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Maija Toropainen

Assessment of natural and outer membrane vesicle (OMV) vaccine induced immunity against *Neisseria meningitidis* serogroup B in an infant rat infection model

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National Public Health Institute,
Department of Vaccines,
Helsinki, Finland
and
University of Helsinki,
Faculty of Biosciences,
Department of Biological and Environmental Sciences

Maija Toropainen

ASSESSMENT OF NATURAL AND OUTER MEMBRANE VESICLE (OMV) VACCINE INDUCED IMMUNITY AGAINST *NEISSERIA MENINGITIDIS* SEROGROUP B IN AN INFANT RAT INFECTION MODEL

ACADEMIC DISSERTATION

To be presented with the permission of the Faculty of Biosciences,
Department of Biological and Environmental Sciences, University of Helsinki,
for public examination in the auditorium no 2402, Viikinkaari 1,
on the 12th of August, 2005, at 12 noon.

National Public Health Institute, Department of Vaccines, Vaccine Immunology Laboratory, Helsinki, Finland

and

University of Helsinki, Faculty of Biosciences, Department of Biological and Environmental Sciences, Division of Animal Physiology, Helsinki, Finland

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Kansanterveyslaitos (KTL)
Mannerheimintie 166
00300 Helsinki
Puh. vaihde (09) 474 41, telefax (09) 4744 8408

Folkhälsoinstitutet
Mannerheimvägen 166
00300 Helsingfors
Tel. växel (09) 474 41, telefax (09) 4744 8408

National Public Health Institute
Mannerheimintie 166
FIN-00300 Helsinki, Finland
Telephone +358 9 474 41, telefax +358 9 4744 8408

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Supervised by

Research Professor Helena Käyhty, PhD
Laboratory of Vaccine Immunology
Department of Vaccines
National Public Health Institute
Helsinki, Finland

Reviewed by

Docent Kirsi Laitinen, PhD
Department of Public Health
University of Helsinki
Helsinki, Finland

and

Professor Seppo Meri, MD
Haartman Institute
Department of Bacteriology and Immunology
University of Helsinki
Helsinki, Finland

Opponents

Professor Seppo Meri, MD
Haartman Institute
Department of Bacteriology and Immunology
University of Helsinki
Helsinki, Finland

and

Professor Per-Erik Saris, PhD
Department of Applied Chemistry and Microbiology
University of Helsinki
Helsinki, Finland

To my family

Maija Toropainen, Assessment of natural and outer membrane vesicle (OMV) vaccine induced immunity against *Neisseria meningitidis* serogroup B in an infant rat infection model
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ABSTRACT

Background: Despite of the amount of work on the meningococcal serogroup B bacteria and immunity to disease caused by them, a satisfactory vaccine for these important pathogens is still missing. Several group B meningococcal vaccines based on outer membrane protein vesicles (OMV) or complexes have been evaluated in phase II and III clinical trials. Although the antibody responses in connection with these phase III clinical protection trials and separate immunogenicity trials have been analyzed extensively by enzyme immunosorbent assay (EIA) and by assays for serum bactericidal (SBA) and opsonophagocidal activity, the specificity and the functional activity of antibodies providing protection against serogroup B disease is still partly open. The lack of reliable laboratory correlates or surrogates for protection has hampered vaccine development against this important pathogen.

Study aims: The main aim of the present study was evaluate the applicability of an infant rat protective activity (IRPA) assay to assess meningococcal serogroup B OMV vaccine responses induced in humans. To this end, pre- and post-vaccination serum samples from teenagers immunized with two doses of either the Norwegian OMV vaccine (MenBvac™), the Cuban OMV vaccine (VA-MENGOC-BC™), or the serogroup A/C capsular PS control vaccine during a previous immunogenicity trial in Iceland were analyzed for IRPA, and the results compared to SBA and EIA data obtained with the same serum set and vaccine efficacy data obtained earlier in different study populations. We also studied the specificity and functional activity of natural antibodies conferring protection in this animal model. Well-characterized Mabs were used to assess the influence of antibody specificity and isotype on protection, and complement component C6 deficient animals to evaluate the importance of complement-mediated bacterial lysis on protection.

Results and conclusions: As compared to the results from rises in anti-OMV IgG levels measured by EIA and to a lesser extent also in SBA titres, the numbers of vaccine responders

detected in IRPA assay were only modest. Thus, although likely to be useful for the pre-clinical evaluation of candidate MenB vaccines, the IRPA assay, as described herein, is probably less suitable for large-scale evaluation of serogroup B OMV vaccine responses in clinical samples. Despite this limitation, the IRPA assay seemed to give some additional value over the SBA assay in that many SBA negative pre-vaccination sera were often IRPA positive though many SBA positive sera remained IRPA negative. In sera taken before vaccination the IRPA against strain 44/76-SL was mainly mediated by serogroup B capsular specific IgM antibody and was independent of complement-mediated bacterial lysis as evident from the lack of SBA *in vitro* and equal protective activity of normal human sera in complement sufficient and C6 deficient animals. Studies with serogroup B capsular specific antibody of mouse origin confirmed the latter finding. These findings were in contrast to PorA protein specific IgG antibodies whose protective activity in C6 deficient animals was severely impaired. A clear connection between the acquisition of natural B-PS specific IgM antibodies and IRPA was also indicated. These results suggest that importance capsular PS specific antibodies on protective immunity against serogroup B disease may have been underestimated.

Keywords: *Neisseria meningitidis B*, OMV vaccines, infant rat infection model

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TIIVISTELMÄ

Tausta: Tällä hetkellä ei ole olemassa tyydyttävää rokotetta B-ryhmän meningokokin aiheuttamia vakavia infektioita vastaan. Useita ulkokalvoproteiinirokotteita on kehitteillä ja näistä osan suojatehoa on jo tutkittu kliinisissä faasi II ja III kokeissa. Vaikka rokotteiden aikaansaamien vasta-aineiden määrällisiä ja laadullisia ominaisuuksia on tutkittu eri menetelmillä, kuten ELISA-menetelmällä, bakterisidia- ja fagosytoosi-testeillä, on suojaavien vasta-aineiden spesifiteetti sekä se, miten vasta-aineet aineet suojaavat B-ryhmän meningokokkitaudeilta, vielä osin selvittämättä. Kliinisen suojatehon laboratoriokorrelaatin puuttuminen on vaikeuttanut rokotekehitystyötä.

