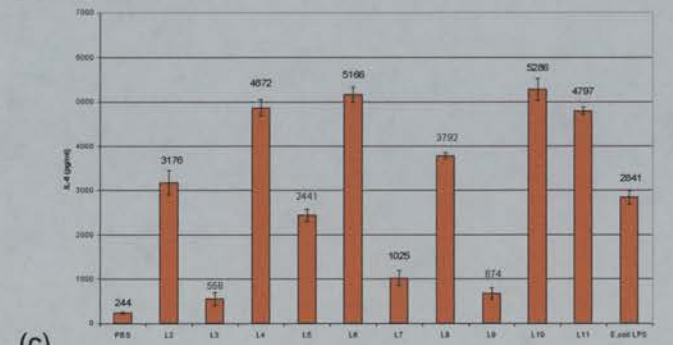
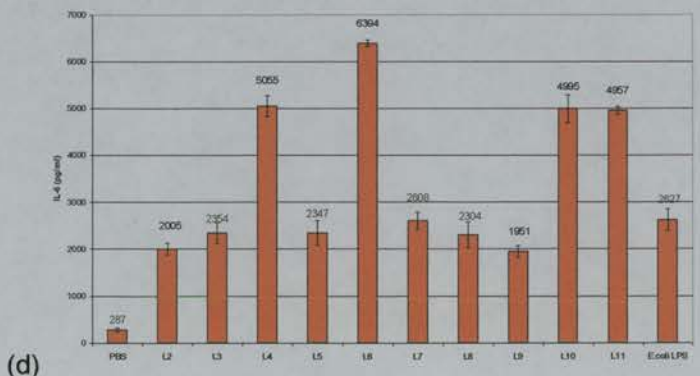


(c)



(d)

6.4 Discussion

6.4.1 Results in relation to objectives

6.4.1.1 Development of model system

The first aim of the study was to develop an *in vitro* model to assess human inflammatory responses to LOS from meningococci and commensal species. Maximal IL-6 and TNFα production was observed between 6-8 hours after exposure of the VD3-differentiated THP-1 cells to meningococcal LOS. THP-1 cells expressing low levels of CD14 responded to the LOS preparations with very low levels of IL-6 and TNFα. VD3-differentiated, CD14-positive THP-1 cells produced significantly higher levels TNFα ($P<0.01$) and IL-6 ($P<0.01$) in response to all endotoxin preparations tested (Figures 6.4 and 6.5).

6.4.1.2 Comparison of inflammatory responses induced by meningococci and commensal species

The second objective was to compare pro-inflammatory responses of THP-1 cells induced by LOS from meningococci with those induced by commensal species. TNFα and IL-6 responses in the model system were used to test the hypothesis that LOS isolated from commensal species would elicit lower levels of pro-inflammatory cytokines than LOS of meningococci. The meningococcal immunotype L3 showed significantly higher TNFα and IL-6 responses compared to LOS of the other isolates tested. The LOS from commensal species elicited lower levels of TNFα than LOS of the L3 immunotype. All results except those for MC2 were significantly higher than the response obtained with commercially available *E. coli* LPS. LOS of NL1, MC1, MC2 and *E. coli* endotoxin elicited significantly lower levels of IL-6 than LOS of the L3 immunotype (Figures 6.5 and 6.6).

6.4.1.3 Assessment of inflammatory responses of meningococcal immunotypes

The third objective was to compare TNFα and IL-6 responses to LOS of different immunotype strains to test the hypothesis that the L(3,7,9) immunotypes obtained from group B and C meningococci had more toxic LOS. Immunotypes L3, L7, and L9 induced significantly higher levels of TNFα and IL-6 compared to strains expressing non-L(3,7,9) immunotypes or endotoxin from *E. coli*. This could partly explain why strains expressing the L(3,7,9) immunotype are isolated more frequently from patients with disease [Jones *et al.*, 1992; Romero & Outschoorn, 1994] (Figures 6.7.a and 6.8.a).

6.4.1.4 Assessment of neutralisation of LOS in the model system

The fourth objective was to use the model to screen for neutralising activities against the LOS of different immunotypes. Treatment of the LOS with pooled human serum significantly reduced cytokine release by all LOS immunotypes. This indicates that antibodies to endotoxin are not only bactericidal but also neutralise the ability of LOS to induce release of pro-inflammatory cytokines from monocytic cells. Immune serum induced in mice to NL1 or L3 strains had narrower ranges of neutralising activity (Figures 6.7.b and 6.8.b).

6.4.2 Methodology

6.4.2.1 Existing models

Several studies investigating the pro-inflammatory responses to meningococcal LOS used either *in vivo* observations in patients with meningococcal disease [Brandtzaeg *et al.*, 1989, 1996], *ex vivo* experiments with peripheral blood mononuclear cells (PBMC) [Cavaillon & Haeffner-Cavaillon, 1986; Petrov *et al.*, 1994; Blondin e*t al.*, 1997; Kalmusova *et al.*, 2000], PMN [Lien *et al.*, 1995; Jenson *et al.*, 1996], *in vivo* mouse studies [Andersen *et al.*, 1997; Quakyi e*t al.*, 1999], or whole blood samples from patients or first degree relatives of patients who died of meningococcal disease [Westendorp *et al.*, 1995; 1997, Gordon *et al.*, 2000].

While these models for the assessment of inflammation reflect the diversity of the inflammatory responses to LOS challenge, some factors might limit their use as a standardised model system for the safety assessment of LOS based vaccines:

Findings from *in vivo* studies using mouse models are only partially transferable to the inflammatory responses in humans. Meningococci are exclusively human pathogens. Patients developing fulminant septicaemia showed plasma levels between 210 and 170,000 ng l^{-1} circulating LOS, and patients with meningitis 25 to 260 ng l^{-1} LOS [Brandtzaeg *et al.*, 1989]. This indicates that relatively small amounts of LOS are associated with the pathology of meningococcal disease in humans. The lethal dose of purified LOS in mice is approximately 10-100 ng LOS/mouse [Petrov *et al.*, 1994; MacKinnon *et al.*, 1992]. The typical weight of a mouse is 20 g which reflects a level of LOS in mice that is 50 times greater than that in humans.

Because of the genetically controlled variability of the inflammatory response of individuals to bacterial antigens and toxins [Westendorp *et al.*, 1995, 1997; Nadel *et al.*, 1996; Gordon *et al.*, 2000; Read *et al.*, 2000], the use of PBMCs and PMNs from individual donors will result in a wide range of inflammatory responses to LOS challenge. The effect of cigarette smoking and viral infections needs to be considered in relation to the results obtained with blood samples from individual donors [Stuart *et al.*, 1989; Cartwright *et al.*, 1991; Hubert *et al.*, 1992; Zorgani *et al.*, 1992; Stanwell-Smith *et al.*, 1994; O'Mahony *et al.*, 1998; Gordon *et al.*, 2000]. For the initial screening of a large range of potential vaccine candidates, a number of donors would have to be included in the screening process.

6.4.2.2 Advantages of the model

The use of the monocyte cell line was considered to have several advantages:

1. THP-1 cells and another monocytic cell line, U937 [Schwende *et al.*, 1996], are readily available through national cell culture collections.
2. Large numbers of cells can be produced within a relatively short period of time for use in studies on inflammatory responses, or protective effects of monoclonal and polyclonal antibodies produced against candidate vaccine antigens or covalescent and normal human serum.
3. The use of cell lines provides a greater reproducibility between different laboratories over time.
4. The method reduces the effects of genetically controlled individual variation in the inflammatory responses and the effects of environmental factors such as exposure to cigarette smoke [Gordon *et al.*, 2000] or effects of recent or current virus infections [Raza *et al.*, 2000].
5. Other factors associated with meningococcal disease can be investigated. These include the exposure to cigarette smoke where water soluble smoke extracts can be co-incubated with the cell line [Raza *et al.*, 1999]. Alternatively, THP-1 cells can be infected with viruses associated with the susceptibility to invasive meningococcal disease [Raza *et al.*, 2000].

6.4.2.3 Limitations of the model:

Limitations of this model include the following:

1. The use of cell lines does not take into account the genetic and environmental variability found in human populations [Westendorp *et al.*, 1997; Read *et al.*, 2000; Gordon *et al.*, 2000].
2. Tumour cell lines are immature cells and do not express all the cell surface antigen phenotypes found in normal human monocytes and macrophages.
3. The involvement of PMN, other leukocytes and antigen presenting cells on the inflammatory process can not be assessed.

6.4.3 Comparison of responses elicited by commensal species

Challenge of VD3-differentiated THP-1 cells with LOS from meningococci and commensal species showed that meningococcal LOS tested induced significantly higher release of cytokines compared with levels obtained with *E. coli* endotoxin. These findings were consistent with reports from a previous study investigating interleukin-1 release after challenge with meningococcal LOS and endotoxins from other Gram-negative species in the absence of LOS specific antibodies [Cavaillon & Haeffner-Cavaillon, 1986]. They support the clinical observations that meningococcal LOS structures are more potent biomodulators compared to endotoxins of enteric bacteria, a key virulence factor contributing to the severity of disease; and CD14-positive monocytic cells might play an important role in the inflammatory response and pathology of meningococcal disease [Petrov *et al.*, 1992].

6.4.3.1 *N. lactamica*

NL1 absorbed bactericidal activity against a broad range of meningococcal immunotype reference strains, isolates from carriers and patients, including strains expressing the L(3,7,9) and L8 immunotypes (Chapter 3). The current study indicates that its LOS induces significantly lower levels of both TNFα and IL-6 compared with LOS from the L3 immunotype. Further assessment of this strain is needed to determine its value as a vaccine candidate and the role of similar commensal strains in the development of natural immunity to meningococcal disease. The ability of antibodies to LOS of NL1 to reduce inflammatory responses to LOS of several meningococcal immunotypes indicates there are cross-reactive components on the LOS structures of both NM and NL. The I-blood group and p^K antigens are structures identified on both these species. NL1 bound monoclonal antibodies specific for L(3,7,9) and L8 epitopes. Other strains of NL also bound immunotype antibodies to L(3,7,9) (23 of 44 strains, 52%) and L8 (8 of 44 strains, 18%) (Table 5.18).

6.4.3.2 *M. catarrhalis*

MC1 has also been shown to absorb bactericidal activity against meningococcal isolates including one expressing the L(3,7,9) immunotype although MC1 did not

bind the monoclonal antibody to this epitope (Chapter 4). It induced higher levels of pro-inflammatory cytokines in VD3 differentiated cells compared to LOS obtained from MC2, immunotype L6, NL1 and *E. coli* endotoxin. Carriage of MC is more frequent than carriage of *Neisseria* species in young infants and children [Harrison *et al.*, 1999; Karalus & Campagnari, 2000], and the structural analysis of MC LOS shows similarities to meningococcal LOS [Vaneechoutte *et al.*, 1990; Rahman & Holme, 1995]. MC is a common causative agent of otitis media in young children, but it is unclear if differences in LOS immunotypes correlate to the severity of MC infections. The findings presented here suggest that the ability of MC to induce inflammation is associated with LOS immunotypes. MC1 bound antibodies to the human blood group antigens p^K, P1 and I similar to MC immunotype C11 [Holme *et al.*, 1990], while MC2 only expressed the p^K antigen (Chapter 5).

Although the L(3,7,9) immunotype was associated with higher levels of inflammatory mediators in experiments with meningococcal LOS immunotype strains, neither MC1 nor MC2 bound the anti-L(3,7,9) antibody. The L(3,7,9) epitope was, however, present on 38 of 126 (30.2%) of the Scottish MC isolates tested. Carriage or infection with MC could induce protective bactericidal antibodies against meningococcal LOS, but its use as a potential vaccine candidate has to be carefully assessed due to the ability of some MC LOS to induce high pro-inflammatory cytokines.

6.4.4 Comparison of responses elicited by NM immunotypes

In this model system, immunotypes L3, L7, L8 and L9 induced significantly higher levels of TNFα and IL-6 compared to other immunotypes. These findings could partly explain why the immunotype L(3,7,9) is most frequently isolated from patients with serogroup B or C meningococcal disease, and other immunotypes are associated with carriage [Jones *et al.*, 1992; Romero & Outschoorn, 1994]. The LOS moieties responsible for different pro-inflammatory responses are not clear. Differences in LOS core, oligosaccharide or lipid A structure might affect the inflammatory responses in the absence of LOS specific antibodies. The oligosaccharide chain length was shown to affect the bioactivity of meningococcal

LOS with mutants expressing short LOS moieties being less active than their wild type forms [Andersen *et al.*, 1997]. Immunotypes L(3,7,9), L2, and L5 express identical oligosaccharide moieties, but their ability to induce inflammatory cytokines varied greatly. Sialylation of the terminal galactose residue might play a role in the ability to induce inflammation. Immunotype reference strains L1, L2, L3, L4, L5, L6, L7, and L8 are thought to be fully or partially sialylated; however, LOS of immunotype L7 induced the highest levels of both TNFα and IL-6 (Figures 6.7 and 6.8).

Immunotype L7 induced the highest levels of cytokines but these were not significantly higher when compared to the structurally related immunotypes L3 and L9. Core antigens might also contribute to induction of these responses. With the exception of immunotype L10 and L11, LOS from strains expressing PEA on the second core heptose induced significantly higher cytokine levels ($P<0.01$) compared to immunotypes expressing either glucose (1→3) or those that lacked a functional group. LOS from immunotypes L(3,7,9) expressing PEA (1→3) HepII core antigen induced the highest levels of cytokines, suggesting that both core structure and α-chain moieties might alter the bioactivity of meningococcal LOS. The lipid A moiety of meningococcal LOS is thought to be heterogenous [Kulshin *et al.*, 1998a & b; Rietschel *et al.*, 1993; Rahman *et al.*, 1998] and differs from the lipid A moiety of MC in composition and chain length of fatty acid. Monophoshorylation found on lipid A from MC and non-pathogenic meningococci is thought to be less toxic compared to diphosphorylated LOS found on meningococcal, but is absent from MC lipid A [Kahler & Stephens, 1998]. There is no published model of the lipid A of NL. With such limited information on the lipid A moieties from meningococci and commensal species, and little published evidence for correlation between lipid A structure and bioactivity, is not possible to assess the findings of this study in relation to the structure of lipid A (8.4).

6.4.6 Assessment of results in relation to vaccine development

In the model system, antibodies found in normal human serum neutralised the pro-inflammatory responses to LOS of each of the meningococcal immunotypes tested. The same serum pool was used to measure bactericidal activity against the immunotype reference strains (Chapters 3 & 4). All meningococcal immunotype reference strains and commensal isolates were killed, ≥ 80 % killing compared to the control to which no serum was added. While complement-mediated bactericidal killing has been used as a major test to assess the protective effect of vaccines for meningococci, the role of neutralising antibodies and opsonic activity in protection against meningococcal disease needs to be considered.

6.4.6.1 Common antigens and neutralising activities

Antibodies elicited in mice by immunisation with L3 or NL1 were able to reduce inflammatory responses against LOS from meningococcal immunotypes with epitopes cross-reactive with the L(3,7,9) immunotype. Co-incubation of LOS with serum obtained from mice vaccinated with the immunotype L3 strain significantly reduced release of TNFα and IL-6 by LOS of L3, L7 and L9 ($P<0.01$) by over 85%. IL-6 release in response to LOS of immunotype L5 and L8 (3,7,9) co-incubated with the immune serum was significantly reduced by 53% and 64%, respectively ($P<0.01$). TNFα release by L5 was reduced by 62% ($P<0.01$) but was not reduced for cells challenged with L8 LOS. The reduction in inflammation of immunotype L5, which shares a terminal saccharide (sialyl) Galβ (1→3) GlcNAcβ (1→3) Galβ(1→4) Glcβ with the L(3,7,9) is evidence that the polyclonal serum contained neutralising antibodies against the terminal oligosaccharide chain, as well as against LOS core antigens. These findings suggest that antibodies to both the oligosaccharide chains and core antigens contribute to neutralising activity.

6.4.6.2 *N. lactamica* LOS vaccine

Antibodies induced by NL1 neutralised inflammatory responses for a broader range of LOS immunotypes than serum obtained by immunising with the L3

meningococcal strain. Co-incubation of LOS with serum obtained from mice vaccinated with NL1 significantly reduced induction of TNFα and IL-6 by LOS from immunotype strains of L2, L3, L5, L7, L8, and L9 ($P<0.01$) by 30-75%. TNFα levels were also reduced in samples challenged with L10, L11 and *E. coli* LOS, but IL-6 levels were not significantly lower in these samples (Table 6.3).

6.4.6.3 LOS as an adjuvant

Endotoxin-depleted or endotoxin-free outer membrane vesicle (OMV) vaccines are poorly immunogenic, and LOS-containing OMV vaccines were more effective at inducing protective antibodies [Alving, 1992; Andersen *et al.*, 1997; Quakyi *et al.*, 1999]. It could be argued that potential vaccine candidates might include LOS from commensal strains that induce neutralising antibodies against the L(3,7,9) immunotypes but induce lower levels of pro-inflammatory cytokines.

6.4.6.4 Potential adverse effects of LOS vaccines

While the paragloboside and I antigen epitopes are common to many immunotypes, vaccines based solely on this structure need to be carefully assessed due to their homology with some host antigens [Mandrell *et al.*, 1988]; 75% of humans express the paragloboside related P1 antigen on their erythrocytes [Naiki & Kato, 1979]. Levels of antibodies to paragloboside, the I antigens need to be assessed in longitudinal studies of infants to determine if they are produced in response to carriage of commensal species expressing these epitopes.

6.5 Conclusions:

1. The THP-1 cell line was useful for screening for induction of pro-inflammatory cytokines by LOS preparations and eliminated the variability of genetic or environmental factors [Westendorp *et al.*, 1995; 1997, Nadel *et al.*, 1996; Gordon *et al.*, 1999, 2000].
2. LOS of meningococci induced higher levels of TNFα and IL-6 than LOS from commensal strains.
3. The LOS from strains expressing the L(3,7,9) moiety as the major immunotype induced significantly higher levels of pro-inflammatory cytokines compared with LOS obtained from other meningococcal immunotypes or commensal species. The results obtained in the *in vitro* model complement the clinical and epidemiological findings and could partly account for the predominance of L(3,7,9) strains among isolates from patients with meningococcal disease.
4. This *in vitro* model could provide a basis for screening vaccine candidates as a first step in toxicity studies or assessment of neutralising antibodies obtained in studies of animal models or human volunteers.

Chapter 7 Opsonophagocytosis of meningococci and commensal species by THP-1 cells

7.1 Introduction

The contributions of commensal species to induction of bactericidal and neutralising antibodies to meningococci were assessed in chapters 3, 4 and 6. In this chapter the effect of cross-reactive antibodies on phagocytosis was assessed.

7.1.1 The role of phagocytosis in meningococcal disease

Some sub-classes of IgG have opsonic functions facilitating phagocytosis and intracellular killing of bacteria by PMN, monocytes, and macrophages through the complement receptor C3 (CD11/18) or IgG affinity receptors (CD16, CD32, CD64) [Halstensen *et al.*, 1989; Guttormsen *et al.*, 1992; Lehmann *et al.*, 1997; Sjursen *et al.*, 1990; Fougerolles *et al.*, 2000]. In the absence of antibodies, meningococci can bind to blood group antigens (Lewis^{x}, Lewis^{a}) found on monocytes. They are ingested but avoid the classical intracellular killing mechanism of lysozyme release and oxidative burst which leads to intracellular survival [Twite *et al.*, 1994]. Opsonin-independent intracellular uptake followed by the oxidative burst was reported to involve the binding of *Neisseria* species to receptors for vitronectin and fibronectin on PMN (CD51, CD41 and CD66) [Dehio *et al.*, 1998].

7.1.2 Phagocytosis and inflammation

Invading meningococci shed outer membrane vesicles (blebs) containing some proteins and LOS [de Voe & Gilchrist, 1973]. PMN are able to phagocytose and kill opsonised meningococci, but they are not able to detoxify endotoxin and release the debris (egestate) of the killed meningococci approximately two hours after phagocytosis [de Voe, 1976, 1982]. Antibodies to meningococcal LOS found in normal human serum can neutralise the inflammatory responses to LOS, but they might also be opsonising in nature [Sjursen *et al.*, 1990]. While IgG1 and IgM are associated with bactericidal action of human serum, the presence of anti-

meningococcal LOS IgG antibodies allows LOS to be detoxified successfully by monocytes through the IgG high affinity FcγRI receptor (CD64) eliciting some release of pro-inflammatory cytokine [Isakov, 1997a & b; Gessner *et al.*, 1998]. Antibody dependent phagocytosis and neutralisation without elicting inflammation is thought to be mediated through the two low affinity IgG receptors FcγRII (CD32) and the FcγRIII receptor (CD16) expressed on natural killer cells, monocytes (FcγRIIIa) and PMN (FcγRIIIb) [Bredius *et al.*, 1994a & b].