Tavoitteet: Tämän tutkimuksen tarkoituksena oli selvittää, voidaanko rotanpoikasen suojatestiä käyttää B-ryhmän meningokokin ulkokalvoproteiinirokotteiden rokotteiden suojatehon mittaamiseen immunisoimalla rotanpoikaset passiivisesti ennen rokottamista ja rokotuksen jälkeen otetuilla ihmisseerumeilla, ja mittaamalla, kuinka hyvin ne suojaavat kokeelliselta meningokokki-infektiolta. Tutkimusrokotteina käytettiin norjalaista (MenBvac™) ja kuubalaista (VA-MENGOC-BC™) ulkokalvoproteiinirokotetta ja kontrollirokotteena meningokokin A/C-polysakkaridirokotetta. Näin saatuja suojatuloksia verrattiin ELISA- ja bakterisidia-testeillä saatuihin tuloksiin sekä tutkimusrokotteiden kliiniseen suojatehoon. Lisäksi tutkimme, mikä on rotanpoikasen infektiomallissa suojaavien luonnollisten vasta-aineiden spesifiteetti ja mahdolliset toimintamekanismit käyttäen apuna komplementin C6-komponentin suhteen puutteellisia eläimiä. Selvitimme myös, onko eri spesifiteetin omaavilla tai eri IgG alaluokan vasta-aineilla eroja niissä mekanismeissa, miten ne suojaavat kokeelliselta meningokokki-infektiolta.

Tulokset ja johtopäätökset: ELISA- ja bakterisidia-testiin verrattuna rotanpoikasen suojatestin kyky mitata rokotevasteita jäi alhaiseksi. Näin ollen se ei näytä soveltuvan sellaisenaan rokotteiden suojatehon arvioimiseen. Sen sijaan se näytti antavan lisäarvoa

bakterisidia-testiin verrattuna siinä, että monet bakterisidia-testissä negatiiviset näytteet osoittautuivat rotanpoikasen suojatestissä positiivisiksi, joskin päinvastaistakin havaittiin. Kun ennen rokottamista otettuja seerumeja tutkittiin tarkemmin, havaittiin, että niiden antama suojavaikutus rotanpoikasen suojatestissä oli B-ryhmän meningokokin kapselia vastaan olevien IgM-luokan vasta-aineiden välittämää. Tämä suoja-vaikutus näytti olevan riippumatonta vasta-aineiden aikaansaamasta, komplementin välittämästä bakteerien hajoamisesta koska osa suojaavista ihmisten seerumeista jäi bakterisidia-testeissä negatiivisiksi. Normaaliseerumit myös suojasivat yhtä hyvin niin komplementin suhteen normaaleissa kuin komplementin C6-komponentin suhteen puutteellisissa eläimissä. Tämä havainto vahvistettiin hiirestä peräisin olevalla kapseli vasta-aineella, joka toisin kuin testatut eri IgG alaluokkien PorA-proteiini vasta-aineet suojasi niin komplementin suhteen normaalit kuin C6-puutteelliset rotat kokeelliselta taudilta. Osoitimme myös ajallisen yhteyden luonnollisten kapseli-vasta-aineiden kehittymisen ja rotanpoikasen suojamallissa mitattavan suojan välillä. Nämä tulokset viittaavat siihen, että luonnollisilla kapseli vasta-aineilla saattaa olla tärkeä rooli puolustuksessa myös B-ryhmän meningokokkitauteja vastaan, toisin kuin on tähän asti yleisesti ajateltu.

Avainsanat: *Neisseria meningitidis B*, ulkokalvoproteiinirokotteet, rotanpoikasen infektiomalli

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ABBREVIATIONS

Ab	Antibody
Al-B-PS	Aluminum hydroxide gel-bound B-PS
Al-C-PS	Aluminum hydroxide gel-bound C-PS
BA	Bactericidal activity
B-PS	Group B polysaccharide
CDC	Centers for Disease Control and Prevention
CEACAM	Carcinoembryonic antigen-related cell adhesion molecule
cfu	Colony forming unit
CI	Confidence interval
C-PS	Group C polysaccharide
CR	Complement receptor
CSF	Cerebrospinal fluid
CV	Coefficient of variation
EIA	Enzyme immunoassay
FbpA	Ferric binding protein A
FcR	Receptor for Fc portion of immunoglobulins
GM	Geometric mean
GMC	Geometric mean concentration
HmbR	Hemoglobin-binding OMP
HMW	High molecular weight
IAV	Influenzae A virus
Ig	Immunoglobulin
IgA	Immunoglobulin class A
IgD	Immunoglobulin class D
IgE	Immunoglobulin class E
IgG	Immunoglobulin class G
IgM	Immunoglobulin class M
i.n.	Intranasal
i.p.	Intraperitoneal

IRPA	Infant rat protective activity
IT	Immunotype
KTL	Kansanterveyslaitos (National Public Health Institute)
LCCD	Late complement component deficiency
LMW	Low molecular weight
LNnT	Lacto-*N*-neotetraose
LOS	Lipooligosaccharide
Mab	Monoclonal antibody
MAC	Membrane attack complex
MCP	Membrane cofactor protein
MLEE	Multilocus enzyme electrophoresis
MLST	Multilocus sequence typing
MV	Microvesicle
NHS	Normal human serum
NIBSC	National Institute for Biological Standards and Control
NIPH	Norwegian Institute of Public Health
NspA	Neisserial surface protein A
NVI	Netherlands Vaccine Institute
O.D.	Optical density
OM	Outer membrane
OMP	Outer membrane protein
OMV	Outer membrane vesicle
Opa	Opacity protein
OPA	Opsonophagocytic activity
PI	Protection index
PMNL	Polymorphonuclear leukocyte
PorA	Porin A (class 1 OMP)
PorB	Porin B (class 2/3 OMP)
PS	Polysaccharide
RB	Respiratory burst
Rmp	Reduction modifiable protein (class 4 OMP)
SBA	Serum bactericidal activity

SDS	Sodium dodecyl sulphate
TbpA	Transferrin-binding protein A
TbpB	Transferrin-binding protein B
WBA	Whole blood assay
VR	Variable region
WRAIR	Walter Reed Army Institute of Research

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles referred to in the text by their Roman numerals:

I Toropainen M., Käyhty H., Saarinen L., Rosenqvist E., Høiby EA., Wedege E., Michaelsen T., Mäkelä PH. The infant rat model adapted to evaluate human sera for protective immunity to group B meningococci. Vaccine 1999;17(20-21):2677-2689.