7.1.3 Avoidance of phagocytosis by NM as a virulence factor

Meningococci possess virulence factors associated with avoidance of opsonisation, phagocytosis or intracellular killing. Over-expression of capsular polysaccharide shields epitopes on the bacterial cell surface from antibodies and complement and is associated with increased bacterial survival *in vivo* [Taylor, 1983; McKinnon *et al.*, 1993; Hammerschmidt *et al.*, 1994; Vogel *et al.*, 1996; Klein *et al.*, 1996; Kahler *et al.*, 1998].

Sialyl-LOS phenotypes are important not only in evading the complement cascade [Hammerschmidt *et al.*, 1994; Mackinnon *et al.*, 1993] but also in resisting complement and anti-LOS antibody mediated phagocytosis [Kim *et al.*, 1992]. The presence of the sialylated LOS phenotypes found on invasive meningococci is linked to the ability of these strains to evade complement mediated killing by masking the immunoactive terminal galactose residue of some immunotypes [Estabrook *et al.*, 1997; Vogel *et al.*, 1997]. Sialylated LOS was found to up-regulate PMN activation markers and was associated with increased injury of epithelial cell lines [Klein *et al.*, 1996].

7.1.4 Aims and objectives

The aim of this part of the study was to test the hypothesis that antibodies directed against commensals can act as opsonins.

The specific objectives were:

1. to develop a flow cytometric method for assessment of CD14-independent, immunoglobulin-dependent phagocytosis using the human monocytic cell line THP-1;
2. to use the THP-1 assay to assess opsonic activity of pooled human serum absorbed with individual strains of commensal species examined in the bactericidal assays.

7.2 Materials and Methods

7.2.1 Preparation of THP-1 cells

The THP-1 cell line (ECACC, Lot/CB 98/K/018 33629) was cultured as described (2.9) in the absence of VD3.

7.2.1.1 Growth of THP-1 cells

For the phagocytosis assays, the THP-1 suspension (50 ml) was washed twice at 300 x *g* in warm RPMI-1640 assay medium supplemented with 1 mM L-glutamine. The cells were resuspended to a final cell concentration of $1x10^6$ ml^{-1} in assay medium and incubated at 37°C for 30 min. The assay medium contained Dulbecco's phosphate buffered saline (pH 7.4) supplemented with 5×10^{-3} M glucose, 9×10^{-4} M $CaCl_2$, and 5×10^{-4} M $MgSO_4$ [Lehmann *et al.*, 1997].

7.2.1.2 Phenotyping of THP-1

THP-1 cells were assessed by flow cytometry for expression of the cell surface markers as described in chapter 2.13 and Chapter 6. Tables 6.1 and 6.2 list the antigens screened, the species of origin, source and dilution of antibodies used.

7.2.2 Preparation of propidium iodide (PI) labelled bacteria

7.2.2.1 Bacterial strains

The strains listed in Table 7.1 were used in these studies. All strains were grown for 18 hr on HBA. The colonies were suspended in 4 ml 2% (v/v) PBS buffered paraformaldehyde and stained with Vindelov's propidium iodide (2.4) for 15 min at RT. The cells were washed three times by centrifugation in PBS.

7.2.2.2 Enumeration of fluorescent bacteria

Prior to use, aliquots of PI-labelled bacteria were counted and the mean fluorescence intensity assessed by flow cytometry. To assess the number of bacteria in relation to a known number of fluorescence alignment beads, a method modified from Antal-Szalmas *et al.* [1997] and Lebaron *et al.* [1998] was used. Immunocheck beads (Coulter) (10 µl containing 5x 10^6 beads) were added to the PI-labelled bacteria (10µl) and diluted to a final volume of 1 ml with PBS and vortexed. The samples were gated on a forward angle light scatter (logFS) and side angle light scatter (logSS) and quantified simultaneously exciting the fluorochromes by an argon laser operating at 488 nm. The emitted green and red fluorescence intensities of the beads were measured between 505 and 545 nm (logFL1) and between 595 and 645 (logFL3), respectively. The suspension of bacteria and beads was simultaneously counted and the number of bacteria determined in relation to the events elicited by the beads as follow:

$$\text{Number of bacteria present in 10 } \mu l^{-1} = \frac{\text{Number of events of PI positive bacteria} * 5x10^6}{\text{Number of events of alignment beads}}$$

The concentration of the bacteria in the original sample was approximated by

Number of bacteria ml^{-1} = number of bacteria present in 10 µl * 100

The numbers of bacteria estimated by flow cytometry were compared to results using a Thoma counting chamber.

To account for day to day variation, the mean fluorescence intensity (MnI) of the fluorescent beads was adjusted daily to a signal reading of 500. The fluorescence intensity of the PI-labelled bacteria was measured using the FL3 log channel and the percentage and MnI recorded. Aliquots of labelled bacteria (10^8 ml^{-1}) were stored in the dark at 4°C for up to one month. Prior to use in the phagocytosis assay, the appropriate volume was removed and warmed to 37°C.

Table 7.1 Bacterial strains used

Code	LOS (Table 2.1)	Blood group epitopes	Major core LOS
NL1	L(3,7,9), L8	I, p^K	
NL3	L(3,7,9)	I, P, P1, paragloboside	
NL7	L(3,7,9), L1	I, P, P1, paragloboside	
L3	L3 (1,8)	I, P, p^K, paragloboside	C3-PEA
L5	L5 (3,7,9)	I, paragloboside	PEA free
L6	L6	I, P, P1	C7-PEA
L7	L7 (3, 8)	I, P, P1, p^K, paragloboside	C3-PEA
L8	L8 (3, 4, 7)	I, p^K, paragloboside	
B:NT:NT	-	I, P, paragloboside	
MC1	-	I, P, P1, p^K	
MC2	-	P1, p^K	
MC27	-	-	

7.2.3 Phagocytosis assay

7.2.3.1 Sera and antibodies

The unabsorbed and absorbed preparations of the pooled human serum tested in Chapters 3, 4, and 6 were used in this part of the study.

7.2.3.2 Opsonising of bacteria

The bacteria (100 µl, 10^8 ml^{-1}) were opsonised with 5% (v/v) of the unabsorbed or absorbed serum for 30 min at 37°C and washed twice in sterile PBS. The PI-labelled bacteria were diluted in opsonising buffer (OB) containing PBS supplemented with $CaCl_2$ (0.13 g) and $MgSO_4$ (0.12 g) per litre PBS.

7.2.3.3 Phagocytosis

THP-1 cells (10^6 ml^{-1}) were pre-warmed (37°C) in the assay medium for 30 min. Opsonised bacteria (100 µl) were added to duplicate samples of THP-1 cells (1 ml) and incubated at 37°C at 100 rpm for 15 min in an orbital incubator (Gallenkamp). Phagocytosis was terminated by adding 3 ml ice cold PBS supplemented with 0.02% EDTA. To assess the optimal number of PI-labelled bacteria, several ratios of bacteria : cells were tested (1:1 to 200:1) and bacteria bound and ingested measured. To quench fluorescence of adherent bacteria on the surface of the THP-1 cells, the suspension was washed once by centrifugation at 300 x *g* in 4 ml of ice cold PBS

supplemented with trypan blue (3 mg l^{-1}) [Bjerknes & Bassøe, 1984]. The pellet was re-suspended for flow cytometric analysis in 1 ml ice cold 2% (w/v) PBS buffered paraformaldehyde. The samples were stored on ice and analysed by flow cytometry within 1 hour.

7.2.4 Flow cytometric analysis

7.2.4.1 THP-1 cell population

THP-1 cells were gated around the FS and SS channels. These gates were used to measure the red fluorescence (logFL3) of phagocytosed PI-labelled bacteria. The percentage and MnI of the positive cell populations showing phagocytosis were recorded. A combination of the two-percent and mean-intensity method to discriminate positive population in flow cytometry were used (Chapter 2.13).

7.2.4.2 Assessment of phagocytosis

The percentage of positive cells in the population (%) was multiplied by the mean fluorescence intensity (MnI) of the positive cell population to provide the mean ingestion index (II). The II was used to compare phagocytosis in populations of cells. A similar method has been used to calculate binding index (BI) for analysis of attachment of bacteria to buccal epithelial cells or cell lines under different experimental conditions [Alkout *et al.*, 1997; El Ahmer *et al.*, 1999a & b; Gordon *et al.*, 1999; Raza *et al.*, 1993]. The results were compared to the numbers of bacteria per cell determined by confocal and fluorescence microscopy (7.2.4.3). The same batch of fluorescence alignment beads (ImmunoCheck, Coulter) was used to calibrate the logFL1, logFL2 and logFL3 fluorescence intensity to 500 to account for day to day variability.

7.2.4.3 Analysis of samples by confocal and fluorescence microscopy

Following fixation, the suspension of cells and bacteria (50 µl) was prepared for confocal and fluorescence microscopy and analysed at the Department of Anatomy (6.2.8).

7.2.5 Statistical analysis

A two-sided paired Mann-Whitney test (confidence interval, 95%) was used to assess the data.

7.3 Results

7.3.1 Detection of cell surface antigens on phagocytic cells

THP-1 cells did not bind antibodies to CD3, CD4, CD8, CD11c, CD14, CD16 or CD25 antibodies. They bound antibodies to the following antigens: CD11b; CD11/18; CD15; CD41; CD45; CD51; CD64; CD77; and H blood group antigen (Table 7.2).

Table 7.2 Expression of cell surface markers on immature THP-1 cells
(mean of 6 experiments, ±SD)

	% positive population
Control	1.0
CD4	4.06 ±1.5
CD8	1.76 ±0.9
CD11/18	99.3 ±0.4
CD11b	45.3 ±2.9
CD11c	5.94 ±1.5
CD14	4.3 ±0.8
CD15	67.5 ±2.1
CD16	1.53 ±0.4
CD25	5.09 ±2.4
CD41	52.5 ±2.8
CD45	87.8 ±6.1
CD51	59.7 ±2.1
CD64	57.9 ±3.1
CD77	37.7 ±4.4
H	80.0 ±8.1

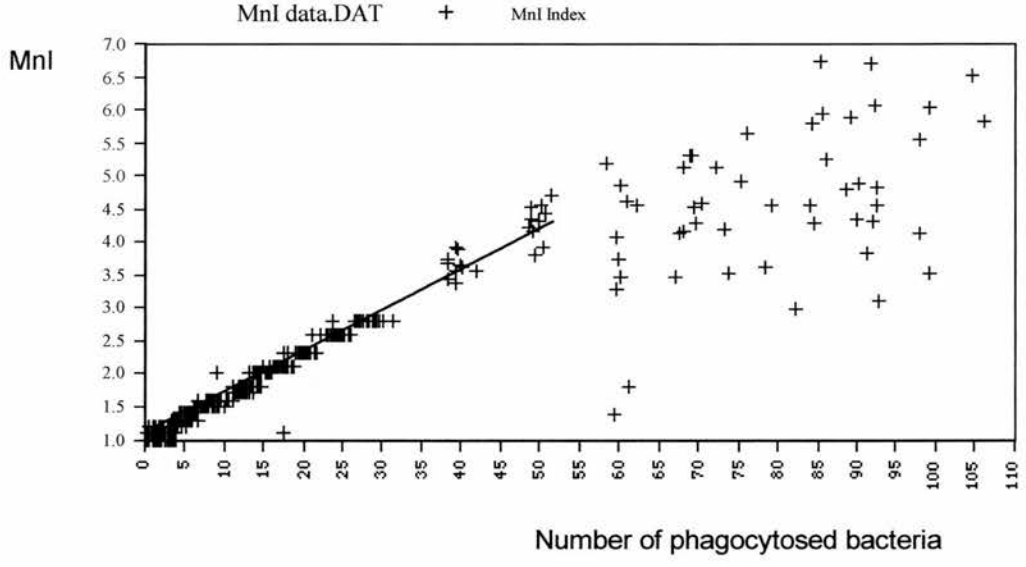
7.3.2 Experimental design

Bacteria : cell ratios were tested ranging from 1:1 to 1:200 bacteria : cell. Quantitative ingestion of bacteria was assessed by the mean fluorescence intensity value (MnI). Increases in the numbers of bacteria ingested correlated with increased MnI (Figure 7.1). To assess the validity of the model, the mean fluorescence intensity index was correlated to the number of phagocytosed PI-labelled bacteria assessed by fluorescence microscopy of 100 THP-1 cells at the individual ratios of bacteria : cell tested [Gardiner, 1997]. The Spearman's correlation coefficient (R^2 = 96.6%) suggested a linear relationship ($P<0.01$) between phagocytosis and the MnI given by the linear regression equation as calculated with Minitab (Figure 7.1):

Number of phagocytosed PI-labelled bacteria = 14.809 * MnI – 14.074

To test if the regression model was not only statistically but also practically linear, the normal plot of residuals and a plot of residuals against fits were used (Minitab). Both supported the linearity of the model between number of neisseriae phagocytosed and the MnI if the numbers of ingested bacteria were between 4 and 75 with a lower limit MnI of 1.3, and an upper limit of 2.8.

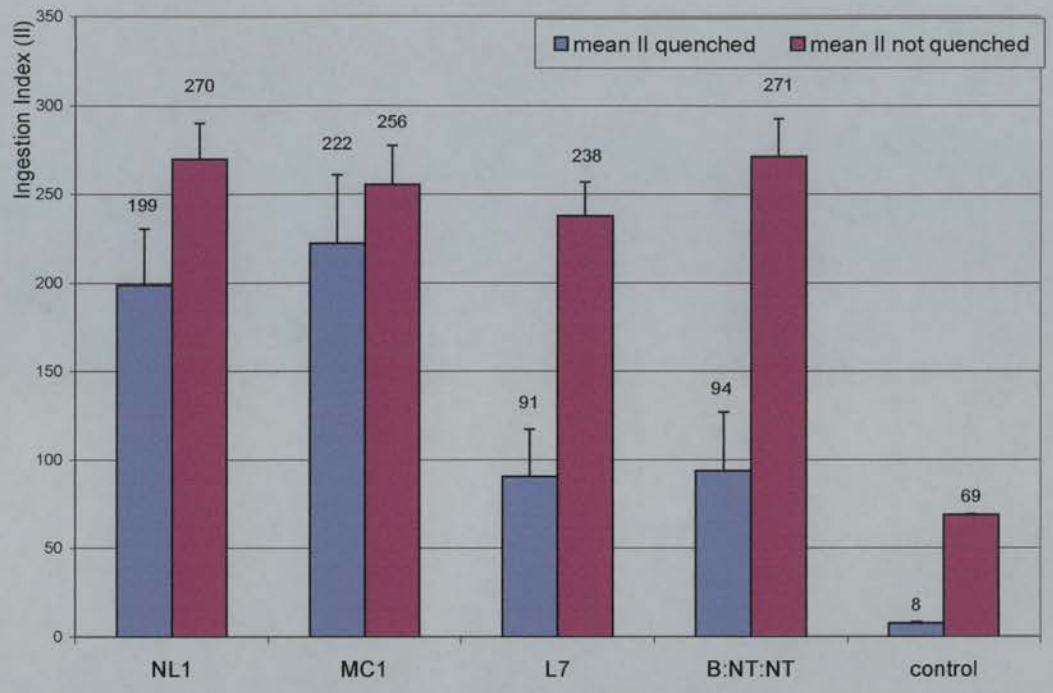
Figure 7.1 Linear relationship (regression) between MnI and number of bacteria per THP-1 cells



7.3.3 Effect of quenching

To discriminate between bound and ingested bacteria, THP-1 cells were assessed by flow cytometry and fluorescence microscopy in the presence and absence of the quenching agent trypan blue (Figure 7.2). All strains tested showed similar ingestion indices in the absence of trypan blue. Quenching of bound bacteria resulted in a significant reduction of the ingestion index of NL1 ($P<0.024$), L7 ($P<0.01$) and B:NT:NT ($P<0.01$), but not MC1 ($P=0.13$). Most of MC1 (87.0%) were ingested, with NL1 showing slightly lower uptake (74.6%). The meningococcal immunotype reference strain L7 (38.1%) and the carrier strain B:NT:NT (34.6%) were phagocytosed in significantly lower number compared to both NL1 and MC1 ($P<0.01$). Quenching reduced the autofluorescence of THP-1 cells (control).

Figure 7.2 Effect of trypan blue quenching on the mean ingestion index after 15 min incubation with opsonised PI-labelled *M. catarrhalis* strains or neisseriae strains



In six independent experiments, the unabsorbed serum pool opsonised all meningococcal and commensal strains tested at a ratio of 50 bacteria : THP-1 cell. Compared to the unquenched samples, the meningococcal immunotype reference strains were ingested by THP-1 cells as follows (percentage in the quenched sample compared to the unquenched control = 100% ± standard error): L3 (41.3% ±4.2); L5 (42.7% ±6.2); L6 (41.9 ±5.3); L7 (38.1% ±7.2); L8 (44.7 ±4.9). The values for the commensal strains were nearly twice that of the meningococcal strains: NL1 (73.6% ±5.6), NL3 (78.1 ±7.7); NL7 (77.5 ±3.5); MC1 (87.0 ±3.3); MC2 (79.3 ±9.1); and MC27 (76.1 ±4.7). Pre-treatment of the bacterial strains with the absorbed complement source used for bactericidal assays in Chapters 3 and 4 resulted in low levels of ingestion of all strains tested, less than 25% compared with the unabsorbed pool.

7.3.3.1 Enumeration of ingested bacteria

In eleven independent experiments the mean intensity was used to calculate the mean number of ingested bacteria per THP-1 cell (± standard error) using the regression equation presented in 7.3.2 (Figure 7.3.a, Table 7.3).

Table 7.3 Mean number of (a) commensals or (b) meningococci ingested per THP-1 cell after 15 min incubation
(mean of eleven independent experiments ± standard deviation)

(a)

NL1	NL3	NL7	MC1	MC2	MC27
25.1 ±4.2	25.5 ±2.9	19.6 ±2.2	29.8 ±4.5	33.3 ±2.0	20.2 ±2.0

(b)

L3	L5	L6	L7	L8	B:NT:NT
10.8 ±3.9	12.9 ±3.5	16.4 ±1.2	12.7 ±5.9	13.6 ±1.8	13.7 ±6.6

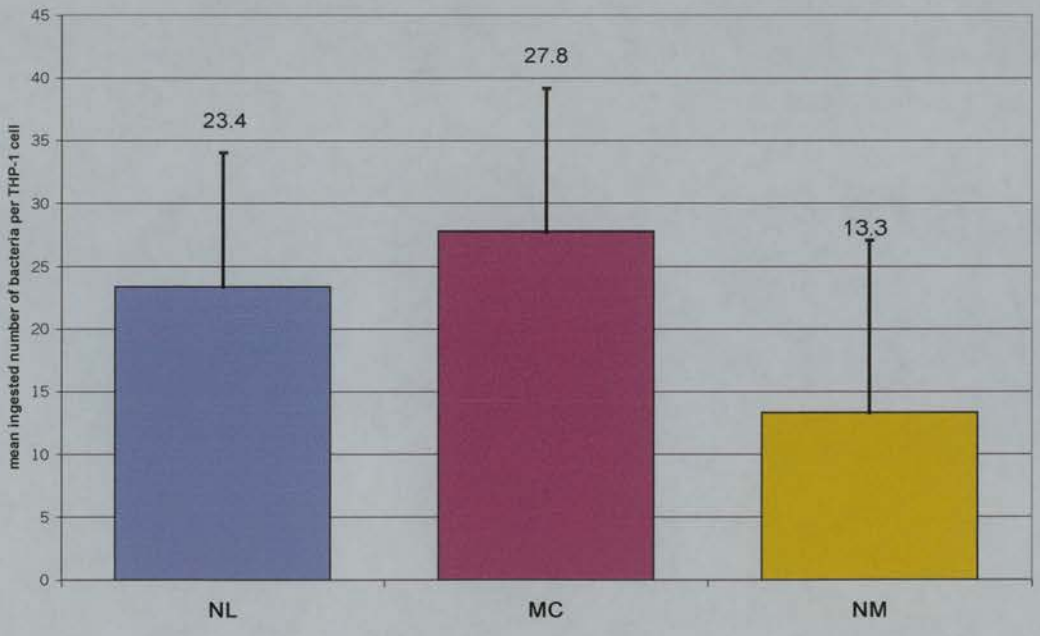
7.3.3.2 Analysis of differences in ingestion of individual strains by species

Mean number of ingested bacteria per THP-1 cell were grouped by species and analysed using two-sided non-parametric Mann-Whitney test (confidence at 95%) to compare differences between the mean number of opsonised meningococci and commensals ingested (Figure 7.3.b): NL mean=23.4, median=22.0, SD=10.66; MC mean=27.78, median=26.4, SD=11.38; NM mean=13.34, median=12.95, SD=13.75.

There were no significant differences between the number of ingested *N. lactamica* and *M. catarrhalis* (P=0.1335). Significantly greater number of NL (P<0.001) or MC (P<0.001) were ingested per THP-1 cell compared to group B meningococci.

Figure 7.3 Mean number of phagocytosed commensals or meningococci per THP-1 cell (a) individual strains; (b) distribution of pooled values by species (mean of eleven independent experiments)

(a)



(b)

7.3.4 The effect of antibody and complement on ingestion of commensal species and meningococci by THP-1 cells

7.3.4.1 *N. lactamica*

Non-opsonised NL1 and NL1 opsonised with the complement source were ingested at a constant rate reaching a maximum ingestion index of approximately 90 after 45 min (Figure 7.4.a). NL1 opsonised with the serum pool showed a linear increase in the ingestion index after 5 min reaching a maximum after 25 min (II=200). NL1 opsonised with the serum pool and the complement source showed a sigmoid relationship after 5 min reaching a plateau after 15 min.

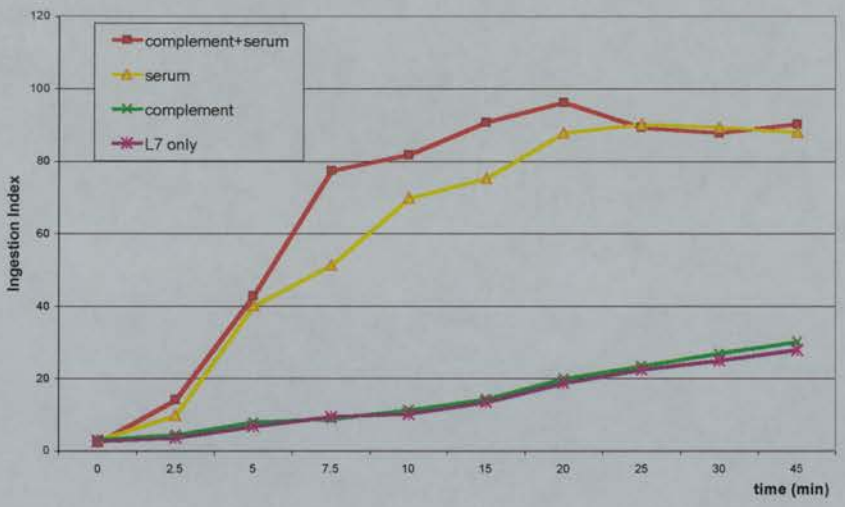
7.3.4.2 *N. meningitidis* L7

Non-opsonised L7 and L7 opsonised with the complement source were ingested reaching a maximum ingestion index of approximately 30 after 45 min (Figure 7.4.b). L7 opsonised with the serum pool showed an increase in the ingestion index after 5 min reaching a maximum of 90 after 20 min. L7 opsonised with the serum pool and the complement source reached a maximum ingestion index of 90 after 15 min.

Time curves for other commensals and meningococci yielded similar results with NL1 representing commensal NL and MC strains, and L7 representing meningococci.

Figure 7.4 The effect of antibody and complement on ingestion of (a) NL1 and (b) L7 meningococci by THP-1 cells
(mean of six independent experiments)

(a)



(b)

7.3.5 Absorption experiments

Three independent experiments with unabsorbed and absorbed sera are summarised in Table 7.4. The ingestion index of the unabsorbed pool co-incubated with the complement source was given a value of 100 and the ingestion index obtained with the absorbed serum is presented as a percentage of the control. A reduction in ingestion index of more than 50% compared with the unabsorbed serum pool was scored as negative (-) reflecting significant reduction in opsonising activity. A reduction of 25 – 50 % was scored as partly absorbed (↓). A reduction of less than 25 % was considered to be positive (+) for opsonic activity. None of the absorbed samples eliminated phagocytic activity completely which indicates that bacteria and/or cell receptors other than immunoglobulin receptors were involved in binding and ingestion of the bacteria.

7.3.5.1 Absorption with *N. lactamica* strains

Absorption with the Scottish strain **NL1** reduced opsonic activity against the following strains: L3 (61%); L5 (12%); L7 (69%); L8 (7%); NL1 (3%); NL3 (5%); NL7 (65%).

Absorption with the Icelandic strain **NL3** reduced opsonic activity against the following strains: L6 (71%); L7 (57%); MC1 (72%); NL1 (11%); and NL3 (8%).

Absorption with the Czech strain **NL7** reduced opsonic activity against the following strains: L6 (55%); NL1 (6%); and NL3 (4%); NL7 (3%).

7.3.5.2 Absorption with *M. catarrhalis* strains

MC1 reduced opsonic activity against NL3 (18%), MC2 (11%) and the homologous strain MC1 (4%). **MC2** reduced the activity against MC1 (13%) and the homologous strain MC2 (9%) only. **MC27** reduced opsonic activity against the meningococcal immunotype L3 (71%) and the homologous strain MC27 (4%).

7.3.5.3 Absorption with *N. meningitidis* strains

Absorption of the pool with immunotype **L3** reduced opsonic activity against the following strains: L3 (8%); L5 (12%); L6 (53%); L7 (8%); L8 (73%); NL1 (55%); NL3 (68%); NL7 (57%); and MC27 (73%).

Absorption with immunotype **L7** reduced opsonic activity against the following strains: L3 (9%); L5 (13%); L7 (8%); L8 (69%); NL1 (61%); NL3 (52%); and NL7 (70%).

Absorption with immunotype **L8** reduced opsonic activity against the following strains: immunotypes L3 (67%); L5 (15%); L7 (57%); L8 (9%); the commensal strains NL1 (7%); NL3 (18%); and NL7 (63%).

Table 7.4: Ingestion indices for phagocytosis of (a) meningococcal immunotype strains and (b) *N. lactamica*, *M. catarrhalis* with the unabsorbed pool and samples of the pool absorbed with meningococcal immunotypes or commensal species
(mean of three independent experiments)

(a)

Test strain	L3		L5		L6		L7		L8	
	%	S	%	S	%	S	%	S	%	S
Unabsorbed pool	100	+	100	+	100	+	100	+	100	+
Absorbed with NL1	61	↓	12	-	96	+	69	↓	7	-
Absorbed with NL3	89	+	91	+	71	↓	57	↓	92	+
Absorbed with NL7	94	+	93	+	55	↓	93	+	99	+
Absorbed with MC1	88	+	92	+	97	+	95	+	96	+
Absorbed with MC2	96	+	87	+	98	+	95	+	95	+
Absorbed with MC27	71	↓	94	+	95	+	96	+	92	+
Absorbed with L3	8	-	12	-	53	↓	8	-	73	↓
Absorbed with L7	9	-	13	-	92	+	8	-	69	↓
Absorbed with L8	67	↓	15	-	91	+	57	↓	9	-

(b)

Test strain	NL1		NL3		NL7		MC1		MC2		MC27	
	%	S	%	S	%	S	%	S	%	S	%	S
Unabsorbed pool	100	+	100	+	100	+	100	+	100	+	100	+
Absorbed with NL1	3	-	5	-	65	↓	95	+	99	+	93	+
Absorbed with NL3	11	-	7	-	4	-	72	↓	97	+	98	+
Absorbed with NL7	6	-	4	-	3	-	94	+	93	+	95	+
Absorbed with MC1	96	+	18	-	94	+	8	-	11	-	91	+
Absorbed with MC2	92	+	92	+	97	+	13	-	9	-	95	+
Absorbed with MC27	93	+	95	+	94	+	91	+	91	+	4	-
Absorbed with L3	55	↓	68	↓	57	↓	87	+	98	+	72	↓
Absorbed with L7	61	↓	52	↓	70	↓	91	+	94	+	98	+
Absorbed with L8	7	-	18	-	63	↓	89	+	93	+	91	+

+ opsonic activity, - no activity ≤25% of unabsorbed pool, ↓ reduction in opsonic activity by 25%>75% of unabsorbed pool

7.4 Discussion

Several studies on phagocytosis of meningococci used polymorphonuclear leukocytes [de Voe & Gilchrist, 1973; de Voe, 1976] and peripheral blood monocytes (PBMC) [Lehmann *et al.*, 1997] or both [Bassøe *et al.*, 2000]. PBMC show variation in their response to meningococcal challenge which might result from: donor variability, different stages of monocyte activation or maturation, differences in the phagocytic response within an PBMC population [Lehmann *et al.*, 1997], the presence of abnormal non-specific functions of PBMC [Nielsen *et al.*, 1988], or contamination with leukocytes or lymphocytes. The method used in this chapter was developed to attempt to reduce some of these problems. Flow cytometry was chosen because it is objective, rapid, and can produce semiquantitiative estimates of the numbers of bacteria per phagocyte and quenching with trypan blue allowed discrimination between surface bound and ingested bacteria.

7.4.1 Method

7.4.1.1 THP-1 phenotype

The high affinity IgG receptor (FcγRI) (CD64) associated with immune phagocytosis is constitutivly expressed on THP-1 cells, as is the complement receptor (C3bR) (CD11/18) [van Furth & van den Berg, 1996].

7.4.1.2 Bacteria : cell ratio

The bacteria count using the flow cytometric method was in general agreement with the enumeration using the Thoma counting method.A linear relationship (r^2=96.6%) was observed between the number of bacteria phagocytosed and the MnI at ratios between 4:1 and 75:1 bacteria per THP-1. These findings are consistent with published PBMC models in which the optimal ratio to assess phagocytosis of meningococci and fluorescent latex beads coated with meningococcal antigens was 1:20 [Lehmann *et al.*, 1997; Bassøe *et al.*, 2000].

7.4.1.3 Discrimination between bound and ingested bacteria

Quenching of bound but not ingested bacteria was an effective way to assess intracellular uptake of PI-labelled bacteria to THP-1 cells, and the results were consistent with other models for the assessment of phagocytosis [Lehmann *et al.*, 1997].

Differences in the binding and ingestion were observed between commensals and group B meningococci. THP-1 cells bound a similar number of all strains tested (approximately 30 – 40 bacteria per cell). THP-1 cells ingested greater numbers of commensal strains after 15 min (20.2 – 33.3) compared to group B meningococci (10.8 – 16.4) ($P<0.01$). These findings are similar to previous observations that capsulate strains associated with invasive meningococcal disease are phagocytosed less efficiently by human monocytic cell lines than non-capsulated meningococci [Kalmusova *et al.*, 2000].

7.4.1.4 Kinetics of opsonophagocytosis of commensals and meningococci

All commensal and meningococcal strains showed similar time curves for ingestion of opsonised bacteria. Maximal ingestion of opsonised bacteria with pooled human serum and complement was observed after 15 – 20 min; 75% of bacteria were ingested after 7.5 min. These findings are consistent with observations using PBMC [Lehmann *et al.*, 1997]. Differences in the number of bacteria ingested depended on the availability of human serum. Low levels of phagocytosis occurred when commensals or meningococci were incubated in the absence of serum or in the presence of the complement source. The kinetics of phagocytosis of serum opsonised bacteria in the presence or absence of complement suggest that both IgG [Sjursen *et al.*, 1990; Lehmann *et al.*, 1997] and complement receptors might be involved in opsonophagocytosis of meningococci and commensal species.

These findings are consistent with previous studies in which complement or properdin deficient individuals showed an increased susceptibility to meningococcal disease due to inefficient phagocytic function [Fijen *et al.*, 2000] or in which

successful phagocytosis of meningococci depended on the presence of human subclasses of IgG and complement using the human monocytic cell line U937 [Aase & Michaelsen, 1994].

7.4.5 Effect on phagocytosis of absorption of pooled human serum by commensals and meningococci

Opsonophagocytic activity of homologous strains was absorbed by all strains tested.

7.4.5.1 *N. lactamica*

All NL strains tested reduced opsonophagocytic activity of the pooled serum against the NL strains tested. Phagocytosis of MC1 was reduced with serum absorbed with NL3. Activity against meningococcal imunotypes L3, L5 and L8 was reduced by absorption with NL1. Phagocytosis of L6 was reduced with the serum absorbed with NL3 or NL7 and phagocytosis of L7 was reduced with serum absorbed with NL1 or NL3. Opsonophagocytic activity against other strains was not affected by absorption with NL strains.

7.4.5.2 M. catarrhalis

Although absorption with MC1 removed bactericidal activity against a number of meningococcal strains (Tables 4.3 & 4.4), the absorbed sera still contained opsonising activity. None of the MC isolates absorbed opsonins for the NL strains tested. MC1 absorbed opsonins against MC2 and MC2 absorbed opsonins against MC1. Absorption of the serum with MC27 reduced phagocytosis of meningococcal immunotype L3. All other strains were not affected by absorption with MC.

7.4.5.3 N. meningitidis

Opsonophagocytic activity for immunotypes L3, L5 and L7 was absorbed by Immunotypes L3, L7, and L8. Opsonins for L3 and L8 were absorbed by

immunotype L3. Activity against NL1 and NL7 was reduced by absorption with L3, L7 or L8. Absorption of serum with L7 or L8 reduced phagocytosis of NL3. Absorption with immunotype L3 reduced opsonising activity against MC27. All other strains were not affected by absorption with meningococci.

Complement-dependent bactericidal activity of pooled human serum correlated with opsonophagocytic activity in most cases (Table 7.5). These findings suggest that antibodies found in normal human serum can be both bactericidal and opsonic in nature.

Table 7.5 Comparison of bactericidal (B) and opsonic (O) activities against (a) meningococcal immunotype reference strains, and (b) commensal species for human pooled serum absorbed by commensal species

(a)

Test strain	L3		L5		L6		L7		L8	
Serum absorbed with	B	O	B	O	B	O	B	O	B	O
Unabsorbed serum	+	+	+	+	+	+	+	+	+	+
NL1	-	↓	-	-	+	+	-	↓	-	-
NL3	+	+	+	+	-	↓	-	↓	+	+
NL7	+	+	+	+	-	↓	-	+	-	+
MC1	+	+	-	+	+	+	+	+	+	+
MC2	+	+	+	+	+	+	+	+	+	+
MC27	+	↓	+	+	+	+	+	+	+	+
Correlation coefficient	0.645		0.645		1		0.73		0.645	

(b)

Test strain	NL1		NL3		NL7		MC1		MC2		MC27	
Serum absorbed with	B	O	B	O	B	O	B	O	B	O	B	O
Unabsorbed serum	+	+	+	+	+	+	+	+	+	+	-	+
NL1	-	-	-	-	+	↓	+	+	+	+	-	+
NL3	-	-	-	-	-	-	+	↓	+	+	-	+
NL7	-	-	-	-	-	-	+	+	+	+	-	+
MC1	+	+	-	-	+	+	-	-	-	-	-	+
MC2	+	+	+	+	+	+	-	-	-	-	-	+
MC27	+	+	-	+	-	+	+	+	+	+	-	-
Correlation coefficient	1		0.73		0.417		0.730		1		na	

+, bactericidal or opsonising activity

-, absence of bactericidal or opsonising activity

7.5 Conclusions

1. The human monocytic cell line THP-1 was a reliable model for the assessment of opsonophagocytic activity of human serum which eliminated the variability due to genetic, maturational and environmental factors.
2. Strains of the commensal species *N. lactamica* and *M. catarrhalis* were more readily phagocytosed in the absence or presence of human serum compared to meningococci.
3. Assessment of functional activity of normal human serum provided evidence that there was a correlation between the presence of bactericidal and opsonic antibodies against most strains tested.
4. The results provide evidence that there are epitopes on commensal strains that can absorb opsonising activity against a variety of group B meningococcal phenotypes; however, there is considerable variability in the cross-reactive antigens between species and among strains within a species.
5. The cross-reactivity between commensals and meningococci suggest that carriage of these induce opsonising antibodies that could contribute to protection against meningococcal disease.

Chapter 8 Discussion

The aims of this study were to assess the role of cross-reactive antigens found on commensal bacteria (NL and MC) in the development of natural immunity to meningococci, and to identify potential vaccine candidates against meningococcal disease caused by serogroup B meningococci that would be safe and effective in children and adults.

The objectives of this study were to address the following questions:

1. Are there antigens on NL that induce antibodies bactericidal for meningococci of different LOS immunotypes? (Chapter 3)
2. Are there antigens on MC that induce antibodies bactericidal for meningococci of different LOS immunotypes? (Chapter 4)
3. Are there oligosaccharide antigens common to NL, MC and meningococcal immunotype reference strains? (Chapter 5)
4. Are the antibodies to NL and MC cross-reactive with meningococci capable of neutralising the bioactivity of LOS? (Chapter 6)
5. Do the antibodies to NL and MC cross-reactive with meningococci have opsonising activities? (Chapter 7)

8.1 Does N. *lactamica* induce antibodies that cross-react with meningococci?

Normal adult serum was absorbed with NL isolates from Scotland, Iceland, the Czech Republic and Greece. The absorbed and unabsorbed serum pools were tested for bactericidal activity against isolates of NL from different areas of Europe and meningococci strains expressing different phenotypes.

The results obtained showed that NL shared antigens with meningococci of different phenotypes including immunotypes L1-L9 of the twelve immunotype reference strains. The results suggested that the isolates tested varied greatly in their phenotype, as has been previously reported for NL [Kim *et al.*, 1989; Bennett *et al.*, 2000]. The Scottish strain NL1 and the Icelandic strain NL3 showed similar absorption patterns, while the isolate from the Czech Republic showed some variation to these two strains. Nevertheless, these three isolates from north and central Europe differed markedly from the Greek isolate tested.

To identify the common antigens, NL and meningococcal strains tested were assessed for their ability to bind monoclonal antibodies used for meningococcal immunotyping [Scholten *et al.*, 1994] and antibodies to blood group antigens. These findings indicate that several carbohydrate moieties homologous with blood group and meningococcal LOS antigens were present on NL. These included terminal oligosaccharide moieties of Ii, paragloboside, P1, and p^K blood group antigens. Meningococcal immunotype structures were also identified. Antibodies to L(3,7,9), the immunotype isolated most often from patients with invasive disease, bound to 75-100% of NL isolates from Scotland, the Czech Republic and Iceland, compared to 33% of isolates from Greece (Table 5.13). Antibodies against immunotype L8 associated with both carrier and disease isolates bound to NL isolates from Scotland (16.7%), Greece (14.8%) and the Czech Republic (50%).

Evidence that NL induced antibodies that are cross-reactive with meningococci was provided in experiments in which immune mouse serum raised against NL1 neutralised inflammatory activity induced by LOS from a broad range of meningococcal immunotypes.

These findings provide evidence that carriage of NL might be associated with the development of natural immunity to meningococcal disease. Phenotypic variations of strains from different geographic regions suggest that these strains induce immune protection that could contribute to herd immunity as illustrated in previous studies of meningococcal isolates from different areas in Europe [Tzanakaki *et al.*, 1993; Krizova *et al.*, 1996]. Epitopes on LOS are the most likely source of cross-reactivity between NL and meningococci. To develop immunity to all major meningococcal immunotypes, exposure to several NL isolates during childhood might be required, and epidemiological studies indicate that children are colonised by genetically different strains [Bennett *et al.*, 2000].

8.2 Does *M. catarrhalis* induce antibodies cross-reactive with meningococci?

The pool of normal adult serum was absorbed with two individual MC isolates from Scotland. The absorption studies with MC1 and MC2 provided evidence for antigens common to MC isolates, NL and meningococcal strains. The two MC strains tested represented different phenotypes and differed in expression of OMP antigens associated with virulence in animal models. MC1 was resistant to antibiotics selective for pathogenic *Neisseriae*, serum resistant and bound in greater numbers to Hep-2 cell line infected with RSV. MC2 was sensitive to selective antibiotics, serum sensitive and bound in significantly lower numbers to RSV-infected cells [El Ahmer *et al.*, 1996, 2000].

MC1 absorbed bactericidal activity against 43% of meningococcal strains tested compared to 17% by MC2 (Chapter 4.4.1). These included immunotype reference strains L1, L4, L5, and L9 and meningococcal isolates from England, Greece, Iceland and Scotland. MC2 absorbed bactericidal activity against Greek and Scottish meningococcal isolates, but did not absorb bactericidal activity against any of the immunotype reference strains, or the meningococcal isolates from England or Iceland.

There was no evidence that MC shared capsular or OMP antigens with meningococci but there are structural similarities between the oligosaccharide moieties of

meningococcal immunotypes and the immunotypes of MC found on different in LOS structures (Table 8.1) (Figure 8.1).

The development of natural immunity to MC is thought to be facilitated by glycoconjugates [Murphy & Bartos, 1989]. Carriage rates of MC are higher in early childhood compared to NL and meningococcal carriage rates combined [Faden *et al.*, 1991; Ejlersten *et al.*, 1994; Harrison *et al.*, 1999] and consecutive carriage of genetically and phenotypically different MC strains are common among young children [Faden *et al.*, 1994].