II Toropainen M., Saarinen L., Wedege E., Bolstad K., Mäkelä PH., Käyhty H. Passive protection in the infant rat protection assay by sera taken before and after vaccination of teenagers with serogroup B meningococcal outer membrane vesicle (OMV) vaccines. Vaccine. In press 2005.

III Toropainen M., Saarinen L., Wedege E., Bolstad K., Michaelsen TE., Aase A., Käyhty H. Protection by natural human immunoglobulin M antibody to meningococcal serogroup B capsular polysaccharide in the infant rat protection assay is independent of complement mediated bacterial lysis. Infect Immun. In press 2005.

IV Toropainen M., Saarinen L., Vidarsson G., Käyhty H. Passive protection by meningococcal PorA outer membrane protein specific mouse-human chimeric IgG1-4 antibodies and a serogroup B capsular polysaccharide specific mouse antibody in complement sufficient and C6 deficient infant rats. Manuscript.

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1 INTRODUCTION

Meningococcal disease is one the most feared infections due to rapidity of onset, propensity to affect infants and young adults, high mortality, serious sequelae, and tendency to spread, sometimes globally. Though the causative organism, *Neisseria meningitidis* or meningococcus, can cause a spectrum of illnesses, two clinically overlapping syndromes, purulent meningitis and sepsis, are by far the most common and most serious presentations.

Meningococci are divided into 12 serogroups on the basis of differences in the structure and antigenicity of their capsular polysaccharides (PSs). Serogroups A, B, and C account for approximately 90% of all cases of meningococcal disease worldwide, group B and C meningococci being the predominant causative agents in industrialized countries. Natural immunity against meningococcal disease develops with age, associated with an increase in serum bactericidal activity (SBA). Thus, induction of bactericidal antibodies has been regarded as evidence of the potential efficacy of putative vaccines. Indeed, because of the lack of an animal model, vaccine candidates considered promising on this basis have been directly subjected to efficacy trials in humans.

Currently, there are capsular PS or conjugate vaccines to combat disease caused by serogroup A, C, Y and W135 meningococci, but no corresponding vaccine is available for serogroup B meningococci. This is due to poor immunogenicity of its capsular PS. Thus, vaccine research against serogroup B disease has focused on non-capsular antigens, mainly outer membrane proteins in the form of outer membrane vesicles (OMVs), or purified recombinant proteins, of which OMV vesicle vaccines have been evaluated in clinical efficacy trials.

The development of serogroup B meningococcal vaccines has been hampered by the lack of reliable laboratory correlates or surrogates for protection. Although the antibody responses in connection with efficacy and separate immunogenicity trials have been analyzed extensively, there is still much uncertainty about the specificity and functional mechanisms of antibodies providing protection against serogroup B disease. In particular, despite the good predictive value of SBA for the protective efficacy of serogroup A and C capsular PS based vaccines the importance of SBA for protection against serogroup B disease remains partly open.

This thesis consists of a series of consecutive studies that have addressed the applicability of an infant rat model of serogroup B meningococcal infection for the assessment of meningococcal serogroup B OMV vaccine responses induced in humans. In the search for vaccine candidates capable of inducing SBA responses, natural immunity to serogroup B meningococcus has been less studied. Thus, the specificity and mechanisms of natural antibodies conferring protective activity to infant rats were also evaluated, and the importance of bacterial lysis on protection afforded by antibodies of different specificity or isotype assessed.

2 REVIEW OF LITERATURE

2.1 Meningococcal disease

2.1.1 The bacterium

Neisseria meningitidis (the meningococcus) is an aerobic, gram-negative, non-motile, non-sporulating coccus that occurs in pairs of cells (diplococcus). It is oxidase and catalase positive and produces acid from glucose and maltose.

Of the genus of *Neisseria*, only *N. gonorrhoea* (the gonococcus) is always considered to be an invader in man, requiring eradication by chemotherapy. The other species occurring in man are regarded as more or less frequently isolated commensals, of which *N. meningitidis* is most often isolated from clinical samples and with gonococcus being the only species with potential to cause severe systemic disease in otherwise healthy individuals. *N. lactamica* shares the nasopharyngeal colonization site with meningococci though they are not associated with invasive disease. Due to severity of meningococcal disease and especially the capability to epidemic spread, in most countries the notification of cases of systemic meningococcal disease to Public Health Authorities is obligatory. Together with collection and characterization of corresponding meningococcal isolates, clinical notifications form the basis for local, national and global epidemiology and disease control.

Traditionally, the characterization of meningococcal strains has been based on bacterial phenotype, i.e. the recognition meningococcal surface structures by monoclonal or polyclonal antibodies raised in experimental animals (mice and rabbits) for these purposes. Thus, the antigenic variability of the capsular polysaccharide (PS) defines the serogroup, class 2/3 or porin B protein (PorB) the serotype, class 1 or porin A protein (PorA) the serosubtype, and lipooligosaccharide (LOS) the immunotype of a meningococcal strain (Frasch *et al.* 1985, Abdillahi and Poolman 1987). Due to the emergence of new PorB and especially PorA variants not recognized by currently available serotyping and serosubtyping reagents, DNA sequencing of variable regions of the respective *porB* and *porA* genes is currently replacing serological typing methods (Russell *et al.* 2004). Other molecular typing methods, indexing more slowly evolving variation in bacterial genome, namely multilocus enzyme

electrophoresis (MLEE) and more recently, multilocus sequence typing (MLST) (Maiden *et al.* 1998), are used to assign meningococcal strains into clonal complexes and have proven useful tools for long-term and global epidemiology.

2.1.2 Epidemiology

Meningococci are divided into 12 serogroups (A, B, C, H, I, K, L, W135, X, Y, Z, 29E) on the basis of differences in the structure and antigenicity of their capsular PS. Serogroups A, B, and C account for approximately 90% of all cases of meningococcal disease worldwide, group B and C meningococci being the predominant causative agents in industrialized countries. From an epidemiological perspective, meningococcal disease is a pleomorphic disease, with incidence rates, serogroup distribution, and the age groups most affected varying according to geographic location and time.