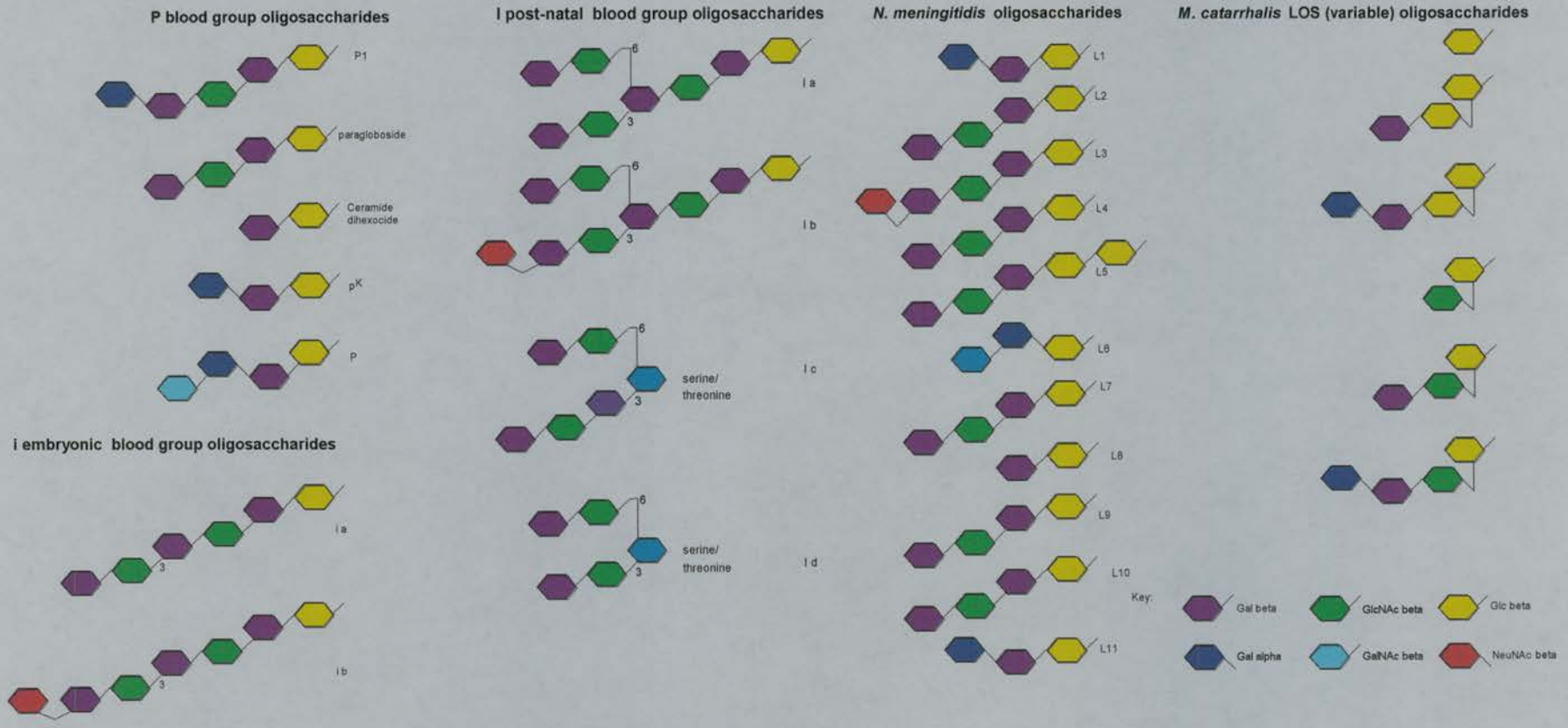
Analysis of the binding of antibodies to blood group antigens and meningococcal immunotypes by WCE showed that the majority of MC isolates from Scotland bound one or more of these antibodies. Most MC strains bound antibodies to p^K, and a third of strains tested bound anti-L(3,7,9) antibodies (Table 5.18). The presence of antibodies to MC in older children, the frequent carriage rate of multiple strains by children, and its cross-reactivity with some of meningococcal antigens provide evidence that MC might be involved in the development of natural immunity to meningococcal disease. The higher frequency of carriage of MC compared to NL and meningococci further suggests that MC might play an important role in the development of antibodies that protect against meningococcal disease.

Table 8.1 Oligosaccharide structures common to human blood group antigens and oligosaccharide chains of LOS of *N. meningitidis* and *M. catarrhalis*

LOS	Terminal oligosaccharide α chain oligomer of the G1 region
L1	Galα (1→4) Galβ (1→4) Glcβ
L11	Galα (1→4) Galβ (1→4) Glcβ
p^k, CD77	Galα (1→4) Galβ (1→4) Glcβ
MC IV	Galα (1→4) Galβ (1→4) Glcα (1→2) Glcβ
P1 blood group	Galα (1→4) Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) Glcβ
MC VII	Galα (1→4) Galβ (1→4) GlcNAcα (1→2)Glcβ
L8	Galβ (1→4) Glcβ
Cer- dihexocide	Galβ (1→4) Glcβ
MC III	Galβ (1→4) Glcα (1→2) Glcβ
P globoside	GalNAcβ (1→3) Galα (1→4) Galβ (1→4) Glcβ
L6	GalNAcβ (1→3) Galα (1→4) Glcβ
I c β adult blood group	Galβ (1→4) GlcNAcβ (1→6) GalNAcβ
I d αβ adult blood group	Galβ (1→4) GlcNAcβ (1→6) GalNAcβ
MC VI	Galβ (1→4) GlcNAcα (1→2) Glcβ
L2	Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) Glcβ
L3	Sialyl→ Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) Glcβ
i b foetal blood group	Sialyl→ Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) Glcβ
I b α adult blood group	Sialyl→ Galβ (1→4) GlcNAcβ (1→3; 1→6) Galβ (1→4) GlcNAcβ (1→4) Galβ (1→4) Glcβ
L4	Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) Glcβ
L5	Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) Glcβ
L7	Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) Glcβ
L9	Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) Glcβ
L10	Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) Glcβ
Paragloboside	Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) Glcβ
i a foetal blood group	Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) Glcβ
I a αβ, I b β adult blood group	Galβ (1→4) GlcNAcβ (1→3; 1→6) Galβ (1→4) GlcNAcβ (1→4) Galβ (1→4) Glcβ
I c α adult blood group	Galβ (1→4) GlcNAcβ (1→3) Galβ (1→6) GalNAcβ
MC V	GlcNAcα (1→2) Glcβ
MC I	Glcβ
MC II	Glcα (1→2) Glcβ

Abbreviations: Gal, galactose; GlcNAc, N-acetylglucosamine; GalNAc, N-acetylgalactosamine ; Glc, glucose; NeuNAc (Sialyl), sialyc acid
MCI-VII identify the variable regions found on MC LOS α and β chains (Chapter 1.5)

Figure 8.1 Oligosaccharide structures common to human blood group antigens and oligosaccharide chains of LOS from *N. meningitidis* and *M. catarrhalis*



8.3 Are antibodies to NL and MC capable of neutralising the bioactivity of LOS?

The majority of work on development of vaccines against meningococcal disease has concentrated on bactericidal activity induced by various candidate antigens. The role that neutralising antibodies might playing in prevention of meningococcal disease has not been considered; however, the damage to the host is mediated by the inflammatory responses to meningococcal antigens, mainly LOS.

Numerous *in vivo* and *in vitro* studies have identified the role of pro- and anti-inflammatory mediators elicited by LOS in mortality or severity of the disease. Endotoxin plasma levels were associated with septic shock (Table 1.1). Serum levels of TNFα correlated with survival, while plasma IL-6 levels were associated with the severity of septic shock in patients with meningococcal disease. Successful neutralisation of free endotoxin through specific or cross-reactive antibodies to meningococcal LOS is a crucial factor in the prevention of septic shock. The LOS of commensals shares a number of oligosaccharide structures with the LOS of meningococcal immunotype strains (Chapter 5) (Figure 8.1). These oligosaccharide structures on commensal species induce neutralising antibodies as suggested by the studies of serum from mice immunised with LOS of strain NL1. If the LOS of commensal species were less toxic than those of meningococci, they might be candidates for vaccine development.

8.3.1 Assessment of release of inflammatory mediators using an *in vitro* model system

A suitable animal model to assess the safety of meningococcal vaccine candidates does not exist. Because of genetically controlled variability of the inflammatory response of individuals to bacterial antigens and toxins [Westendorp *et al.*, 1995, 1997; Nadel *et al.*, 1996; Gordon *et al.*, 1999; Read *et al.*, 2000], an *in vitro* model using the human monocytic cell line THP-1 was developed to assess the induction of cytokines by LOS preparations from meningococci, NL and MC.

The model was considered to have several advantages over models using whole blood or peripheral blood mononuclear cells (PBMC). Whole blood from a variety of donors might contain antibodies able to neutralise the effects of endotoxin challenge. Environmental factors such as exposure to cigarette smoke [Gordon *et al.*, 2000] and viral infections [Raza *et al.*, 2000] were shown to have an effect on the inflammatory response. Monocytic cell lines are readily available through national cell culture collections, sufficient cells can be produced within a relatively short period and the use of cell lines would result in greater reproducibility between different laboratories.

CD14 expression on THP-1 cells can be induced by VD3 allowing the assessment of a wide range of biological functions: toxicity studies; the effect of natural antibodies in normal or convalescent serum from donors of different age groups or ethnic origin; the neutralising activity of antibodies to potential vaccine candidates on inflammatory responses; studies on opsonophagocytosis and intracellular killing.

In vitro models have limitations. The use of cell lines does not take into account genetic and environmental factors that contribute to susceptibility to or severity of the infection. Monocytic tumour cell lines express immature phenotypes and do not express all the cell surface antigens found on PBMC. The involvement of other leukocytes in inflammatory responses to endotoxin challenge are absent.

The *in vitro* model fulfilled the criteria set out in the objectives of this part of the study to assess CD14-dependent inflammatory responses to meningococcal and commensal LOS, and to assess the neutralising activity of normal human serum and immune mouse serum in endotoxin challenge assays.

8.3.2 Comparison of inflammatory responses elicited by LOS from meningococci and commensal species

Meningococcal immunotype L3 induced significantly higher levels of TNFα and IL-6 compared to LOS obtained from NL and MC (Figures 6.4 and 6.5). NL1 absorbed bactericidal activity against a broad range of meningococcal immunotype reference strains including strains expressing the L(3,7,9) immunotype, and isolates from

carriers and patients (Chapter 3). The ability of mouse antibodies to LOS of NL1 to reduce inflammatory responses to LOS of several meningococcal immunotypes indicated there are similar oligosaccharide components on NM and NL. The I-blood group and p^K antigens are structures common to many strains of these species. NL1 bound monoclonal antibodies specific for L(3,7,9) and L8 epitopes. Other strains of NL also bound immunotype antibodies to L(3,7,9) (23 of 44 strains, 52%) and L8 (8 of 44 strains, 18%) (Table 5.18).

MC1 absorbed bactericidal activity against meningococcal isolates expressing a variety of oligosaccharide phenotypes (Chapter 4). Its LOS induced higher levels of pro-inflammatory cytokines compared to LOS obtained from MC2, immunotype L6, NL1 and *E. coli* endotoxin. MC is a common causative agent of otitis media, but it is unclear if differences in LOS immunotypes correlate with the severity of MC infections. Further assessment of MC strains is needed to determine their value as vaccine candidates, and the role of these commensal strains in the development of natural immunity to meningococcal disease.

Although the L(3,7,9) immunotype was associated with higher levels of inflammatory mediators in experiments with meningococcal immunotype strains, neither MC1 nor MC2 bound the anti-L(3,7,9) antibody. The L(3,7,9) epitope was, however, present on 38 of 126 (30.2%) of the Scottish MC isolates tested. Carriage or infection with MC could induce protective bactericidal antibodies against meningococcal LOS, but its use as a potential vaccine candidate has to be carefully assessed due to the ability of some MC LOS to induce levels of pro-inflammatory cytokines equivalent by those elicited by meningococci.

8.3.3 Comparison of inflammatory responses elicited by LOS of meningococcal immunotypes

In this model system, immunotypes L3, L7, L8 and L9 induced significantly higher levels of TNFα and IL-6 compared to other immunotypes. These findings could partly explain why the immunotype L(3,7,9) is most frequently isolated from patients with serogroup B or C meningococcal disease, but other immunotypes are identified

among strains obtained from asymptomatic carriers [Jones *et al.*, 1992; Romero & Outschoorn, 1994]. The LOS moieties responsible for different pro-inflammatory responses are not clear. Differences in LOS core, oligosaccharide or lipid A structure might alter the inflammatory responses in the absence of LOS-specific antibodies. The oligosaccharide chain length was shown to affect the bioactivity of meningococcal LOS with mutants expressing short LOS moieties being less active than their wild type forms [Andersen *et al.*, 1997]. Immunotypes L(3,7,9), L2, and L5 express identical oligosaccharide moieties, but their ability to induce inflammatory cytokines varied greatly. Sialylation of the terminal galactose residue might play a role in the ability to induce inflammation. Immunotype reference strains L1, L2, L3, L4, L5, L6, L7, and L8 are thought to be fully or partially sialylated; however, LOS of immunotype L7 induced the highest levels of both TNFα and IL-6 (Figures 6.6 and 6.7).

Immunotype L7 induced the highest levels of cytokines, but these were not significantly higher when compared to the structurally related immunotypes L3 and L9. Core antigens might also contribute to induction of these responses. With the exception of immunotypes L10 and L11, LOS from strains expressing PEA on the second core heptose induced significantly higher cytokine levels ($P<0.01$) compared to immunotypes expressing either glucose (1→3) or those that lacked a functional group (5.3.9). LOS from immunotypes L(3,7,9) expressing PEA (1→3) HepII core antigen induced the highest levels of cytokines, suggesting that both core structure and α-chain moieties might alter the bioactivity of meningococcal LOS.

8.4 Lipid A

The lipid A moiety of meningococcal LOS is thought to be heterogenous with different phosphorylation patterns in the trans-membrane moiety [Kulshin *et al.*, 1992; Rietschel *et al.*, 1993; Rahman *et al.*, 1998a & b].

The fatty chain composition differs between meningococcal LOS and LOS from serogroup A *M. catarrhalis* (Table 8.2) (Figure 8.2). Meningococcal LOS lacks 10

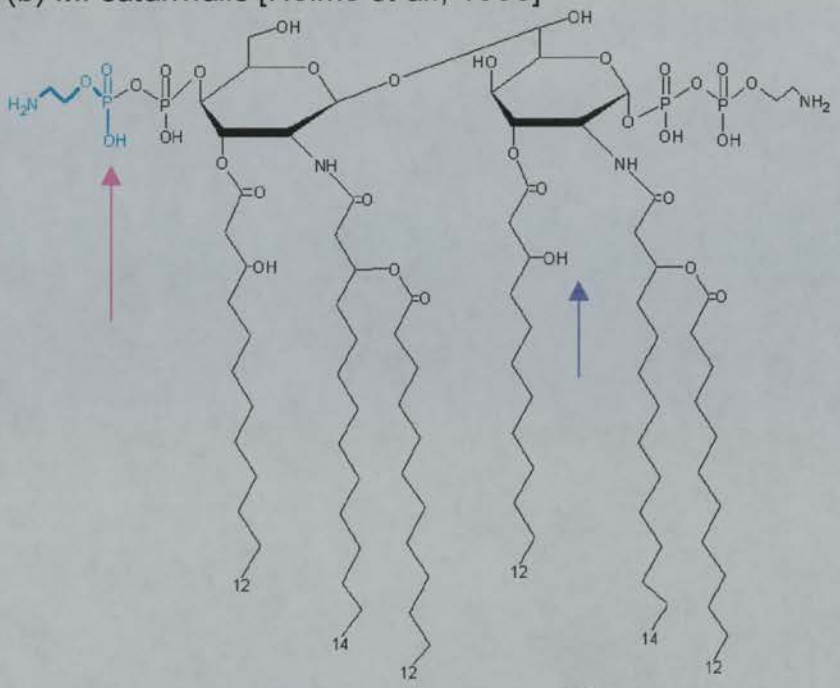
carbon fatty acid chains (0:10) and contains hydroxylated C14 chains (0:14-OH) that are absent in MC lipid A. The structure of LOS from *N. lactamica* has not been published. The chemical composition of LOS from NL [Wieseman & Caird, 1977] indicates the presence of fatty acid chains with 10, 12 and 14 carbons in a ratio of 3 to 1 to 2. NM lipid A with six fatty acid chains compared to seven found in MC. The degree of phosphorylation of lipid A is thought to be associated with its toxicity. Meningococcal lipid A contains of one or two phosphate groups on the first glucosamine, while MC has only one phosphate group. It is thought that monophosphorylated LOS structures are less bioactive compared to diphoshorylated LOS [Kahler & Stephens, 1998]. Differences in phosphorylation, fatty acid chain length and composition might contribute to lower biological activity of LOS of commensal MC and NL compared to meningococcal LOS. These differences might be exploited in the choice of LOS vaccine candidates against meningococcal disease.

Table 8.2 Predicted ratio of the composition of lipid acid chain length found in meningococcal and commensal lipid A

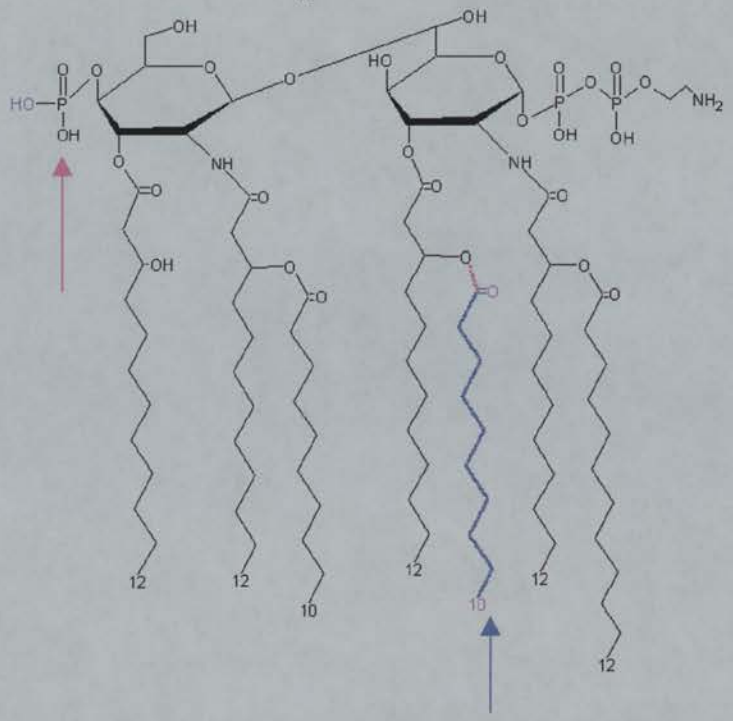
Lipid acid chain length	0:10	0:12	0:14 -OH
N. meningitidis	0	4	2
M. catarrhalis	2	5	0
N. lactamica	3	1	2

Figure 8.2 Structure of lipid A from (a) *N. meningitidis* [Kahler & Stephens, 1998] and (b) *M. catarrhalis* [Holme *et al.*, 1990]

(a)



(b)



meningococcal lipid A may have one or two phosphate groups on the first glucosamine, while MC has only one phosphate group. (red arrow).

MC LOS contains an additional 0:10 lipid acid moiety compared to meningococcal lipid A (blue arrow)

8.5 Are the antibodies to *N. lactamica* and *M. catarrhalis* cross-reactive with meningococci associated with opsonophagocytic activity?

Phagocytosis has a dual role within the immune system:
Invading meningococci can be effectively removed and killed by professional phagocytes [Lehmann *et al.*, 1997] and monocytic phagocytes are able to detoxify opsonised bacterial endotoxin.
Phagocytes are involved in the development of cellular immunity to bacterial infections. Monocytes, tissue, spleen and lymph node macrophages, and Kupffer cells are important antigen presenting cells (APC) able to phagocytose invading bacteria.

The first aim of this part of the study was to develop an *in vitro* model to assess the opsonophagocytic activity of human serum. The undifferentiated monocytic cell line THP-1 expressed the high affinity IgG receptor (FcγR1, CD64) and the complement receptor (C3bR, CD11/18) associated with phagocytosis [van Furth & van den Berg, 1996]. The use of an *in vitro* model reduced variations phagocytic responses within PBMC populations [Nielsen *et al.*, 1988; Lehman *et al.*, 1997], and there was a high degree of control over the phenotype of the cells and experimental conditions.

An optimal bacteria : cell ratio of 50 : 1 was found to yield results similar to other methods which used phagocytic cells isolated from human blood [Lehmann *et al.*, 1997; Bassøe *et al.*, 2000]. Quenching was an effective method to discriminate between external binding and intracellular uptake of PI-labelled bacteria [Lehmann *et al.*, 1997].

THP-1 cells ingested a greater number of non-capsulate commensal strains compared to serogroup B meningococci. Commensal strains of NL and MC were more readily phagocytosed in the absence or presence of human serum compared to meningococci. These finding were comparable to previous observations that capsulate meningococci resist uptake by human phagocytes compared to non-capulate meningococci [Kalmusova *et al.*, 2000] or other bacterial species [Bassøe &

Bjerknes, 1985]. Maximal ingestion of opsonised bacteria was observed between 15 – 20 min, and assessment of functional activity of normal human serum provided evidence that there was a correlation between the presence of bactericidal and opsonic antibodies (7.4.5) (Table 7.5).

The model system supported the key role of immunoglobulin and complement dependent phagocytosis in meningococcal disease [Aase & Michaelsen, 1994; Fijen *et al.*, 2000]. Absorption of normal human serum with meningococcal immunotype strains and commensals provided evidence that serum contained natural, cross-reactive, functional antibodies to carbohydrate antigens expressed on NL, MC and a variety of serogroup B meningococci.

The presence of functional cross-reactive antibodies between commensal and meningococcal strains capable of mediating complement dependent-bactericidal activity and phagocytosis provide further evidence that carriage of commensal bacteria contributes to protection against meningococcal disease.

8.6 Choosing candidates for vaccine development

8.6.1 LOS based vaccines

NL1 induced antibodies in mice able to neutralise the pro-inflammatory response against a wide range of meningococcal immunotypes. Bactericidal and opsonising activity against the corresponding immunotypes was absorbed from the human serum pool with NL1. This suggests that antigens present on NL1 might be prospective candidates for a vaccine against meningococcal disease. NL1 bound antibodies to immunotypes L(3,7,9) and L8 associated most frequently with meningococcal disease, but its LOS induced significantly lowerlevels of pro-inflammatory cytokines than LOS from meningococcal immunotype strains expressing the L(3,7,9) or L8 epitopes.

In order to develop a vaccine candidate against all meningococcal immunotypes, antigens present on additional NL isolates such as NL3 or NL7 should be considered to complement those on NL1. Toxicity tests on the LOS of the NL3 and NL7 LOS phenotypes using human leukocytes should be carried out as a further step if an injectable vaccine is to be considered.

8.6.2 Live vaccines

Young children are often carriers of commensal species. The use of live non-pathogenic NL or MC strains expressing phenotypes with antigens cross-reactive to meningococci might be considered. While NL rarely causes disease, MC is the third most common isolate from children with otitis media. The potential for causing disease is much greater and makes MC a less promising candidate for live vaccines. Ethical considerations and public opinion are also major challenges in pursuing such an approach. NL1 complemented by the Icelandic or the Czech NL isolates might be candidates to cover the major LOS immunotypes of serogroup B.

Due to the lack of suitable animal models for the assessment of mucosal immunity induced by bacterial carriage, further investigations into carriage of commensal species and development of natural immunity to meningococcal disease are crucial.

The main benefit of such a vaccine would be the mimicing the natural development of protective immunity to meningococcal disease in young children who are most susceptible to meningococcal disease.

8.6.3 Commensal LOS as adjuvants for serogroup B OMP vaccines

Endotoxin-depleted or endotoxin-free outer membrane vesicle (OMV) vaccines based on meningococcal OMP are poorly immunogenic, and LOS-containing OMV are more effective at inducing protective antibodies [Alving, 1992; Andersen *et al.*, 1997; Quakyi *et al.*, 1999]. Substituting commensal LOS for meningococcal LOS might boost antibodies to the protein antigens, inducing neutralising antibodies against the L(3,7,9) immunotypes but induce lower levels of pro-inflammatory cytokines.

Several studies have shown that OMV protein vaccines with low LOS content (<0.01%) could be applied directly to the mucosal membrane of animals and human volunteers eliciting both serum and secretory antibodies [Oftung *et al.*, 1999; Haneberg *et al.*, 1998 a & b]. This route of vaccination might be an alternative way using OMV vaccines obtained from *N. lactamica* isolates.

8.7 Future work

This study provided evidence that there are oligosaccharide antigens on the commensals *N. lactamica* and *M. catarrhalis* cross-reactive with those on *N. meningitidis*. Further assessment of cross-reactive natural antibodies and antibodies in immune mouse serum are required to identify the molecules involved and the epitopes recognised by natural antibodies. Of particular interest are structures identified by the immunotypes L8 or L(3,7,9) monoclonal antibodies as these epitopes are found on >90% of isolates from patients with disease. In addition to bactericidal protection, assessment of neutralising and opsonophagocytic antibodies need to be considered.

8.7.1 Antigens

Little is known about the heterogenicity of meningococcal lipid A, and how this structural diversity might affect the toxicity of LOS *in vivo* (8.4). Diverse, truncated or mutant lipid A moieties can easily be assessed for induction of cytokine levels using the THP-1 model. Structural analysis of lipid A in commensals and meningococci could yield insights into its role in pathogenesis. This might identify a vaccine candidate that contains cross-reactive core or oligosaccharide epitopes, but that is less toxic that natural meningococcal LOS.

8.7.2 Epidemiology of commensal isolates

Epidemiological studies in different countries are needed to compare commensal species for expression of cross-reactive oligosaccharide antigens. These studies are particularly important in relation to the findings that commensals from different European regions were significantly different in their phenotypes. Population movements following the opening of the borders with Eastern Europe might lead to introduction of new meningococcal and commensal strains. Greece which has migrants from Balkan, middle East and ex-Soviet block countries should be one focus for these studies.

8.7.3 Antibodies blood group antigens

Further investigations into antibodies that recognise carbohydrate antigens similar to blood group antigens are required. Assessment of antibodies to Ii and P antigens for their interaction with meningococci and commensal species is needed to determine if they correlate with bactericidal, opsonophagocytic, or neutralising activity.

8.7.4 Longitudinal studies

The need for longitudinal studies on development of serum and salivary antibodies against NM is crucial to establish the role that carriage of commensals plays in the development of protection against meningococcal disease in children and young

adults. The systematic screening of isolates, sera and saliva from infants, children and teenagers from different geographic regions for evidence of antibodies to commensals and meningococci is currently planned with colleagues across Europe. The study should include the assessment of neutralising, opsonophagocytic and bactericidal activity against NM strains causing disease in the corresponding regions.

8.7.5 The main questions to be addressed in future studies are:

- Is reduction in carriage of NL and MC with increasing age associated with increased susceptibility to NM as a result of lower levels of antibodies to the commensal strains?
- Does continuous or repeated carriage maintain natural immunity? The increase in risk of meningococcal disease during late puberty might be related to the loss of natural booster effects of mucosal carriage of NL and MC as well as changes in social behaviour and exposure to new strains of meningococci.
- What effect does the conjugate vaccine for serogroup C meningococci have on carriage of commensals?
- If vaccines against MC are introduced to prevent otitis media, what effect would this have on carriage rates of meningococci and commensal species?

Chapter 9 References

1. Aase A., and Michaelsen T.E. (1994) Opsonophagocytic activity induced by chimeric antibodies of the four human IgG subclasses with or without help from complement. *Scand.J.Immunol.* **39**(6): p. 581-587;
2. Abdillahi H., and Poolman J.T. (1988) Typing of group-B Neisseria meningitidis with monoclonal antibodies in the whole-cell ELISA. *J.Med.Microbiol.* **26**(3): p. 177-180;
3. Achtman M. (1991) Clonal properties of meningococci from epidemic meningitis. *Trans.R.Soc.Trop.Med.Hyg.* **85** (suppl. 1): p. 24-31;
4. Aebi C., Maciver I., Latimer J.L., Cope L.D., Stevens M.K., Thomas S.E., McCracken G.H. Jr., and Hansen E.J. (1997) A protective epitope of Moraxella catarrhalis is encoded by two different genes. *Infect.Immun.* **65**(11):4367-4377;
5. Ala'Alden D.A., Powell N.B., Wall R.A., and Borriello S.P. (1993) Localization of the meningococcal receptor for human transferrin. *Infect.Immun.* **61**: p. 751-759;
6. Alkout A.M., Blackwell C.C., Weir D.M., Poxton I.R., Elton R.A., Luman W., and Palmer K. (1997) Isolation of a cell surface component of Helicobacter pylori that binds H type 2, Lewis(a), and Lewis(b) antigens. Gastroenterology **112**(4): p. 1179-1187;
7. Alving C.R. (1992) Lipopolysaccharide, lipid A and liposomes containing lipid A as immunologic adjuvants. *Immunobiol.* **6**: p. 2499-2506;
8. Andersen B.M., and Solberg O. (1988) Endotoxin liberation associated with growth, encapsulation and virulence of Neisseria meningitidis. *Scand.J.Infect.Dis.* **20**(1): p. 21-31;
9. Anderson B.M., and Solberg O. (1988) Endotoxin liberation associated with growth, encapsulation and virulence of *Neisseria meningitidis*. *Scand.J.Infect.Dis.* **20**: 21-31;
10. Anderson B.M., Solberg O., Bryn K., Froholm L.O., Gaustad P., Hoiby E.A., Kristiansen B.E., and Bovre K. (1987) Endotoxin liberated from *Neisseria meningitidis* isolated from carriers and clinical cases. *Scand.J.Infect.Dis.* **19**: p. 409-419;
11. Antal-Szalmas P., van Strijp J.A.G., Weersin A.J.L., Verhoef J., and van Kessel K.P.M. (1997) Quantitation of surface CD14 on human monocytes and neutrophils. *J. Leuk.Biol.* **61**: p. 721-728;
12. Apicella M.A., Shero M., Jarvis G.A., Griffiss J.M., Mandrell R.E., and Schneider H. (1987) Phenotypic Variation in Epitope Expression of the *Neisseria gonorrhoeae* Lipooligosaccharide. *Infect.Immun.* **55**(8): p. 1755-1761;
13. Artenstein M.S., Gold R., Zimmerly J.G., Wyle F.A., Branche W.C. Jr., and Harkins C. (1970) Cutaneous reactions and antibody response to meningococcal group C polysaccharide vaccines in man. J.Infect.Dis. **121**(4): p. 372-377;
14. Aspinall G.O., McDonald A.G., Raju T.S., Pang H., Mills S.D., Kurjanczyk L.A., and Penner J.L. (1992) Serological diversity and chemical structures of Campylobacter

jejuni low-molecular-weight lipopolysaccharides. *J.Bacteriol.* **174**(4): p. 1324-1332;

15. Bailly P., Piller F., Gillard B., Veyrieres A., Marcus D., and Cartron J.P. (1992) Biosynthesis of the blood group Pk and P1 antigens by human kidney microsomes. *Carbohydr.Res.* **228**(1): p. 277-287;
16. Bartoloni A., Norelli F., Ceccarini C., Rappuoli R., and Costantino P. (1995) Immunogenicity of meningococcal B polysaccharide conjugated to tetanus toxoid or CRM197 via adipic acid dihydrazide. *Vaccine* **13**(5): p. 463-470;
17. Bassoe C.F., Smith I., Sornes S., Halstensen A., and Lehmann A.K. (2000) Concurrent measurement of antigen- and antibody-dependent oxidative burst and phagocytosis in monocytes and neutrophils. *Methods* **21**(3): p. 203-220;
18. Bennett J.S., Jolley K.A., Griffiths D., Crook D.W., and Maiden M.C.J. (2000) Population study of *Neisseria lactamica* in infants. 12th International Pathogenic Neisseria Conference, Galveston, Texas, 12-17 November 2000, Poster #228;
19. Bhattacharjee A.K., Moran E.E., Ray J.S., and Zollinger W.D. (1988) Purification and characterization of H.8 antigen from group B *Neisseria meningitidis*. *Infect.Immun.* **56**: p. 773-778;
20. Bhattacherjee A.K., Moran E.E., and Zollinger W.D. (1990) Antibodies to meningococcal H.8 (Lip) antigen fail to show bactericidal activity. *Can.J.Microbiol.* **36**: p. 117-122;
21. Bjerknes R., and Bassøe C.F. (1984) Phagocyte C3-mediated attachment and internalization: flow cytometric studies using a fluorescence quenching technique. *Blut* **49**(4): p. 315-323;
22. Bjerre A., Ovstebo R., Kierulf P., Halvorsen S., and Brandtzaeg P. (2000) Fulminant meningococcal septicemia: dissociation between plasma thrombopoietin levels
23. Blackwell C.C., and Law J.A. (1981) Typing of non-serogroupable Neisseria meningitidis by means of sensitivity to R-type pyocines of Pseudomonas aeruginosa. *J.Infect.* **3**(4): p. 370-378;
24. Blackwell C.C., Jonsdottir K., Weir D.M., Hanson M.F., Cartwright K.A., Stewart J., Jones D., and Mohammed I. (1989) Blood group, secretor status and susceptibility to bacterial meningitis. *FEMS Microbiol.Immunol.* **1**(6-7): p. 351-356;
25. Blakebrough I.S., Greenwood B.M., Whittle H.C., Bradley A.K., and Gilles H.M. (1982) The epidemiology of infections due to *Neisseria meningitidis* and *Neisseria lactamica* in a northern Nigerian community. *J.Infect.Dis.* **146**(5): p. 626-37;
26. Blondin C., Le Dur A., Cholley B., Caroff M., and Haeffner-Cavaillon N. (1997) Lipopolysaccharide complexed with soluble CD14 binds to normal human monocytes. *Eur.J.Immunol.* **27**(12): p. 3303-3309;
27. Boslego J., Garcia J., Cruz C., Zollinger W., Brandt B., Ruiz S., Martinez M., Arthur J., Underwood P., and Silva W (1995) Efficacy, safety, and immunogenicity of a meningococcal group B (15:P1.3) outer membrane protein vaccine in Iquique, Chile.

Chilean National Committee for Meningococcal Disease. *Vaccine* **13**(9): p. 821-829;

28. Bradford M.M. (1976) A rapid and sensitive method for quantitation of microgram quantities of protein utilising the principle of protein-dye binding. *Analyt.Biochem.* **72**: p. 248-254;
29. Brandtzaeg P., Kierulf P., Gaustad P., Skulberg A., Bruun J.N., Halvorsen S., and Sorensen E. (1989) Plasma endotoxin as a predictor of multiple organ failure and death in systemic meningococcal disease. *J.Infect.Dis.* **159**: p. 195-203;
30. Brandtzaeg P., Osnes L., Ovstebo R., Joo G.B., Westvik A.B., and Kierulf P. (1996) Net inflammatory capacity of human septic shock plasma evaluated by a monocyte-based target cell assay: identification of interleukin-10 as a major functional detectivator of human monocytes. *J.Exp.Med.* **184**(1): p. 51-60;
31. Brandtzaeg P., Ovstebo R., and Kierulf P. (1994) Bacteremia and compartmentalization of LPS in meningococcal disease. *J.Endotoxin Res.* **1**(suppl.1): p. 7;
32. Bredius R.G., Fijen C.A., De Haas M., Kuijper E.J., Weening R.S., Van de Winkel J.G., and Out T.A. (1994a) Role of neutrophil Fc gamma RIIa (CD32) and Fc gamma RIIIb (CD16) polymorphic forms in phagocytosis of human IgG1- and IgG3-opsonized bacteria and erythrocytes. *Immunology.* **83**(4): p. 624-630;
33. Bredius R.G.M., Derkx B.H.F., Fijen C.A.P., de Wit T.P.M., de Haas M., Weening R.S., van de Winkel J.G.J., and Out T.A. (1994b) Fcγ receptor Iia (CD32) polymorphism in fulminant meningococcal septic shock in children. *J.Infect.Dis.* **170**: p. 848-853;
34. Brown K.E., Anderson S.M., and Young N.S. (1993) Erythrocyte P antigen: cellular receptor for B19 parvovirus. *Science* **262**: p. 114-117;
35. Butch A.W., and Nahm M.H. (1992) Functional properties of human germinal center B cells. *Cell.Immunol.* **140**(2): p. 331-344;
36. Calandra T., Echtenacher B., Roy D.L., Pugin J., Metz C.N., Hultner L., Heumann D., Mannel D., Bucala R., and Glauser M.P. (2000) Protection from septic shock by neutralization of macrophage migration inhibitory factor. *Nat.Med.* **6**(2): p. 164-170;
37. Cannon J.G., Black W.J., Nachamkin I., and Stewart P.W. (1984) Monoclonal antibody that recognizes an outer membrane antigen common to the pathogenic Neisseria species but not to most nonpathogenic Neisseria species. *Infect.Immun.***43**(3): p. 994-999;
38. Cartwright K.A., Jones D.M., Smith A.J., Stuart J.M., Kaczmarski E.B., and Palmer S.R. (1991) Influenza A and meningococcal disease. *Lancet* **338**(8766): p. 554-557;
39. Cartwright K.A., Stuart J.M., Jones D.M., and Noah N.D. (1987) The Stonehouse survey: nasopharyngeal carriage of meningococci and *Neisseria lactamica*. *Epidemiol.Infect.* **99**(3): p. 591-601;
40. Catlin B.W. (1990) *Branhamella catarrhalis*: an organism gaining respect as a pathogen. *Clin Microbiol Rev.* **3**(4): p. 293-320;

41. Catlin B.W., and Reyn A. (1982) *Neisseria gonorrhoeae* isolated from disseminated and localised infections in pre-penicillin era. Auxotypes and antibacterial drug resistances. *Br.J.Vener.Dis.* **58**(3): p. 158-165;

42. Cavaillon J.M., and Haeffner-Cavaillon N. (1986) Polymyxin-B inhibition of LPS-induced interleukin-1 secretion by human monocytes is dependent upon the LPS origin. *Mol.Immunol.* **23**(9): P. 965-969;

43. Ceesay S.J., Allen S.J., Menon A., Todd J.E., Cham K., Carlone G.M., Turner S.H., Gheesling L.L., DeWitt W., and Plikaytis B.D. (1993) Decline in meningococcal antibody levels in African children 5 years after vaccination and the lack of an effect of booster immunization. *J.Infect.Dis.* **167**(5): p. 1212-1216;

44. Coen P.G., Cartwright K., and Stuart J. (2000) Mathematical modelling of infection and disease due to *Neisseria meningitidis* and *Neisseria lactamica*. *Int.J.Epidemiol.* **29**(1): p. 180-188;

45. Cope L.D., Lafontaine E.R., Slaughter C.A., Hasemann C.A. Jr., Aebi C., Henderson F.W., McCracken G.H. J.r, and Hansen E.J. (1999) Characterization of the *Moraxella catarrhalis* uspA1 and uspA2 genes and their encoded products. J.Bacteriol. **181**(13): p. 4026-4034;

46. Craven D.E., Shen K.T., and Frasch C.E. (1982) Natural bactericidal activity of human serum against *Neisseria meningitidis* isolates of different serogroups and serotypes. *Infect Immun.* **37**(1): p.132-137;

47. Crissan H.A., and Steinkamp J.A. (1999) Cytochemical techniques for multivariate analysis of DNA and other cellular constituents. In Flow Cytometry and Sorting. M.R. Melamed, T. Lindmo, and M.L. Mendelson, editors. Wiley-Liss, New York, p. 227-247

48. Damas P., Reuter A., Gysen P., Demonty J., Lamy M., and Franchimont P. (1989) Tumor necrosis factor and interleukin-1 serum levels during severe sepsis in humans. Crit.Care Med. **17**(10): p. 975-978;

49. Damas P., Ledoux D., Nys M., Vrindts Y., de Groote D., Franchimont P., and Lamy M. (1992) Cytokine serum level during severe sepsis in human IL-6 as a marker of severity. *Ann.Surg.* **215**(4): p. 356-362;

50. Damas P., Canivet J.L., de Groote D., Vrindts Y., Albert A., Franchimont P., and Lamy M. (1997) Sepsis and serum cytokine concentrations. *Crit.Care Med.* **25**(3): p. 405-412;

51. Davies A.L., O'Flanagan D., Salmon R.L., AND Coleman T.J. (1996) Risk factors for *Neisseria meningitidis* carriage in a school during a community outbreak of meningococcal infection. *Epidemiol.Infect.* **117**(2): p. 259-266;

52. De Voe I.W. (1976) Egestion of degraded meningococci by polymorphonuclear leukocytes. *J.Bacteriol.* **125**(1): p. 258-266;

53. De Voe I.W. (1982) The meningococcus and mechanisms of pathogenicity. *Microbiol.Rev.* **46**(2): p. 162-190;

54. De Voe I.W., and Gilchrist J.E. (1973) Release of endotoxin in the form of cell wall blebs during in vitro growth of *Neisseria meningitidis*. *J.Exp.Med.* **138**(5): p. 1156-1167;

55. De Voe I.W., and Gilchrist J.E. (1975) Pili on meningococci from primary cultures of nasopharyngeal carriers and cerebrospinal fluid of patients with acute disease. *J.Exp.Med.* **141**(2): p. 297-305;

56. De Waal Malefyt R., Abrams J., Bennett B., Figdor C.G. and de Vries J.E. (1991) Interleukin 10 (IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. *J.Exp.Med.* **174**: p.1209-1220

57. Dehio C., Gray-Owen S.D., and Meyer T.F. (1998) The role of neisserial Opa proteins in interactions with host cells. *Trends in Microbiol.* **6**(12): p. 489-495;

58. Delahooke D.M., Barclay G.R., and Poxton I.R.. (1995) Tumor necrosis factor induction by an aqueous phenol-extracted lipopolysaccharide complex from *Bacteroides* species. *Infect.Immun.*; **63**(3): p. 840-846;