It has been estimated that (excluding epidemics) approximately 500,000 cases of invasive meningococcal disease occur annually worldwide, with over 50,000 deaths (WHO 1998). In most countries, the annual attack rates vary from < 1 to 5 per 100,000 populations but may increase up to 1000 or more especially during serogroup A epidemics (WHO 1998). The disease is most common among infants and young children under 5 years of age, with a secondary smaller peak occurring among adolescents 15-19 years of age; approximately one-third of the cases of sporadic meningococcal disease occur in adults >19 years of age (EU-IBIS 2002). Epidemics tend to shift the age distribution, with older children, teenagers and young adults also affected (Peltola *et al.* 1982).

The risk of epidemic disease differs between serogroups through mechanisms that are poorly understood. Serogroup A meningococcus has historically been the main cause of large epidemics and is still the dominating serogroup during both endemic and epidemic periods in sub-Saharan Africa in the so called African meningitis belt extending from Senegal in the west to Ethiopia in the east. In this area, epidemics caused mainly by serogroup A meningococci, and more recently serogroup W135 (Taha *et al.* 2002), occur in irregular cycles every 8 to 12 years, last for two to three dry seasons, fading away during the intervening rainy seasons (WHO 1998). Since the epidemic in Finland during 1973-74, serogroup A disease has been extremely rare in Europe though recently, a rise in the number

of cases of serogroup A meningococcal disease in Moscow has been reported (Lawrence and Handford 2003).

Serogroup C typically causes smaller outbreaks or epidemics, and with serogroup B meningococcus is the prevalent cause of endemic disease in most industrialized countries. During last years, high or increasing proportions of group C strains, mainly belonging to hypervirulent ST-11/ET-37 clonal complex first identified in Canada in 1986 (Ashton *et al.* 1991), have been reported from several European countries including Belgium (De Schrijver and Maes 2003), the Czech republic (Krizova and Musilek 1995), the Netherlands (de Greeff *et al.* 2003), Republic of Ireland (Fogarty 1997), Spain (Alcala *et al.* 2002) and the United Kingdom (Kaczmarski 1997, Ramsay *et al.* 1997). This lead to nationwide vaccination campaigns in several countries and the accelerated introduction of the newly developed serogroup C conjugate vaccine (Miller *et al.* 2001, de Greeff *et al.* 2003, De Schrijver and Maes 2003, Salleras *et al.* 2003).

Serogroup B meningococci differ from serogroups A and C in disease epidemiology. In contrast to serogroup A and C epidemics, which usually resolve in 1 to 3 years, serogroup B epidemics begin more slowly, usually reach lower country-wide rates of 5 to 20 cases per 100 000 population per year and may persist for 5 to 10 years or longer, as seen in past decades in Cuba (Sierra *et al.* 1991), Norway (Lystad and Aasen 1991), Brasilia (Sacchi *et al.* 1998a), areas of Chile (Cruz *et al.* 1990), and more recently, in New Zealand (Baker *et al.* 2001).

2.1.3 Carriage

It has been estimated that approximately 10% of the population carry meningococci in their nasopharynx (Cartwright *et al.* 1987); yet only a minority of carriers develop invasive disease. Carriage is most common in young adults, less so in young children and relatively rare in adult populations (Cartwright *et al.* 1987). In infants and toddlers, the carriage of *N. lactamica*, a related non-pathogenic organism, predominates (Gold *et al.* 1978, Cartwright *et al.* 1987, Bakir *et al.* 2001).

Meningococcal carriage is a dynamic process, with high rate of turnover and apparently multiple carriage periods occurring throughout the life (Ala'Aldeen *et al.* 2000). The duration of carriage is also highly variable (Ala'Aldeen *et al.* 2000), with the median time of

approximately 4 months (Gold *et al.* 1978). If systemic meningococcal disease occurs, it usually manifests within days after the acquisition of a new *N. meningitidis* strain in nasopharynx (Edwards *et al.* 1977) thought longer carriage period before development of invasive disease has also been reported (Ala'Aldeen *et al.* 2000). Despite the increased risk of invasive meningococcal disease after the acquisition of meningococci in the nasopharynx, at population level the fluctuations in overall carriage rates have not proven very useful in the prediction of outbreaks or emerging epidemics. This reflects the fact that in sharp contrast to meningococcal strains isolated from patients that belong to few hyperinvasive lineages only, the majority strains isolated from carriers are highly diverse, comprising genotypes rarely or never recovered from patients and having thus limited pathogenic potential (Caugant *et al.* 1988, Jolley *et al.* 2000, Yazdankhah *et al.* 2004). During circulation in human populations, new lineages of meningococci with increased capacity to cause invasive disease occasionally arise from carriage strains thorough genetic recombination and mutation, with capacity to spread, sometimes globally (Caugant *et al.* 1986).

2.1.4 Adhesion, colonization of human nasopharynx, and invasion

Humans are the only known reservoir of meningococci. Thus the pathogen is transmitted only by aerosol or direct contact with respiratory secretions of patients or healthy human carriers. After access to respiratory track, meningococci adhere selectively to microvilli of non-ciliated columnar epithelial cells and colonize the nasopharynx (Stephens *et al.* 1983). The initial attachment of meningococci is mediated by long filamentous pili (Stephens and McGee 1981, Virji *et al.* 1992) that specifically recognize complement regulatory protein CD46 (or membrane cofactor protein, MCP) on the apical surface of human epithelial cells (Källström *et al.* 1997). This interaction between the pathogen and human host is highly specific, as evidenced by the inability of piliated strains to bind cells of non-human origin (Virji *et al.* 1993b). Adhesion of meningococci to host cells leads to down-regulation of pili and capsular polysaccharide synthesis (Deghmane *et al.* 2002). This allows a more intimate contact to host cells to be established by colony opacity-associated outer membrane proteins Opa and OpcA which both facilitate meningococcal attachment to and invasion of human cultured epithelial and endothelial cells but only when present in un-encapsulated background (Virji *et al.* 1993a). Transient carriage with mild or no symptoms rather than meningitis and/or septicemia is the most common outcome of meningococcal infection. The illness results when the

organism penetrates the respiratory mucosa and enters the bloodstream of a susceptible host. Once entered the blood stream, the bacteria gains access to other parts of body and central nervous system by crossing the blood-brain barrier. The mechanisms by which meningococci cross the blood-brain barrier composed of tight junction forming epithelial and/or endothelial cells are poorly understood but similar to initial contact with nasopharyngeal epithelial cells, the interaction between pili and CD46 receptor is likely to be involved (Pron *et al.* 1997).