59. Dell A., Azadi P., Tiller P., and Thomas-Oates J. (1990) Analysis of oligosaccharides epitopes of meningococcal lipopolysaccharides by fast-atom-bombardment mass spectrometry. *Carbohydr.Res.* **200**: p. 59-76;

60. Delogu G., Casula M.A., Mancini P., Tellan G., and Signore L. (1995) Serum neopterin and soluble interleukin-2 receptor for prediction of a shock state in gram-negative sepsis. *J.Crit.Care* **10**(2): p. 64-71;

61. Devi S.J., Schneerson R., Egan W., Vann W.F., Robbins J.B., and Shiloach J. (1991) Identity between polysaccharide antigens of Moraxella nonliquefaciens, group B Neisseria meningitidis, and Escherichia coli K1 (non-O acetylated). *Infect.Immun.* **59**(2): p. 732-736;

62. Di Fabio J.L., Michon F., Brisson J.R., and Jennings H.J. (1990) Structures of the L1 and L6 core oligosaccharide epitopes of *Neisseria meningitidis*. *Can.J.Chem.* **68**: p. 1029-1034;

63. Dunn K.L., Virji M., and Moxon E.R. (1995) Investigations into the molecular basis of meningococcal toxicity for human endothelial and epithelial cells: the synergistic effect of LPS and pili. *Microb.Pathog.* **18**(2): p. 81-96;

64. Edebrink P, Jansson PE, Rahman MM, Widmalm G, Holme T, Rahman M, and Weintraub A. (1994) Structural studies of the O-polysaccharide from the lipopolysaccharide of *Moraxella (Branhamella) catarrhalis* serotype A (strain ATCC 25238). *Carbohydr Res.* **257**(2): p. 269-284;

65. Edebrink P., Jansson P.E., Rahman M.M., Widmalm G., Holme T., and Rahman M. (1995) Structural studies of the O-antigen oligosaccharides from two strains of *Moraxella catarrhalis* serotype C. *Carbohydr Res.* **266**(2): p. 237-61;

66. Edebrink P., Jansson P.E., Widmalm G., Holme T., and Rahman M. (1996) The structures of oligosaccharides isolated from the lipopolysaccharide of *Moraxella*

catarrhalis serotype B, strain CCUG 3292. *Carbohydr Res.* **295**: p. 127-146;

67. Edgar J.D., Wilson D.C., McMillan S.A., Crockard A.D., Halliday M.I., Gardiner K.R., Rowlands B.J., Halliday H.L., and McNeill T.A. (1994) Predictive value of soluble immunological mediators in neonatal infection. *Clin.Sci.(Colch.)* **87**(2): p. 165-171;
68. El Ahmer O.R., Raza M.W., Ogilvie M.M., Blackwell C.C., Weir D.M., AND Elton R.A. (1996) The effect of respiratory virus infection on expression of cell surface antigens associated with binding of potentially pathogenic bacteria. *Adv.Exp.Med.Biol.* **408**: p. 169-177;
69. El Ahmer O.R., Essery S.D., Saadi A.T., Raza M.W., Ogilvie M.M., Weir D.M., and Blackwell C.C. (1999a) The effect of cigarette smoke on adherence of respiratory pathogens to buccal epithelial cells. *FEMS Immunol. Med. Microbiol.* **23**(1): p. 27-36;
70. El Ahmer O.R., Raza M.W., Ogilvie M.M., Weir D.M., and Blackwell C.C. (1999b) Binding of bacteria to Hep-2 cells infected with influenza A virus. *FEMS Immunol.Microbiol.* **23**: p. 331-341;
71. El Ahmer O.R., Amyes S.G.B., and Blackwell C.C. (2000) Surface antigens of *Moraxella (Neisseria) catarrhalis* in relation to antibiotic resistance and serum sensitivity. Proceedings of the 12th International Pathogenic Neisseria Conference, Galveston, Texas, USA USA 12-17 November 2000, Poster *#069;*
72. Engel A., Steinbach G., Kern P., and Kern W.V. (1999) Diagnostic value of procalcitonin serum levels in neutropenic patients with fever: comparison with interleukin-8. Scand.J.Infect.Dis. **31**(2): p. 185-9;
73. Enright M.C., and McKenzie H. (1997) *Moraxella (Branhamella) catarrhalis*--clinical and molecular aspects of a rediscovered pathogen. *J.Med.Microbiol.* **46**(5): p. 360-371;
74. Estabrook M.M., Griffiss J.M., and Jarvis G.A. (1997) Sialylation of *Neisseria meningitidis* lipooligosaccharide inhibits serum bactericidal activity by masking lacto-N-neotetraose. *Infect.Immun.* **65**: p. 4436-4444;
75. Evans J.R., Artenstein M.S., and Hunter D.H. (1968) Prevalence of meningococcal serogroups and description of three new groups. *Am.J.Epidemiol.* **87**(3): p. 643-646;
76. Evans J.S., Yost S., Maiden M.C.J., and Feavers I.M., Yost S.E., and Maiden M.C.J. (1994) Molecular analysis of meningococcal serosubtyping antibodies. Proceedings of the Ninth International Pathogenic Neisseria Conference, 26-30 September 1994, Winchester, England: p. 314-316;
77. Faden H., Waz M.J., Bernstein J.M., Brodsky L., Stanievich J., and Ogra P.L. (1991) Nasopharyngeal flora in the first three years of life in normal and otitis-prone children. *Ann.Otol.Rhinol.Laryngol* **100**(8): p. 612-615;
78. Faden H., Hong J.J., and Pahade N. (1994) Immune response to *Moraxella catarrhalis* in children with otitis media: opsonophagocytosis with antigen-coated latex beads. *Ann.Otol.Rhinol.Laryngol.* **103**(7): p. 522-524;

79. Fernandez de Cossio M.E., Ohlin M., Llano M., Selander B., Cruz S., del Valle J., and Borrebaeck C.A. (1992) Human monoclonal antibodies against an epitope on the class 5c outer membrane protein common to many pathogenic strains of *Neisseria meningitidis*. *J.Infect.Dis.* **166**: p. 1322-1328;

80. Ferreirós C.M., Ferrón L., and Criado M.T. (1994) *In vivo* human immune response to transferrin binding protein 2 and other iron-regulated protein of *Neisseria meningitidis*. *FEMS Immunol.Med.Microbiol.* **8**: p. 63-68;

81. Ferrón L., Ferreirós C.M., Criado M.T., and Pintor M. (1992) Immunogenicity and antigenic heterogeneity of a human transferrin-binding protein in *Neisseria meningitidis*. *Infect.Immun.* **60**(7): p. 2887-2892;

82. Fijen C.A., Bredius R.G., Kuijper E.J., Out T.A., De Haas M., De Wit A.P., Daha M.R., and De Winkel J.G. (2000) The role of Fcgamma receptor polymorphisms and C3 in the immune defence against *Neisseria meningitidis* in complement-deficient individuals. Clin.Exp.Immunol. **120**(2): p. 338-345;

83. Finne J., Finne U., Deagostini-Bazin H., and Goridis C. (1983) Occurrence of alpha 2-8 linked polysialosyl units in a neural cell adhesion molecule. *Biochem.Biophys.Res.Commun.* **112**(2): p. 482-487;

84. Fougerolles de A.R., Chi-Rosso G., Bajardi A., Gotwals P., Green C.D., and Koteliansky V.E. (2000) Global expression analysis of extracellular matrix-integrin interactions in monocytes. *Immunity* **13**(6): p. 749-758;

85. Frasch C.E., and Peppler M.S. (1982) Protection against group B *Neisseria meningitidis* disease: preparation of soluble protein and protein-polysaccharide immunogens. *Infect.Immun.* **37**: p. 271-280;

86. Frasch C.E., Zollinger W.D., and Poolman J.T. (1985a) Proposed Scheme for Identification of Serotypes of *Neisseria meningitidis*, in: The Pathogenic *Neisseriae* G.K. Schoolnik (ed.) Proceedings of the fourth international symposium, American Society for Microbiology, Washington D.C. p. 519-524

87. Frasch C.E., Zollinger W.D., and Poolman J.T. (1985b) Serotype antigens of *Neisseria meningitidis* and a proposed scheme for designation of serotypes. *Rev.Infect.Dis.* **7**: p. 71-91;

88. Frieling J.T., van Deuren M., Wijdenes J., van der Meer J.W., Clement C., van der Linden C.J., and Sauerwein R.W. (1995) Circulating interleukin-6 receptor in patients with sepsis syndrome. *J.Infect.Dis.* **171**(2): p. 469-472;

89. Frosch M., Muller D., Bousset K., and Müller A. (1992) Conserved outer membrane protein of *Neisseria meningitidis* involved in capsular expression. *Infect.Immun.* **60**: p. 798-803;

90. Gamain A., Beurret M., Michon F., Brisson J.-R., and Jennings H.J. (1992) Structure of the L2 lipopolysaccharide core oligosaccharides of *Neisseria meningitidis*. *J.Biol.Chem.* **267**: p. 992-925;

91. Gardiner W.P. (1997) Statistics for the Biosciences. Prentice Hall Europe, Hertfordshire;
92. Gessner J.E., Heiken H., Tamm A., and Schmidt R.E. (1998) The IgG Fc receptor family. *Ann.Hematol.* **76**: p. 231-248;
93. Gold R, Goldschneider I., Lepow M.L., Draper T.F., and Randolph M. (1978) Carriage of *Neisseria meningitidis* and *Neisseria lactamica* in infants and children. *J.Infect.Dis.* **137**(2): p. 112-121;
94. Goldblatt D., Turner M.W., and Levinsky R.J. (1990) *Branhamella catarrhalis*: antigenic determinants and the development of the IgG subclass response in childhood. *J.Infect.Dis.* **162**(5): p. 1128-1135;
95. Goldschneider I., Gotschlich E.C., and Artenstein M.S. (1969) Human immunity to the meningococcus. I. The role of humoral antibodies; II. Development of natural immunity. *J Exp Med.*; 129(6): p.1307-48;
96. Gordon A.E., Al Madani O., Weir D.M., Busuttil A., and Blackwell C.C. (1999) Cortisol levels and control of inflammatory responses to toxic shock syndrome toxin-1 (TSST-1): the prevalence of night-time deaths in sudden infant death syndrome (SIDS). *FEMS Immunol Med Microbiol.* **25**(1-2): p. 199-206;
97. Gordon A.E, El-Ahmer O.R., Weir D.M., Raza M.W., and Blackwell C.C. (2000) Why is cigarette smoke a risk factor for meningococcal disease? Proceedings of the 12th International Pathogenic Neisseria Conference, Galveston, Texas, USA USA 12-17 November 2000, Poster *#202;*
98. Gotschlich E.C. (1972) Enteric bacteria cross-reactive with *Neisseria meningitidis* groups A and C and *Diplococcus pneumoniae* types I and 3. *Infect.Immun.* **6**(5): p. 651-656;
99. Gotschlich E.C., Liu T.Y., and Artenstein M.S. (1969) Human immunity to the meningococcus. 3. Preparation and immunochemical properties of the group A, group B, and group C meningococcal polysaccharides. *J.Exp.Med.* **129**(6): p. 1349-1365;
100. Grados O., and Ewing W.H. (1970) Antigenic relationship between *Escherichia coli* and *Neisseria meningitidis*. *J.Infect.Dis.* **122**(1): p. 100-103;
101. Gratama J.W., D'Hautcourt J.L., Mandy F., Rothe G., Barnett D., Janossy G., Papa S., Schmitz G. and Lenkei R. (1998) Flow cytometric quantitation of immunofluorescence intensity: problems and perspectives. European Working Group on Clinical Cell Analysis. *Cytometry* **33**(2): p. 166-178;
102. Griffiss J.M., O'Brien J.P., Yamasaki R., Williams G.D., Rice P.A., and Schneider H. (1987) Physical heterogeneity of neisserial lipooligosaccharides reflects oligosaccharides that differ in apparent molecular weight, chemical composition, and antigenic expression. *Infect.Immun.* **55**(8): p. 1792-1800;
103. Griffiss J.M., Brandt B., Engstrom J., Schneider H., Zollinger W., and Gibson B. (1994) Structural relationships and sialylation among meningococcal lipopolysaccharide

(LOS) serotypes. in Proceedings of the Ninth International Pathogenic *Neisseriae* Conference; 26-30 September 1994, Winchester, England: p. 12;

104. Gu X.X., Tsai C.M.,and Karpas A.B. (1992) Production and characterization of monoclonal antibodies to type 8 lipooligosaccharide of *Neisseria meningitidis*. *J.Clin.Microbiol.* **30**(8): p. 2047-2053;
105. Gu X.-X., Chen J., Barenkamp S.J., Robbins J.B., Tsai C.-M., Lim D.J., and Battey J. (1998) Synthesis and characterization of lipooligosaccharide-based conjugates as vaccine candidates for *Moraxella (Branhamella) catarrhalis*. *Infect.Immun.* **66**(5): p. 1891-1897;
106. Guttormsen H.K., Bjerknes R., Naess A., Lehmann V., Halstensen A., Sornes S., and Solberg C.O. (1992) Cross-reacting serum opsonins in patients with meningococcal disease. *Infect.Immun.* **60**(7): p. 2777-2783;
107. Hager H., Verghese A., Alvarez S., and Berk S.L. (1987) *Branhamella catarrhalis* respiratory infections. *Rev.Infect.Dis.* **9**(6): p. 1140-1149;
108. Hakomori S. and Kannagi R. (1986) Carbohydrate antigens in higher animals in: Handbook of Experimental Immunology (ed.) D.M. Weir, Blackwell Scientific;
109. Halstensen A., Sjursen H., Vollset S.E., Froholm L.O., Naess A., Matre R., and Solberg C.O. (1989) Serum opsonins to serogroup B meningococci in meningococcal disease. *Scand.J.Infect.Dis.* **21**(3): p. 267-276;
110. Hammerschmidt S., Birkholz C., Zahringer U., Robertson B.D., van Putten J., Ebeling O., and Frosch M. (1994) Contribution of genes from the capsule gene complex (cps) to lipooligosaccharide biosynthesis and serum resistance in *Neisseria meningitidis*. *Mol.Microbiol.* **11**(5): p. 885-896;
111. Hancock I., and Poxton I.R. (1988) Modern Microbiological Methods: Bacterial Cell Surface techniques John Wiley & Sons, Chichester, UK;
112. Haneberg B., Dalseg R., Wedege E., Hoiby E.A., Haugen I.L., Oftung F., Andersen S.R., Naess L.M., Aase A., Michaelsen T.E., and Holst J. (1998a) Intranasal administration of a meningococcal outer membrane vesicle vaccine induces persistent local mucosal antibodies and serum antibodies with strong bactericidal activity in humans. *Infect.Immun.* **66**(4): p. 1334-1341;
113. Haneberg B., Dalseg R., Oftung F., Wedege E., Hoiby E.A., Haugen I.L., Holst J., Andersen S.R., Aase A., Meyer Naess L., Michaelsen T.E., Namork E., and Haaheim L.R. (1998b) Towards a nasal vaccine against meningococcal disease, and prospects for its use as a mucosal adjuvant. *Dev.Biol.Stand.* **92**: p. 127-133;
114. Harlow E., and Lane D. (1988) Antibodies: A laboratory mannual. Cold Spring Harbor Laboratory, New York;
115. Harrison L.M., Morris J.A., Telford D.R., Brown S.M., and Jones K. (1999) The nasopharyngeal bacterial flora in infancy: effects of age, gender, season, viral upper respiratory tract infection and sleeping position. *FEMS Immunol.Med.Microbiol.* **25**(1-

2): p. 19-28;

116. Hayrinen J., Jennings H., Raff H.V., Rougon G., Hanai N., Gerardy-Schahn R., and Finne J. (1995) Antibodies to polysialic acid and its N-propyl derivative: binding properties and interaction with human embryonal brain glycopeptides. *J.Infect.Dis.* **171**(6): p. 1481-1490;
117. Hazelzet J.A., van der Voort E., Lindemans J., der Heerdt P.G., and Neijens H.J. (1994) Relation between cytokines and routine laboratory data in children with septic shock and purpura. *Intensive Care Med.* **20**(5): p. 371-374;
118. Helminen M.E., McIver I., Paris J., Latimer J.L., Lumbley S.L., Cope L.D., McCracken G.H., and Hansen E.J. (1993) A mutation affecting expression of a major outer membrane protein of *Moraxella catarrhalis* alters serum resistance and survival *in vivo*. *J.Infect.Dis.* **168**: p. 1194-1201;
119. Herbert D.A., and Ruskin J. (1981) Are the "nonpathogenic" Neisseriae pathogenic? *Am.J.Clin.Pathol.* **75**(5): p. 739-743;
120. Hitchcock P.J. (1989) Unified nomenclature for pathogenic *Neisseria* species. *Clin.Microbiol.Rev.* B: supplement S64-S65
121. Hodgetts T.J., Brett A., and Castle N. (1998) The early management of meningococcal disease. *J.Accid.Emerg.Med.* **15**(2): p. 72-76;
122. Holme T., Rahman M., Jansson P.E., and Widmalm G. (1990) The lipopolysaccharide of *Moraxella catarrhalis* structural relationships and antigenic properties. *Eur.J.Biochem.* **265**(2): p. 524-529;
123. Holten E., Bratlid D., and Bovre K. (1978) Carriage of *Neisseria meningitidis* in a semi-isolated arctic community. *Scand.J.Infect.Dis.* **10**(1): p. 36-40;
124. Holzheimer R.G., Capel P., Cavaillon J.M., Cainzos M., Frileux P., Haupt W., Marie C., Muller E., Ohmann C., Schoffel U., Lopez-Boado M.A., Sganga G., Stefani A., and Kronberger L. (2000) Immunological surrogate parameters in a prognostic model for multi-organ failure and death. *Eur.J.Med.Res.* **5**(7): p. 283-294;
125. Horsburgh T., Martin S. and Robson A.J. (2000) The application of flow cytometry to histocompatibility testing. *Transpl.Immunol.* **8**(1): p. 3-15;
126. Hubert B., Watier L., Garnerin P., and Richardson S. (1992) Meningococcal disease and influenza-like syndrome: a new approach to an old question. *J.Infect.Dis.* **166**(3): p. 542-545;
127. Isakov N. (1997b) ITIMs and ITAMs. The Yin and Yang of antigen and Fc-receptor-linked signaling machinery. *Immunol.Res.* **16**: p. 85-100;
128. Isakov N. (1997a) Immunoreceptor tyrosine-based activation motif (ITAM), a unique module linking antigen and Fc receptors to their signaling cascades. *J.Leukoc.Biol.* **61**(1): p. 6-16;
129. James S.Y., Williams M.A., Kelsey S.M., Newland A.C., and Colston K.W. (1997) The role of vitamin D derivates and retinoids in the differenciation of human leukaemia

cells. *Biochem.Pharm.* **54**: p. 625-637;

130. Jennings H.J., Johnson K.G., and Kenne L. (1983) The structure of an R-type oligosaccharide core obtained from some lipopolysaccharides of *Neisseria meningitidis*. Carbohydr.Res. **121**: p. 233-241;
131. Jennings H.J., Lugowski C., and Ashton F.E. (1984) Conjugation of meningococcal lipopolysaccharide R-type oligosaccharides to tetanus toxoid as a route to a potential vaccine against group B *Neisseria meningitidis*. *Infect.Immun.* **43**: p. 407-412;
132. Jennings M.P., Srikhanta Y.N., Moxon E.R., Kramer M., Poolman J.T., Kuipers B., and van der Ley P. (1999) The genetic basis of the phase variation repertoire of lipopolysaccharide immunotypes in *Neisseria meningitidis*. *Microbiology* **145** (Pt 11): p. 3013-3021;
133. Jensen T.J., Kharazmi A., Shand G., Nielsen H., and Tvede M. (1996) Immunological properties of meningococcal lipopolysaccharide from serogroups A, B & C. *APMIS*. **104**(1): p. 54-60;
134. Jones D.M., Borrow R., Fox A.J., Gray S., Cartwright K.A., and Poolman J.T. (1992) The lipooligosaccharide immunotype as a virulence determinant in *Neisseria meningitidis*. *Microb.Pathog.* **13**: p. 219-224;
135. Jousimies-Somer H.R., Savolainen S., and Ylikoski J.S. (1989) Comparison of the nasal bacterial floras in two groups of healthy subjects and in patients with acute maxillary sinusitis. *J.Clin.Microbiol.* **27**(12): p. 2736-2743;
136. Kahler C.M., and Stephans D.S. (1998) Genetic basis for biosynthesis, structure, and function of meningococcal lipooligosaccharide (endotoxin) *Crit.Rev.Microbiol.* **24**(4): p. 281-334;
137. Kahler C.M., Martin L.E., Shih G.C., Rahman M.M., Carlson R.W., and 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.* **66**(12): p. 5939-5947;
138. Kalmusova J., Novotny J., Hulinska D., Musilek M., and Kriz P. (2000) Interactions of invasive and noninvasive strains of *Neisseria meningitidis* with monkey epithelial cells, mouse monocytes and human macrophages. New.Microbiol **23**(2): p. 185-200;
139. Karalus R., and Campagnari A. (2000) *Moraxella catarrhalis*: a review of an important human mucosal pathogen. *Microbes Infect.* **2**(5): p. 547-559;
140. Kim J.J., Mandrell R.E., Hu Z., Westerink M.A., Poolman J.T., and Griffiss J.M. (1988) Electromorphic characterization and description of conserved epitopes of the lipooligosaccharides of group A *Neisseria meningitidis*. *Infect.Immun.* **56**(10): p. 2631-2638;
141. Kim J.J., Mandrell R.E., and Griffiss J.M. (1989) *Neisseria lactamica* and *Neisseria meningitidis* share lipooligosaccharide epitopes but lack common capsular and class 1, 2, and 3 protein epitopes. *Infect.Immun.* **57**(2): p. 602-608;

142. Kim J.J., Zhou D., Mandrell R.E., and Griffiss J.M. (1992) Effect of exogenous sialylation of the lipooligosaccharide of *Neisseria gonorrhoeae* on opsonophagocytosis. *Infect.Immun.* **60**(10): p. 4439-4442;

143. Klein N.J., Ison C.A., Peakman M., Levin M., Hammerschmidt S., Frosch M., and Heyderman R.S. (1996) The influence of capsulation and lipooligosaccharid structure on neutrophil adhesion molecule expression and endothelial injury by Neisseria meningitidis. *J.Infect.Dis.* **173**: p. 172-179;

144. Klingman K.L., and Murphy T.F. (1994) Purification and characterization of a high-molecular-weight outer membrane protein of *Moraxella (Branhamella) catarrhalis*. *Infect.Immun.* **62**(4): p. 1150-1155;

145. Kogan G., Uhrin D., Brisson J.R., and Jennings H.J. (1997) Structural basis of the *Neisseria meningitidis* immunotypes including the L4 and L7 immunotypes. *Carbohydr.Res.* **298**(3): p. 191-199;

146. Kornelisse R.F., Hazelzet J.A., Hop W.C., Spanjaard L., Suur M.H., van der Voort E., and de Groot R. (1997) Meningococcal septic shock in children: clinical and laboratory features, outcome, and development of a prognostic score. *Clin.Infect.Dis.* **25**(3): p. 640-646;

147. Kremastinou J., Tzanakaki G., Velonakis E., Voyiatzi A., Nickolaou A., Elton R.A., Weir D.M., and Blackwell C.C. (1999a) Carriage of *Neisseria meningitidis* and *Neisseria lactamica* among ethnic Greek school children from Russian immigrant families in Athens. *FEMS Immunol.Med.Microbiol.*; **23**(1): p.13-20;

148. Kremastinou J., Tzanakaki G., Pagalis A., Theodondou M., Weir D.M., and Blackwell C.C. (1999b) Detection of IgG and IgM to meningococcal outer membrane proteins in relation to carriage of *Neisseria meningitidis* or *Neisseria lactamica*. *FEMS Immunol.Med.Microbiol.* **24**(1): p.73-78;

149. Krizova P., Musilek M., Danielova V., and Holubova J. (1996) New serotype candidate of *Neisseria meningitidis*. *Cent.Eur.J.Public Health.* **4**(3): p. 169-172;

150. Kulshin V.A., Zahringer U., Lindner B., Frasch C.E., Tsai C.M., Dmitriev B.A., and Rietschel E.T. (1992) Structural characterisation of the lipid A component of pathogenic *Neisseria meningitidis*. *J.Bacteriol.* **174**(6): p. 1793-1800;

151. Lafontaine E.R., Wagner N.J., and Hansen E.J. (2001) Expression of the Moraxella catarrhalis UspA1 protein undergoes phase variation and is regulated at the transcriptional level. *J.Bacteriol.* **183**(5): p. 1540-1551;

152. Lampariello F., and Aiello A. (1998) Complete mathematical modeling method for the analysis of immunofluorescence distributions composed of negative and weakly positive cells. *Cytometry* **32**: p. 241-254;

153. Lebaron P., Parthuisot N., and Catala P. (1998) Comparison of blue nucleic acid dyes for flow cytometric enumeration of bacteria in aquatic systems. *Appl.Environ.Microbiol.* **64**: p. 1725;

154. Lee B.C. (1994) Isolation and characterization of the haemin binding proteins from *Neisseria meningitidis*. *Microbiology* **140**: p. 1473-1480;

155. Lehmann A.K., Halstensen A., Holst J., and Bassøe C.-F. (1997) Functional assays for evaluation of serogroup B meningococcal structures as mediators of human opsonophagocytosis. *J.Immunol.Methods* **200**: p. 55-68;

156. Lenkei R., Gratama J.W., Rothe G., Schmitz G., D'Hautcourt J.L., Arekrans A., Mandy F. and Marti G. (1998) Performance of calibration standards for antigen quantitation with flow cytometry. *Cytometry* **33**(2): p. 188-196;

157. Lien E., Liabakk N.B., Johnsen A.C., Nonstad U., Sundan A., and Espevik T. (1995) Polymorphonuclear granulocytes enhance lipopolysaccharide-induced soluble p75 tumor necrosis factor receptor release from mononuclear cells. *Eur.J.Immunol.* **25**(9): p. 2714-2717;

158. Lifely M.R, and Wang Z. (1988) Immune responses in mice to different noncovalent complexes of meningococcal B polysaccharide and outer membrane proteins. *Infect.Immun.* **56**(12): p. 3221-3227;

159. Lüderitz O., Staub A.M., and Westphal O. (1966) Immunochemistry of O and R antigens of *Salmonella* and related *Enterobacteriaceae*. *Bacteriol.Rev.* **30**(1): p. 192-255;

160. Mackinnon F.G., Gorringe A.R., Funnell S.G., and Robinson A. (1992) Intranasal infection of infant mice with Neisseria meningitidis. *Microb.Pathog.* **12**(6): p. 415-420;

161. Mackinnon F.G., Borrow R., Gorringe A.R., Fox A.J., Jones D.M., and Robinson A. (1993) Demonstration of lipooligosaccharide immunotype and capsule as virulence factors for *Neisseria meningitidis* using an infant mouse intranasal infection model. *Microb.Pathog.* **15**(5): p. 359-366;

162. Mackinnon F.G., Ho Y., Blake M.S., Michon F., Chandraker A., Sayegh M.H., and Wetzler L.M. (1999) The role of B/T costimulatory signals in the immunopotentiating activity of neisserial porin. *J.Infect.Dis.* **180**(3):755-761;

163. Mäkelä P.H., Kayhty H., Weckstrom P., Sivonen A., and Renkonen O.V. (1975) Effect of group-A meningococcal vaccine in army recruits in Finland. *Lancet* **2**(7941): p. 883-886;

164. Mandrell R.E., Griffiss J.M., and Macher B.A. (1988) Lipooligosaccharides (LOS) of *Neisseria gonorrhoeae* and *Neisseria meningitidis* have components that are immunochemically similar to precursors of human blood group antigens. Carbohydrate sequence specificity of the mouse monoclonal antibodies that recognize crossreacting antigens on LOS and human erythrocytes. *J.Exp.Med.* **168**(1): p.107-126;

165. Mandrell R.E., Kim J.J., John C.M., Gibson B.W., Sugai J.V., Apicella M.A., Griffiss J.M., and Yamasaki R. (1991) Endogenous sialylation of the lipooligosaccharides of *Neisseria meningitidis*. *J.Bacteriol.* **173**(9): p. 2823-32;

166. Mandrell R.E., and Apicella M.A. (1993) Lipo-oligosaccharides (LOS) of mucosal

pathogens: molecular mimicry and host-modification of LOS. *Immunobiology*. **187**(3-5): p. 382-402;

167. Marcus D.M., Naiki M., and Kundu S.K. (1976) Abnormalities in the glycosphingolipid content of human Pk and p erythrocytes. *Proc.Natl.Acad.Sci. U S A* **73**(9): p. 3263-3267;
168. Marsh W.L. and Jenkins W.J. (1960) Anti-i: a new cold antibody. *Nature* **188**: p. 753-755;
169. Marsh W.L. (1961) Anti-i, a cold antibody defining the Ii relationship in human red cells. *Br.J.Haematol.* **7**: p. 200-209;
170. Martin C., Saux P., Mege J.L., Perrin G., Papazian L., and Gouin F. (1994) Prognostic values of serum cytokines in septic shock. *Intensive Care Med.* **20**(4): p. 272-277;
171. Martin D., Cadieux N., Hamel J., and Brodeur B.R. (1997) Highly conserved *Neisseria meningitidis* surface protein confers protection against experimental infection. *J.Exp.Med.* **185**(7): p. 1173-1183;
172. Maskell D., Allen A., Khan S., and Servos S. (1995) Molecular genetic manipulation of nonenteric bacterial pathogens to synthesise altered lipopolysaccharide molecules. *Prog.Clin.Biol.Res* **392**: p. 35-47;
173. Maslanka S.E., Gheesling L.L., Libutti D.E., Donaldson K.B.J., Harakeh H.S., Dykes J.K., Arhin F.F., Devi S.J.N., Frash C.E., Huang J.C., Kriz-Kuzemenska P., Lemmon R.D., Lorange M., Peeters C.C.A.M., Quataert S., Tai J.Y., Carlone G.M., and The Multilaboratory Study Group (1997) Standardization and a Multilaboratory Comparison of *Neisseria meningitidis* Serogroup A and C Serum Bactericidal Assay. *Clin.Diagn.Lab.Immunol.* **4**(2), p. 156-167;
174. Masoud H., Perry M.B., and Richards J.C. (1994) Characterization of the lipopolysaccharide of *Moraxella catarrhalis*. Structural analysis of the lipid A from *M. catarrhalis* serotype A lipopolysaccharide. *Eur.J.Biochem.* **220**(1): p. 209-216;
175. Masson L., and 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.* **47**(2): p. 465-471;
176. Mattsby-Baltzer I., Lindgren K., Lindholm B. and Edebo L. (1991) Endotoxin shedding by enterobacteria: free and cell-bound endotoxin differ in *Limulus* activity. *Infect.Immun.* **59**(2): p. 689-695;
177. McMichael A.J., Rust N.A., Pilch J.R., Sochynsky R., Morton J., Mason D.Y., Ruan C., Tobelem G., and Caen J. (1981) Monoclonal antibody to human platelet glycoprotein I. I. Immunological studies. *Br.J.Haematol.* **49**(4): p. 501-509;
178. McMichael J.C. (2000) Progress toward the development of a vaccine to prevent *Moraxella (Branhamella) catarrhalis* infections. *Microbes.Infect.* **2**(5): p. 561-568;
179. McNeil G., Virji M., and Moxon E.R. (1994) Interaction of *Neisseria meningitidis* with human monocytes. *Micob.Pathog.* **16**: p. 153-163;

180. Michon F., Beurret M., Gamian A., Brisson J.-R., and Jennings H.J. (1990) Structure of the L5 lipopolysaccharide core oligosaccharide of *Neisseria meningitidis*. *J.Biol.Chem.* **265**: p. 7243-7247;

181. Mietzner T.A., Barnes R.C., JeanLouis Y.A., Shafer W.M., and Morse S.A. (1986) Distribution of an antigenically related iron-regulated protein among the Neisseria spp. *Infect.Immun.* **51**(1): p. 60-68;

182. Mihalcu F., Pasolescu O., Levenet I., and Iacob A. (1994) Meningococcal disease in Romania 1971-1992. Proceedings of the Ninth International Pathogenic Neisseria Conference, 26-30 September 1994, Winchester, England: p. 389-390;

183. Moraes de J.C., Perkins B.A., Camargo M.C., Hidalgo N.T., Barbosa H.A., Sacchi C.T., Landgraf I.M., Gattas V.L., Vasconcelos H.D, and Gral I.M. (1992) Protective efficacy of a serogroup B meningococcal vaccine in Sao Paulo, Brazil. *Lancet* **340**(8827): p. 1074-1078;

184. Munkley A., Tinsley C.R., Virji M., and Heckels J.E. (1991) Blocking of bactericidal killing of *Neisseria meningitidis* by antibodies directed against class 4 outer membrane protein. *Microb.Pathog.* **11**: p. 447-452;

185. Murphy T.F., and Bartos L.C. (1989) Surface-exposed and antigenically conserved determinants of outer membrane proteins of *Branhamella catarrhalis*. *Infect.Immun.* **57**(10): p. 2938-2941;

186. Murphy T.F. (1996) *Branhamella catarrhalis*: epidemiology, surface antigenic structure, and immune response. *Microbiol.Rev.* **60**(2): p. 267-279;

187. Nadel S., Newport M.J., Booy R., and Levin M. (1996) Variation in the tumor necrosis factor-α gene promotor region may be associated with death from meningococcal disease. *J.Infect.Dis.* **174**: p. 878-880;

188. Naiki M. and Kato M. (1979) Immunological Identification of blood group pK antigen on normal human erythrocytes and isolation of anti-pK with different affinity. *Vox Sang.* **37**: p. 30-38;

189. Nielsen H., Ronne-Rasmussen J.O., and Willumsen L. (1988) Blood-monocyte functions in acute bacterial meningitis. *APMIS* **96**(4): p. 289-293;

190. Nikaido H., Naide Y., and Makela P.H. (1966) Biosynthesis of O-antigenic polysaccharides in *Salmonella*. Ann.N.Y.Acad.Sci. **133**(2): p. 299-314;

191. Nuernberger W., Platonov A., Stannigel H., Beloborodov V.B., Michelmann I., von Kries R., Burdach S., and Gobel U. (1995) Definition of a new score for severity of generalized *Neisseria meningitidis* infection. Eur.J.Pediatr **154**(11): p. 896-900;

192. Oftung F., Naess L.M., Wetzler L.M., Korsvold G.E., Aase A., Hoiby E.A., Dalseg R., Holst J., Michaelsen T.E., and Haneberg B. (1999) Antigen-specific T-cell responses in humans after intranasal immunization with a meningococcal serogroup B outer membrane vesicle vaccine. *Infect.Immun.* **67**(2): p. 921-927;

193. Olsen S.F., Djurhuus B., Rasmussen K., Joensen H.D., Larsen S.O., Zoffman H., and Lind I. (1991) Pharyngeal carriage of *Neisseria meningitidis* and *Neisseria lactamica* in households with infants within areas with high and low incidences of meningococcal disease. *Epidemiol.Infect.* **106**(3): p. 445-457;

194. O'Mahony L., Holland J., Jackson J., Feighery C., Hennessy T.P. and Mealy K. (1998) Quantitative intracellular cytokine measurement: age-related changes in proinflammatory cytokine production. *Clin.Exp.Immunol.* **113**(2): p. 213-219;

195. Osborn M.J. (1966) Biosynthesis and structure of the core region of the lipopolysaccharide in *Salmonella typhimurium*. *Ann.N.Y.Acad.Sci.* **133**(2): p. 375-383;

196. Padron J., Bebelagua Y., Lastre M., Lapinet J., Zayas C., Quintero Y., Diaz M., and Perez O. (1999) Nitric oxide participates in the immune response against *Neisseria meningitidis* serogroup B. FEMS Immunol.Med.Microbiol. **25**(4): p. 385-389;

197. Pavliak V., Brisson J.-R., Michon F., Uhrin D., and Jennings H.J. (1993) Structure of the sialylated L3 lipopolysaccharide of *Neisseria meningitidis*. *J.Biol.Chem.* **268**: p. 14146-14152;

198. Peltola H., Makela H., Kayhty H., Jousimies H., Herva E., Hallstrom K., Sivonen A., Renkonen O.V., Pettay O., Karanko V., Ahvonen P., and Sarna S. (1977) Clinical efficacy of meningococcus group A capsular polysaccharide vaccine in children three months to five years of age. *N.Engl.J.Med.* **297**(13): p. 686-691;

199. Petrov A.B., Kolenko V.M., Koshkina N.V., Zakirov M.M., Bugaev L.V., Semenova I.B., Wiertz E.J., and Poolman J.T. (1994) Non-specific modulation of the immune response with liposomal meningococcal lipopolysaccharide: role of different cells and cytokines. Vaccine. 12(12): p. 1064-1070;

200. Petrov A.B., Semenov B.F., Vartanyan Y.P., Zakirov M.M., Torchilin V.P., Trubetskoy V.S. (1992) Toxicity and immunogenicity of *Neisseria meningitis* lipopolysaccharide incorporated into liposomes. *Infect.Immun.* **60**: p.3897-3903

201. Pettersson A., Kuipers B., Pelzer M., Verhagen E., Tiesjema R.H., Tommassen J., and Poolman J.T. (1990) Monoclonal antibody against the 70-kilodalton iron-regulated protein of *Neisseria meningitidis* are bactericidal and strain specific. *Infect.Immun.* **58**: p. 3036 – 3041.

202. Pinner R.W., Onyango F., Perkins B.A., Mirza N.B., Ngacha D.M., Reeves M., DeWitt W., Njeru E., Agata N.N., and Broome C.V. (1989) Epidemic meningococcal disease in Nairobi, Kenya, 1989. The Kenya/Centers for Disease Control (CDC) Meningitis Study Group. *J.Infect.Dis.* **166**(2): p. 359-364;

203. Pizza M., Scarlato V., Masignani V., Giuliani M.M., Arico B., Comanducci M., Jennings G.T., Baldi L., Bartolini E., Capecchi B., Galeotti C.L., Luzzi E., Manetti R., Marchetti E., Mora M., Nuti S., Ratti G., Santini L., Savino S., Scarselli M., Storni E., Zuo P., Broeker M., Hundt E., Knapp B., Blair E., Mason T., Tettelin H., Hood D.W., Jeffries

A.C., Saunders N.J., Granoff D.M., Venter J.C., Moxon E.R., Grandi G., and Rappuoli R. (2000) Identification of vaccine candidates against serogroup B meningococcus by whole-genome sequencing. *Science.* **287**(5459): p.1816-1820.

204. Plested J.S., Makepeace K., Jennings M.P., Gidney M.A., Lacelle S., Brisson J., Cox A.D., Martin A., Bird A.G., Tang C.M., Mackinnon F.M., Richards J.C., and Moxon E.R. (1999) Conservation and accessibility of an inner core lipopolysaccharide epitope of *Neisseria meningitidis*. *Infect.Immun.* **67**(10): p. 5417-5426;
205. Poolman J.T., de Marie S., and Zanen H.C. (1980) Variability of low-molecular-weight, heat-modifiable outer membrane proteins of *Neisseria meningitidis*. *Infect.Immun.* **30**(3): p. 642-648;
206. Poolman J.T., Hopman C.T., and Zanen H.C. (1985) Colony variants of *Neisseria meningitidis* strain 2996 (B:2b:P1.2): influence of class-5 outer membrane proteins and lipopolysaccharides. *J.Med.Microbiol.* **19**(2): p. 203-209;
207. Poolman J.T., Hopman C.T.P. and Zanen H.C. (1982) Problems in the definition of meningococcal serotypes. *FEMS Microbiol.Lett.* **13**: p. 339-348;
208. Prokop O. and Uhlenbruck G. (1965) Human blood and serum groups. McClaren and Sons, London;
209. Quakyi E.K., Frasch C.E., Buller N., and Tsai C.M. (1999) Immunization with meningococcal outer-membrane protein vesicles containing lipooligosaccharide protects mice against lethal experimental group B *Neisseria meningitidis* infection and septic shock. *J.Infect.Dis.* **180**(3): p. 747-754;
210. Race R.R. and Sanger R. (1975) Blood Groups in Man, 6th edn. Backwell Scientific Publications, Oxford;
211. Rahman M., Holme T., Jonsson I., and Krook A (1995) Lack of serotype-specific antibody response to lipopolysaccharide antigens of *Moraxella catarrhalis* during lower respiratory tract infection. *Eur.J.Clin.Microbiol.Infect.Dis.* **14**: p. 297-304;
212. Rahman M., and Holme T. (1996) Antibody response in rabbits to serotype-specific determinants in lipopolysaccharides from *Moraxella catarrhalis*. *J.Med.Microbiol.* **44**(5): p. 348-354;
213. Rahman M.M, Stephens D.S., Kahler C.M., Glushka J., and Carlson R.W. (1998a) The lipooligosaccharide (LOS) of Neisseria meningitidis serogroup B strain NMB contains L2, L3, and novel oligosaccharides, and lacks lipid-A 4'-phosphate substituent. *Carbohydr.Res.* **307**(3-4): p. 311-324;
214. Rahman M.M., Stephens D.S., Kahler C.M., Gluska J., and Carlson R.W. (1998b) The structural heterogeneity of the lipooligosaccharide (LOS) of *Neisseria meningitidis* serogroup B: substituents of the inner core. *Carbohyd.Res.* **307**: p. 311-324;
215. Raza M.W, Ogilvie M.M., Blackwell C.C., Stewart J., Elton R.A., and Weir D.M. (1993) Effect of respiratory syncytial virus infection on binding of *Neisseria meningitidis* and *Haemophilus influenzae* type b to a human epithelial cell line (HEp-2).