2.1.5 Clinical aspects

Systemic meningococcal infection is a bacteremic disease often associated with secondary infection of skin, meninges and other parts of the body. Though meningococci can cause a spectrum of illnesses, two clinically overlapping syndromes, purulent meningitis and bloodstream infection (meningococcemia), are by far the most common presentations, septic shock with extremely rapid onset being the most devastating form of the disease (Cartwright and Ala'Aldeen 1997, Munro 2002). Other manifestations include septic arthritis, pneumonia, purulent pericarditis and conjunctivis. Most untreated cases of meningococcal meningitis and especially septicemia are fatal. Despite efficient anti-microbial treatment and intensive supportive care the case fatality rate from invasive disease has not significantly improved during past decades, and the mortality from meningococcal disease has stabilized to about 7-9% overall, varying from 2-3% in uncomplicated meningitis to 50% or more in septic shock (Cartwright and Ala'Aldeen 1997). A nearly equal number of survivors (5%) sustain permanent neurological sequelae, mainly deafness (Cartwright and Ala'Aldeen 1997).

Meningococcal disease is typically of rapid onset and thus treatment should commence as soon as the diagnosis is suspected. In most parts of world, meningococci are still highly susceptible to penicillin, which is usually the choice of antimicrobial drug for treatment. Other drugs such as rifampicin are required to eradicate nasopharyngeal colonization and thus, to prevent relapse. As the risk of secondary cases among close contacts such as family members is relatively high, chemoprophylaxis combined with vaccination, if available, is usually recommended. There are capsular PS or conjugate vaccines against serogroups A, C, Y and W135 meningococci, but no vaccine available for serogroup B meningococci.

The factors that affect the balance between asymptomatic carriage and bacterial invasion are poorly understood. From the bacterial side, the virulence of the colonizing strain and from the host side, the state of innate and specific, i.e. adaptive immunity are the most obvious ones likely to be involved. Additional factors such as interference by viral infections or environmental factors such climatic conditions or smoking that damage integrity of the respiratory mucosa, and hence, its effectiveness as a barrier to invasion, may also predispose the host to systemic infection (Moore *et al.* 1990, Fischer *et al.* 1997, Scholten *et al.* 1999).

2.1.6 Genetic factors associated with disease susceptibility

It has been estimated that host genetic factors contribute to approximately one third of the increased risk of disease in siblings of affected cases compared with the risk of disease in general population (Haralambous *et al.* 2003) but the genetic polymorphisms behind this finding has not been systemically studied. Variation in host genetic factors may contribute to disease at different stages, affecting susceptibility, severity, and/or outcome of infectious disease (Emonts *et al.* 2003). From genetic association studies, defects in sensing, recognizing, opsonophagocytic, and lytic pathways, such Toll-like receptor 4 polymorphism (Smirnova *et al.* 2003), hypogammaglobulinemia (Salit 1981), congenital IgM deficiency (Hobbs *et al.* 1967), certain Fcγ receptor genotypes (Platonov *et al.* 1998, van der Pol *et al.* 2001, Domingo *et al.* 2002), and especially, defects in classical, alternative, or lectin complement pathways (Ross and Densen 1984, Hibberd *et al.* 1999) are the most obvious factors involved in genetically established susceptibility to and/or severity of meningococcal infections.

2.2 Meningococcal surface structures: virulence factors and potential vaccine antigens

Apparently the most important virulence factor of meningococcus is its enormous genomic plasticity that allows the pathogen to adapt to different ecological niches and resist varying non-specific and specific host defense mechanisms it encounters at different stages of infection. Not only the antigenic structure but also the expression of a number of meningococcal surface components thought to be of importance for virulence varies through processes of transformation, mutation and phase variation (Maiden 1993, Feil *et al.* 1999, Snyder *et al.* 2001). This variety presents the most important challenge to serogroup B

meningococcal vaccine development against which efforts to develop capsular PS-based vaccines has been unsuccessful (Wyle *et al.* 1972).

2.2.1 Antigenic and phase variability

Meningococci are transformable, i.e. naturally competent for DNA uptake, enabling a mechanism for inter-strain or even inter-species horizontal genetic exchange and antigenic variability. This process is the primary mode of DNA change in *Neisseria* (Feil *et al.* 1999) and has been implicated in the acquisition of *sodC* from *Haemophilus influenzae* (Kroll *et al.* 1998), alteration of *penA* resulting in penicillin resistance (Spratt *et al.* 1992), antigenic variation of PorA (Feavers *et al.* 1992a) and Opa proteins (Hobbs *et al.* 1994), and capsular switching (Swartley *et al.* 1997). Mutations in coding regions provide additional variability and are responsible for the micro-heterogeneity of OMPs, such PorA and PorB protein minor variants.

In addition to antigenic variability, many neisserial genes involved in host-parasite interactions are subject to phase variation (Tettelin *et al.* 2000, Snyder *et al.* 2001), which can be defined as high frequency (10^{-2} to 10^{-4}/cell/generation), reversible on-and-off switching of gene expression, contributing to both transmissibility and invasiveness of the organism (de Vries *et al.* 1996). By comparative whole-genome analyses, over 100 putative phase-variable genes in the pathogenic *Neisseria* species has been identified (Snyder *et al.* 2001). These include the genes for pilus (Rytkönen *et al.* 2004), opacity proteins (Kawula *et al.* 1988), capsular PS (Hammerschmidt *et al.* 1996b), LOS (Jennings *et al.* 1999) and HmbR, a hemoglobin-binding outer membrane protein (Richardson and Stojiljkovic 1999). The genetic basis for this variation depends on the presence of iterative DNA motifs, especially homopolymeric tracts, which act as sites of hypermutation. Mutations in tracts situated within promoters can alter the degree of gene expression by regulating transcription (e.g. PorA and OpcA) while mutations in tracts within coding regions result in on-off switching (e.g. Opa) of gene expression at translation level depending on whether the downstream sequences are moved in or out of frame for expression. Depending on genes in question, the latter may result in on-and-off switching of protein expression or in the case of multiple genes involved in LOS biosynthesis, alteration of LOS structure.

2.2.2 General characteristics of the cell envelope

As a typical gram-negative bacterium, the meningococcal cell envelope is composed of three layers: an inner phospholipid cytoplasmic membrane, a thin middle peptidoglycan layer, and an outer membrane (OM) composed of phospholipids, LOS and numerous proteins embedded on it (Fig. 1). In pathogenic strains, isolated from systemic infections, the OM is further surrounded by a capsule composed of high-molecular weight anionic polysaccharide. Most research has been focused on the capsule and OM components since these are the sites where meningococcus interacts with the human host and potential vaccine antigens as well as antigenic variability exist.

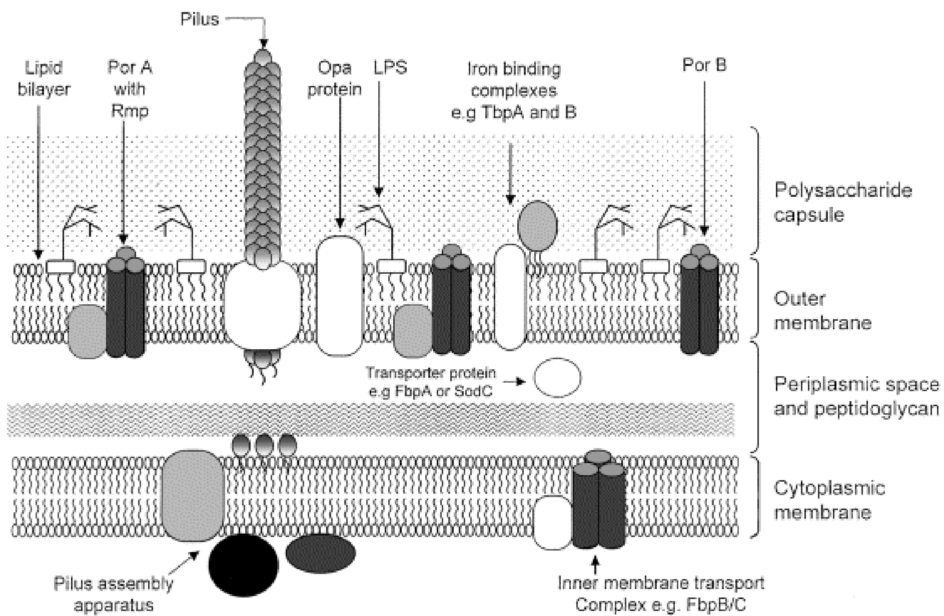

Figure 1. Structure of meningococcal cell envelope. Reprinted from Vaccine, Vol 20, Morley SL and Pollard AJ. Vaccine prevention of meningococcal disease, coming soon? Pages 666-687, Copyright (2001), with permission from Elsevier.

2.2.3 Polysaccharide capsule

Capsule is the major virulence factor of meningococcus. With the exception of serogroup A meningococci, all the disease associated serogroups B, C, Y and W135 have sialic acid (*N*-acetyl neuraminic acid) in their capsular polysaccharides. This confers the organism

resistance to host alternative complement pathway mediated attack through mechanisms that are not fully understood (Jarvis and Vedros 1987, Ram *et al.* 1999), partly due to the presence of another potentially sialylated molecule, i.e. LOS, on bacterial surface. *In vitro*, sialic acids of both capsule and LOS are prerequisite for bacterial survival in normal human serum (Vogel *et al.* 1997, Kahler *et al.* 1998) and confer resistance to phagocytosis (Estabrook *et al.* 1992). Likewise, in an animal model of meningococcal infection, expression of both sialic acids has been reported to be indispensable for bacterial survival (Vogel *et al.* 1996).

Like many other virulence factors, the expression of capsular PS is phase variable (Hammerschmidt *et al.* 1996a, Hammerschmidt *et al.* 1996b), allowing the bacteria to vary from adherent, serum sensitive, un-encapsulated phenotype to less-adherent, serum resistant encapsulated phenotype. Colonization is favored by the absence of the capsule, un-encapsulated mutants adhering to human buccal epithelial cells or nasopharyngeal organ cultures in greater numbers than the encapsulated parent (Stephens *et al.* 1993). This is in strict contrast to systematic spread: encapsulation is a prerequisite for bacterial survival in the blood. In contrast to case isolates that are frequently encapsulated, approximately 40-50% of the strains isolated from carriers lack capsule and hence are not serologically groupable (Caugant *et al.* 1988, Ala'Aldeen *et al.* 2000, Yazdankhah *et al.* 2004).

In serogroup B and C meningococci, the capsular PSs are a homopolymers of N-acetyl-neuraminic acid. The major difference between these two PS lies in the linkage between the sialic acid residues, which is α2-8 in serogroup B and α2-9 in serogroup C meningococci. The serogroup C PS (C-PS) is also usually *O*-acetylated (Borrow *et al.* 2000) whereas the serogroup B PS (B-PS) is always de-*O*-acetylated (Jennings *et al.* 1977). These differences are sufficient to alter the immunological properties of B-PS profoundly. Thus, by contrast to C-PS, the purified B-PS is only poorly immunogenic in man (Wyle *et al.* 1972). This has been attributed to immunologic tolerance arising from structural and antigenic similarities between B-PS and polysialosyl glycopeptides in fetal and adult neural and extra-neural tissues (Finne *et al.* 1983a), and exemplifies another mechanism by which pathogenic bacteria may circumvent host immunity, i.e. molecular mimicry.