Epidemiol.Infect. **110**(2): p. 339-347;

216. Raza M.W., El Ahmer O.R., Ogilvie M.M., Blackwell C.C., Saadi A.T., Elton R.A., and Weir D.M. (1999) Infection with respiratory syncytial virus enhances expression of native receptors for non-pilate *Neisseria meningitidis* on HEp-2 cells. *FEMS Immunol.Med.Microbiol.* **23**(2): p. 115-124;
217. Raza M.W., Blackwell C.C., Elton R.A., and Weir D.M. (2000) Bactericidal activity of a monocytic cell line (THP-1) against common respiratory tract bacterial pathogens is depressed after infection with respiratory syncytial virus. *J.Med.Microbiol.* **49**: p.227-233;
218. Read R.C., Zimmerli S., Broaddus V.C., Sanan D.A., Stephens D.S., and Ernst J.D. (1996) The (α2,8) linked polysialic acid capsule of group B *Neisseria meningitidis* modifies multiple steps during interaction with human macrophages. *Infect.Immun.* **64**: p. 3210-3217;
219. Read R.C., Camp N.J., di Giovine F.S., Borrow R., Kaczmarski E.B., Chaudhary A.G., Fox A.J., and Duff G.W. (2000) An interleukin-1 genotype is associated with fatal outcome of meningococcal disease. *J.Infect.Dis.* **182**(5): p. 1557-1560;
220. Reingold A.L., Broome C.V., Hightower A.W., Ajello G.W., Bolan G.A., Adamsbaum C., Jones E.E., Phillips C., Tiendrebeogo H., and Yada A. (1985) Age-specific differences in duration of clinical protection after vaccination with meningococcal polysaccharide A vaccine. *Lancet* **2**(8447): p. 114-118;
221. Reller L.B., MacGregor R.R., and Beaty H.N. (1973) Bactericidal antibody after colonization with *Neisseria meningitidis*. *J.Infect.Dis.* **127**(1): p. 56-62;
222. Richmond P., Goldblatt D., Fusco P.C., Fusco J.D., Heron I., Clark S., Borrow R., and Michon F. (1999) Safety and immunogenicity of a new Neisseria meningitidis serogroup C-tetanus toxoid conjugate vaccine in healthy adults. *Vaccine* **18**(7-8): p. 641-646;
223. Rietschel E.T., Kirikae T., Schade F.U., Ulmer A.J., Holst O., Brade H., Schmidt G., Mamat U., Grimmecke H.-D., Kusumoto S. and Zähringer U. (1993) The chemical structure of bacterial endotoxin in relation to bioactivity. *Immunobiol.* **187**: p. 169-190;
224. Rio del M.A., Chrane D., Shelton S., McCracken G.H. Jr., and Nelson J.D. (1983) Ceftriaxone versus ampicillin and chloramphenicol for treatment of bacterial meningitis in children. *Lancet* **1**(8336): p. 1241-1244;
225. Rixen D., Siegel J.H., and Friedman H.P. (1996) "Sepsis/SIRS," physiologic classification, severity stratification, relation to cytokine elaboration and outcome prediction in posttrauma critical illness. *J.Trauma* **41**(4): p. 581-598;
226. Robbins J.B., Myerowitz L., Whisnant J.K., Argaman M., Schneerson R., Handzel Z.T., and Gotschlich E.C. (1972) Enteric bacteria cross-reactive with *Neisseria meningitidis* groups A and C and *Diplococcus pneumoniae* types I and 3. *Infect.Immun.* **6**(5): p.

651-656;

227. Robbins A., and Freeman P. (1988) Obstacles to developing vaccines for the Third World. *Sci.Am.* **259**(5): p. 126-133;
228. Robinson J.P. (1993) Handbook of Flow Cytometry Methods, Wiley-Liss, New York;
229. Rodriguez A.P., Dickinson F., Baly A., and Martinez R. (1999) The epidemiological impact of antimeningococcal B vaccination in Cuba. *Mem.Inst.Oswaldo Cruz.* **94**(4): p. 433-440;
230. Romero J.D., and Outschoorn I.M (1994) Current status of meningococcal group B vaccine candidates: capsular or noncapsular? *Clin.Micrbiolo.Rev.* **7**(4): p. 559-575;
231. Romero J.D., and Outschoorn I.M. (1997) The immune response to the capsular polysaccharide of *Neisseria meningitidis* group B. *Zentralbl.Bakteriol.* **285**(3): p. 331-340;
232. Rosenqvist E., Harthug S., Froholm L.O., Hoiby E.A., Bovre K., and Zollinger W.D. (1988) Antibody responses to serogroup B meningococcal outer membrane antigens after vaccination and infection. *J.Clin.Microbiol.* **26**(8): p. 1543-1548;
233. Rosenqvist E., Hoiby E.A., Wedege E., Bryn K., Kolberg J., Klem A., Ronnild E., Bjune G., and Nokleby H. (1995) Human antibody responses to meningococcal outer membrane antigens after three doses of the Norwegian group B meningococcal vaccine. *Infect.Immun.* **63**(12): p. 4642-4652;
234. Ross S.C., Berberich H.M., and Densen P. (1985) Natural serum bactericidal activity against *Neisseria meningitidis* isolates from disseminated infections in normal and complement-deficient hosts. *J.Infect.Dis.* **152**(6): p. 1332-1335;
235. Roth J., Zuber C., Komminoth P.J., Scheidegger E.P., Warhol M.J., Bitter-Suermann D., and Heitz P.U. (1993) Expression of polysialic acid in tumors and its significance for tumor growth. In Polysialic acid. From microbes to man. Roth J. Rutishauser U., and Troy F.A. (eds.) Birkhäuser Verlag, Basel: p. 335-348;
236. Saez-Nieto J.A., Dominguez J.R., Monton J.L., Cristobal P., Fenoll A., Vazquez J., Casal J., and Taracena B. (1985) Carriage of *Neisseria meningitidis* and *Neisseria lactamica* in a school population during an epidemic period in Spain. *Hyg. (Lond.)* **94**(3): p. 279-288;
237. Schneider H., Hale T.L., Zollinger W.D., Seid R.C. Jr., Hammack C.A., and Griffiss J.M. (1984) Heterogeneity of molecular size and antigenic expression within lipooligosaccharides of individual strains of *Neisseria gonorrhoeae* and *Neisseria meningitidis*. *Infect.Immun.* **45**(3): p. 544-549;
238. Scholten R.J., Kuipers B., Valkenburg H.A., Dankert J., Zollinger W.D., and Poolman J.T. (1994) Lipo-oligosaccharide immunotyping of *Neisseria meningitidis* by a whole-cell ELISA with monoclonal antibodies. *J.Med.Microbiol.* **41**(4):236-43
239. Schweda E.K., Jonasson J.A., and Jansson P.E. (1995) Structural studies of lipooligosaccharides from *Haemophilus ducreyi* ITM 5535, ITM 3147, and a fresh

clinical isolate, ACY1: evidence for intrastrain heterogeneity with the production of mutually exclusive sialylated or elongated glycoforms. *J.Bacteriol.* **177**(18): p. 5316-5321;

240. Schwende H., Fitzke E., Ambs P., and Dieter P. (1996) Differences in the state of differentiation of THP-1 cells induced by phorbol ester and 1,25-dihydroxyvitamin D3. *J.Leukoc.Biol.* **59**(4): p. 555-561;
241. Serke S., van Lessen A., and Huhn D. (1998) Quantitative fluorescence flow cytometry: a comparison of the three techniques for direct and indirect immunofluorescence. *Cytometry* **33**(2): p. 179-187;
242. Siddiqui B, and Hakomori S. (1973) A ceramide tetrasaccharide of human erythrocyte membrane reacting with anti-type XIV pneumococcal polysaccharide antiserum. *Biochim.Biophys.Acta.* **330**(2): p. 147-155;
243. Simmons G., Martin D., Stewart J., and Bremner D. (2000) Carriage of *Neisseria lactamica* in a population at high risk of meningococcal disease. *Epidemiol.Infect.* **125**(1): p. 99-104;
244. Sjursen H., Wedege E., Rosenqvist E., Naess A., Halstensen A., Matre R., and Solberg C.O. (1990) IgG subclass antibodies to serogroup B meningococcal outer membrane antigens following infection and vaccination. APMIS. 98(12): p. 1061-1069;
245. Slotman G.J. (2000) Prospectively validated prediction of organ failure and hypotension in patients with septic shock: the Systemic Mediator Associated Response Test (SMART). *Shock* **14**(2): p. 101-106;
246. Smith K.B., and Ellis S.A. (1999) Standardisation of a procedure for quantifying surface antigens by indirect immunofluorescence. *J.Immunol.Methods* **228**(1-2): p. 29-36;
247. Stanwell-Smith R.E., Stuart J.M., Hughes A.O., Robinson P., Griffin M.B., and Cartwright K. (1994) Smoking, the environment and meningococcal disease: a case control study. *Epidemiol.Infect.* **112**(2): p. 315-328;
248. Stephens D.S., and McGee Z.A. (1981) Attachment of *Neisseria meningitidis* to human mucosal surfaces: influence of pili and type of receptor cell. *J.Infect.Dis* **143**(4): p. 525-532;
249. Stephens D.S., and Farley M.M (1991) Pathogenic events during infection of the human nasopharynx with *Neisseria meningitidis* and *Haemophilus influenzae*. *Rev.Infect.Dis.* **13**: p. 22-33;
250. Stimson E., Virji M., Makepeace K., Dell A., Morris H.R., Payne G., Saunders J.R., Jennings M.P., Barker S., and Panico M. (1995) Meningococcal pilin: a glycoprotein substituted with digalactosyl 2,4-diacetamino-2,4,6-trideoxyhexose. Mol.Microbiol. 17: p. 1201-1214;
251. Storm G., Wilms H..P, and Crommelin D.J. (1991) Liposomes and biotherapeutics. *Biotherapy* **3**(1): p. 25-42;

252. Strait R.T., Kelly K.J., and Kurup V.P. (1999) Tumor necrosis factor-alpha, interleukin-1 beta, and interleukin-6 levels in febrile, young children with and without occult bacteremia. *Pediatrics* **104**(6): p. 1321-1326;

253. Stuart J.M., Cartwright K.A., Robinson P.M., and Noah N.D. (1989) Effect of smoking on meningococcal carriage. *Lancet* **2**(8665): p. 723-725;

254. Suakkonen K.M., Leinonen M., Abdillahi H., and Poolman J.T. (1989) Comparative evaluation of potential components for group B meningococcal vaccine by passive protection in the infant rat and in vitro bactericidal assay. *Vaccine* **7**: p. 325-328;

255. Taylor P.W. (1983) Bactericidal and bacteriolytic activity of serum against gram-negative bacteria. *Microbiol.Rev.* **47**(1): p. 46-83;

256. Troncoso G., Sanchez S., Moreda M., Criado M.T., and Ferreiros C.M. (2000) Antigenic cross-reactivity between outer membrane proteins of *Neisseria meningitidis* and commensal *Neisseria* species. *FEMS Immunol.Med.Microbiol.* **27**(2): p.103-109;

257. Tsai C.M., and Civin C.I. (1991) Eight lipooligosaccharides of *Neisseria meningitidis* react with a monoclonal antibody which binds lacto-N-neotetraose (Gal beta 1-4 GlcNAc beta 1-3 Gal beta 1-4 Glc). *Infect.Immun.* **59**(10): p. 3604-3609;

258. Tsuchiya S., Yamabe M., Yamaguchi Y., Kobayashi Y., Konno T., and Tada K. (1980) Establishment and characterization of a human monocytic leukemia cell line (THP-1). *Int.J.Cancer* **26**(2): p. 171-176;

259. Turner R., and Hendley J.O. (1982) Detection of meningococcal carriage by throat culture. *J.Infect.Dis.* **145**(6): p.914;

260. Twite S.J., Blackwell C.C., Raza M.W., Saadi A.T., Essery S.D., and Weir D.M. (1994) Are monocyte the "Trojan horses" of meningococcal disease? Proceedings of the Ninth International Pathogenic *Neisseriae* Conference; 26-30 September 1994, Winchester, England: p 284-286;

261. Tzanakaki G., Blackwell C.C., Kremastinou J., Weir D.M., Mentis A., and Fallon R.J. (1993) Serogroups, serotypes and subtypes of *Neisseria meningitidis* isolated from patients and carriers in Greece. *J.Med.Microbiol.* **38**(1): p. 19-22;

262. Tzanakaki, G., Kriz, P., Kremastinou, J., Musilek, M., Smart, L.E. and Blackwell C.C. (1997) Reactivity of the new monoclonal antibody "22" with meningococcal strains isolated from patients and carriers in Greece. FEMS Immunology Medical Microbiology 19:1-5;

263. van der Ley P., Hamstra H.J., and Steeghs L. (1998) Modification of lipid A biosynthesis in *Neisseria meningitidis.* In: Abstracts of the Eleventh International Pathogenic Neisseria Conference, Nassif X., Quentin-Millet M.-J., and Taha M.K. Eds., Nice, 27;

264. van der Ley P., Heckels J.E., Hoogerhout P., and Poolman J.T. (1991) Topology of outer membrane porins in pathogenic *Neisseria* spp. *Infect.Immun.* **59**: p. 2963-2971;

265. van der Ley P., van der Biezen J., and Poolman J.T. (1995) Construction of *Neisseria*

meningitidis strains carrying multiple chromosomal copies of the porA gene for use in the production of a multivalent outer membrane vesicle vaccine. *Vaccine* **13**(4): p. 401-407;

266. van Deuren M., van der Ven-Joongekrijg J., Bartelink A.K.M., van Dalen R., Sauerwein R.W., and van der Meer J.W.M. (1995) Correlation between proinflammatory cytokines and antiinflammatory mediators and the severety of disease in meningococcal infections. J.Infect.Dis. 172: p. 433-439;
267. van Furth R., and van den Berg B.M. (1996) in: Weir's Handbook of Immunological Methods, 8th edition, Blackwell Scientific, London;
268. van Hare G.F., Shurin P.A., Marchant C.D., Cartelli N.A., Johnson C.E., Fulton D., Carlin S., and Kim C.H. (1987) Acute otitis media caused by *Branhamella catarrhalis*: biology and therapy. *Rev.Infect.Dis.* **9**(1): p. 16-27;
269. Vaneechoutte M., Verschraegen G., Claeys G., and Van Den Abeele A.M. (1990) Serological typing of *Branhamella catarrhalis* strains on the basis of lipopolysaccharide antigens. *J.Clin.Microbiol.* **28**(2): p. 182-187;
270. Vedros N.A., and Jarvis G.A. (1987) Sialic acid of group B *Neisseria meningitidis* regulates alternative complement pathway activation. *Infect.Immun.* **55**(1): p. 174-180;
271. Verheul A.F., Snippe H., and Poolman J.T. (1993) Meningococcal lipopolysaccharides: virulence factor and potential vaccine component. *Microbiol.Rev.* **57**(1): p. 34-49;
272. Vieusseux G. (1805) Mémoire sur la maladie qui a regné á Genéve au printemps de 1805. *J.Med.Pharm.* **11**: p. 163;
273. Vindelov L.L. (1977) Microfluorometic analysis of nuclear DNA in cells from tumours and cell suspensions. *Virchow's Arch. (Cell.Pathol.)* **24**: p. 227-242;
274. Vogel U., Hammerschmidt S., and Frosch M. (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.* **185**: p. 81-87;
275. Vogel U., Weinberger A., Frank R., Muller A., Kohl J., Atkinson J.P., and Frosch M. (1997) Complement factor deposition and serum resistance in isogenic capsule and lipooligosaccharide sialic acid mutants of serogroup B *Neisseria meningitidis*. *Infect.Immun.* **65**: p. 4022-4029;
276. Waage A., Halstensen A., and Espevik T. (1987) Association between tumor necrosis factor in serum and fatal outcome in patients with meningococcal disease. *Lancet* **i**: p. 355-357;
277. Waage A., Brandtzaeg P., Halstensen A., Kierulf P., and Espevik T. (1989) The complex pattern of cytokines in serum from patients with meningococcal septic shock. Association between interleukin 6, interleukin 1 and fatal outcome. *J.Exp.Med.* **169**: p. 333-338;
278. Wahdan M.H., Sallam S.A., Hassan M.N., Abdel Gawad A., Rakha A.S., Sippel J.E.,

Hablas R., Sanborn W.R., Kassem N.M., Riad S.M., and Cvjetanovic B. (1977) A second controlled field trial of a serogroup A meningococcal polysaccharide vaccine in Alexandria. *Bull.World Health Organ.* **55**(6): p. 645-651;

279. Wakarchuk W., Gilbert M., Martin A., Wu Y., Brisson J.-R., Thibault P., and Richards J.C. (1998) Structure of an α2,6-sialylated lipooligosaccharide from *Neisseria meningitidis* immunotype L1. *Eur.J.Biochem.* **254**: p. 626-633;
280. Warner L., Ermak T., and Griffiss J.M. (1987) Mucosal and serum immunity following commensal enteric colonization. Adv.Exp.Med.Biol. **216B**: p. 959-964;
281. Weir D.M., and Srewart J. (1997) Immunology 8th edition, Churchill Livingston, Pearsin Professional, Edinburgh;
282. Westendorp R.G., Langermans J.A., de Bel C.E., Meinders A.E., Vandenbroucke J.P., van Furth R., and 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.* **171**(4): p. 1057-1060;
283. Westendorp R.G., Langermans J.A., Huizinga T.W., Elouali A.H., Verweij C.L., Boomsma D.I., and Vandenbroucke J.P. (1997) Genetic influence on cytokine production and fatal meningococcal disease. *Lancet.* **349**(9046): p. 170-173;
284. Westphal O., and Luderitz O. (1954) Chemische Erforschung von Lipopolysacchariden gramnegativer Bacterien. *Angew.Chem.* **66**: p. 407-417;
285. Wiels J., Fellous M., and Tursz T. (1981) Monoclonal antibody against a Burkitt lymphoma-associated antigen. *Proc.Natl.Acad.Sci.U.S.A.* **78**(10): p. 6485-6488;
286. Wiener A.S. (1973) Blood group distributions. *Ann.Intern.Med.* **79**(1): p. 137;
287. Wieseman G.M., and Caird J.D. (1977) Composition of the lipopolysaccharide of *Neisseria gonorrhoeae. Infect.Immun.* **16**(2): p. 550-556;
288. World Health Organisation (1976) requirements for meningococcal polysaccharide vaccine (requirements for biological substances no. 23). *W.H.O.Tech.Rep.Ser.*, **594**: p. 72-73;
289. Wyle F.A., Artenstein M.S., Brandt B.L., Tramont E.C., Kasper D.L., Altieri P.L., Berman S.L., and Lowenthal J.P. (1972) Immunologic response of man to group B meningococcal polysaccharide vaccines. *J.Infect.Dis.* **126**(5): p. 514-521;
290. Yount W.J., Dorner M.M., Kunkel H.G., and Kabat E.A. (1968) Studies on human antibodies. VI. Selective variations in subgroup composition and genetic markers. *J.Exp.Med.* 127: p. 633-646;
291. Zaleski Lipooligosaccharide P(k) (Galalpha1-4Galbeta1-4Glc) epitope of *Moraxella catarrhalis* is a factor in resistance to bactericidal activity mediated by normal human serum.
292. Zollinger W.D. and Mandrell R.E. (1977) Outer-membrane protein and lipopolysaccharide serotyping of *Neisseria meningitidis* by inhibition of a solid phase radioimmunoassay. *Infect.Immun.* **18**: p. 424-433;

293. Zollinger W.D., Mandrell R.E., Griffiss J.M., Altieri P., and Berman S. (1979) Complex of meningococcal group B polysaccharide and type 2 outer membrane protein immunogenic in man. *J.Clin.Invest.* **63**(5): p. 836-848;

294. Zollinger W.D. and Mandrell R.E. (1983) Importance of Complement Source in Bactericidal Activity of Human and Murine Monoclonal Antibody to Meningococcal Group B Polysaccharide. *Infect.Immun.* **40**(1): p. 257-264;

295. Zollinger W.D., Moran E.E., and Brandt B.L. (2000) Epitope specificity of bactericidal antibodies to meningococcal L3,7 lipooligosaccharide (LOS). 12th International Pathogenic Neisseria Conference, Galveston, Texas, USA 12-17 November 2000, Poster #144;

296. Zorgani A.A., Stewart J., Blackwell C.C., Elton R.A., Weir D.M. (1992) Secretor status and humoral immune responses to *Neisseria lactamica* and *Neisseria meningitidis*. *Epidemiol.Infect.* **109**(3): p. 445-452.

297. Zorgani A.A., James V.S., Stewart J., Blackwell C.C., Elton R.A., and Weir D.M. (1996) Serum bactericidal activity in a secondary school population following an outbreak of meningococcal disease: effects of carriage and secretor status. *FEMS Immunol Med Microbiol.* **14**(2-3): p.73-81;