2.2.4 Lipooligosaccharide (LOS)

LOS is the major constituent of the outer leaflet of the meningococcal OM and responsible for the physical integrity and proper functioning of the membrane. It is a glycolipid composed of three portions: i) an innermost lipid moiety called lipid A, ii) an inner core oligosaccharide structure composed 2-keto-3-deoxy-octulosonic acid and heptoses, the latter sugars being variably substituted with iii) short polysaccharide side chains but lacking an O-antigen characteristic to enteric gram-negative bacteria. The lipid A portion anchors the LOS into the outer leaflet of OM, and is responsible for the toxicity of this molecule due to its ability to up-regulate a number of inflammation mediators either directly or indirectly, leading ultimately to septic shock accompanied by disseminated intra-vascular coagulation and multiple organ failure (Brandtzaeg *et al.* 2001). The inner core OS structure is common to most if not all immunotypes, with most of the variability lying in the short polysaccharide side chains (Griffiss *et al.* 1987) and forming the basis of division of meningococci into 12 different immunotypes (ITs) (Verheul *et al.* 1993). ITs L1 thorough L9 are found within group B and C meningococci and ITs L8 through L11 within group A meningococci, with ITs L10 and L11 most often associated with the latter serogroup (Verheul *et al.* 1993).

Similarly to many other meningococcal genes, the *lgt* genes responsible for LOS biosynthesis are subject to phase variation, most strains having potential to express several alternative terminal LOS structures (Jennings *et al.* 1999, Berrington *et al.* 2002). In contrast nasopharyngeal isolates that usually express the L8 IT, invasive isolates express lacto-*N*-neotetraose (LNnT) containing LOS IT (Jones *et al.* 1992). This structure, antigenically identical to carbohydrate moieties of glycosphingolipids present in many human cells (Mandrell *et al.* 1988), is present on many ITs, including L2, L3, L5, L7, and L9, and can be endogenously (Mandrell *et al.* 1991) or exogenously sialylated (Estabrook *et al.* 1997). Besides its suggested role for virulence through molecular mimicry (Mandrell and Apicella 1993, Moran *et al.* 1996), sialylation of LNnT confers the organism resistance to killing through all the three complement pathways, irrespective of capsular phenotype (Estabrook *et al.* 1997, Vogel *et al.* 1997, Jack *et al.* 1998, Jack *et al.* 2001).

2.2.5 Major outer membrane proteins (OMPs)

Meningococci express a number of major and minor OMPs, some of which can serve as targets for bactericidal, opsonic and/or protective antibodies (Saukkonen *et al.* 1989) and are thus important serogroup B vaccine candidates. On the basis of differences in molecular weights, five different classes (1 to 5) of major OMPs, named so because of their abundance in OM, are recognized (Tsai *et al.* 1981).

Class 1 or porin A (PorA) proteins are porins with slight cation selectivity (Tommassen *et al.* 1990). They are present in the OM as trimers, and function as pores through which small hydrophilic solutes can pass in a diffusion-like process (Nikaido 1992). PorA proteins are generally present in most meningococcal strains but the expression of this protein varies considerably (van der Ende *et al.* 1995, van der Ende *et al.* 2000); occasionally, the protein may be absent (Newcombe *et al.* 1998, van der Ende *et al.* 1999). Recently, an outbreak of meningococcal disease caused by PorA-deficient C meningococci has been described (van der Ende *et al.* 2003).

The *porA* genes responsible for expression of PorA protein in meningococci of different subtypes have been cloned and sequenced (Barlow *et al.* 1989, McGuinness *et al.* 1990, Maiden *et al.* 1991). On the basis of nucleotide sequence, a 16-stranded β-barrel topology model has been proposed, with eight hydrophilic loops (L1-L8) exposed to the cell surface (van der Ley *et al.* 1991, Derrick *et al.* 1999) (Fig. 2). Most of the structural diversity is confined to two discrete variable regions (VR1 and VR2) in the predicted surface-exposed loops 1 and 4 (Maiden *et al.* 1991, van der Ley *et al.* 1991), respectively. The major structural differences in these two variable regions independently generate two separate subtype-specific antigenic determinants and form the basis of the serosubtyping (Frasch *et al.* 1985, Abdillahi and Poolman 1988) and PorA VR typing scheme (Russell *et al.* 2004). Within these schemes, the PorA is assigned by the prefix "P1.", followed by numbers separated by commas that describe the subtype designation. Thus, a meningococcal strain with B:15:P1.7,16 phenotype has subtype determinants P1.7 and P1.16 in the VR1 and VR2, respectively.

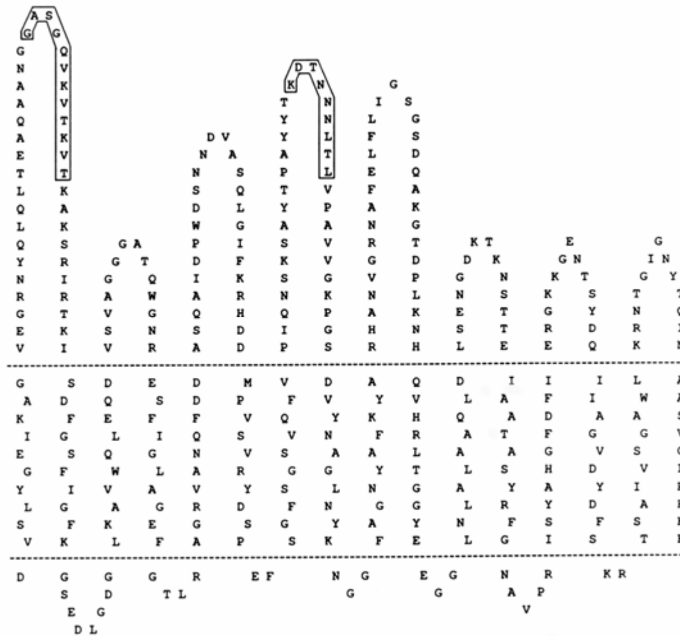

Figure 2. Topology model of meningococcal PorA protein. Amphipathic β-sheets form the outer membrane spanning regions, with eight hydrophilic loops exposed to cell surface. Reprinted from Meningococcal Disease, Cartwright K ed. Surface structures and secreted products of meningococci, Poolman JT *et al*. Pages 21-34, Copyright (1995), with permission from John Wiley & Sons Ltd.

PorA is immunogenic in humans following infection (Mandrell and Zollinger 1989, Guttormsen *et al.* 1994, Wedege *et al.* 1998) or vaccination (Wedege and Froholm 1986, Rosenqvist *et al.* 1995, Tappero *et al.* 1999) and the antibodies induced exhibit both bactericidal (Rosenqvist *et al.* 1995, Milagres *et al.* 1998, Tappero *et al.* 1999) and opsonic functions (Lehmann *et al.* 1999) thought to be of importance for host protection against systemic disease. However, due considerable inter-strain antigenic variation, a multivalent vaccine is needed to increase vaccine coverage if PorA is to be used as a single vaccine antigen.

Class 2/3 or porin B (PorB) proteins are the most abundant proteins of the OM, where they function as anion-selective porins (Tommassen *et al.* 1990). They are structurally related to PorA proteins, with eight surface-exposed hydrophilic loops separated by conserved membrane spanning β-sheets (van der Ley *et al.* 1991, Derrick *et al.* 1999). All

meningococcal strains stably express either of the two mutually exclusive PorB proteins (Tsai *et al.* 1981), PorB2 or PorB3, encoded by alternate alleles present at the *porB* locus. In Europe, majority of current serogroup B disease isolates express PorB3 (EU-IBIS).

The *porB* genes responsible for expression of PorB protein in meningococci of different serotypes have been cloned and sequenced (Feavers *et al.* 1992b, Bash *et al.* 1995, Sacchi *et al.* 1998b). Most of the structural diversity is confined to four discrete variable regions (VR1 through VR4) in the predicted surface-exposed loops 1, 5, 6, and 7, respectively (Feavers *et al.* 1992b, Bash *et al.* 1995), of the proposed porin structure, and forms the basis of division of meningococci into different serotypes (Frasch *et al.* 1985) and PorB VR types (Sacchi *et al.* 1998b).

PorB protein is highly immunogenic in humans following infection and vaccination (Guttormsen *et al.* 1993, Delvig *et al.* 1997, Wedege *et al.* 2000) but the protective activity of these antibodies may be limited due to poor accessibility of especially PorB3 epitopes on intact, live bacteria (Michaelsen *et al.* 2001). Indeed, by contrast to allelic PorB2 and the PorA protein (Brodeur *et al.* 1985, Saukkonen *et al.* 1987, Saukkonen *et al.* 1989), monoclonal antibodies to PorB3 are less frequently bactericidal and failed to confer protection *in vivo* in an infant rat model of meningococcal infection (Saukkonen *et al.* 1987, Saukkonen *et al.* 1989).

Class 4 or reduction modifiable protein (Rmp) differs from other major OMPs in terms that it is both constitutively expressed as well as antigenically invariable (Lytton and Blake 1986). It is a structural, periplasmic protein involved in the maintenance of cell envelope integrity by stabilizing the oligomeric forms of a number of OMPs (Jansen *et al.* 2000, Prinz and Tommassen 2000) and linking outer membrane to peptidoglycan layer (Grizot and Buchanan 2004). As might be predicted from its periplasmic location, antibodies to Rmp are neither bactericidal nor opsonic (Rosenqvist *et al.* 1999), making it less attractive candidate for meningococcal vaccines.

Class 5 or colonial opacity-associated Opa and OpcA proteins are a group of heat-modifiable proteins that are functionally related but genetically and structurally distinct. Both proteins mediate meningococcal adhesion to and invasion of epithelial and endothelial cells,

Opa proteins mainly by binding to members of the carcinoembryonic antigen-related cell adhesion molecule (CEACAM) family (Virji *et al.* 1996) while OpcA protein interacting indirectly via vitronectin with β-integrins on endothelial cells (Virji *et al.* 1994) and directly with heparan sulphate proteoglycans on epithelial cells (de Vries *et al.* 1998).

Opa proteins are encoded by a family of three to four genes that undergo high-frequency on-off phase (Stern and Meyer 1987, Kawula *et al.* 1988, Woods and Cannon 1990) and antigenic variation of expression (Hobbs *et al.* 1994, Malorny *et al.* 1998), a single strain of *N. meningitidis* being able to express from none up to several distinct Opa proteins due to genetic rearrangements within the pentameric repeat units in coding region of the amino-terminal leader peptide (Stern and Meyer 1987). On the basis of nucleotide sequence, a two-dimensional eight-stranded β-sheet topology model has been proposed, with four hydrophilic loops (L1-L4) exposed to the cell surface (Malorny *et al.* 1998). Loops 2 and 3 are highly variable in sequence (Malorny *et al.* 1998) and involved in the interaction with CEACAM receptors on host epithelial cells (Virji *et al.* 1996, de Jonge *et al.* 2003).

OpcA protein (formerly know as 5C or Opc) differs from Opa proteins in terms of that only one functional gene (*opcA*) is present in meningococcal genome (Zhu *et al.* 1999); in some epidemic clones it may be absent (Seiler *et al.* 1996). By contrast to highly variable *opa* genes, *opcA* shows only limited sequence variability (Seiler *et al.* 1996). Nevertheless, due to variation in the length of a polycytidine strecth in the promoter region, the expression of OpcA is hypervariable at transcriptional level, leading to bacteria with different OpcA levels on their OM (Sarkari *et al.* 1994). On the basis of nucleotide sequence, a two-dimensional eight-stranded β-sheet topology model has been proposed, with 10 transmembrane strands and five hydrophilic loops (L1-L5) exposed to the cell surface (Merker *et al.* 1997). In the crystal structure, the loops protrude from the β-barrel and form a positively charged crevice that have been suggested to be the binding site to negatively charged host proteoglycan polysaccharide (Prince *et al.* 2002).

Although the Opa and especially the OpcA protein have been shown to be highly immunogenic and able to induce bactericidal antibodies in humans following infection or vaccination (Rosenqvist *et al.* 1993, Danelli *et al.* 1996, Milagres *et al.* 1998), the high degree

of phase and/or antigenic variability has been considered the major impediment to vaccine application.

The minor OMPs are discussed later in the "Other serogroup B vaccine candidates" section.

2.2.6 Other surface structures

Pili are long filamentous multimeric proteins protruding from the OM to bacterial surface. In encapsulated strains, pili are essential for adhesion of meningococci to human epithelial and endothelial cells (Stephens and McGee 1981, Virji *et al.* 1991). Similarly to a number of other meningococcal surface structures, also pili undergo extensive antigenic and phase variation (Tinsley and Heckels 1986, Nassif *et al.* 1993, Rytkönen *et al.* 2004), and hence, are considered less attractive vaccine candidates.

2.3 Immunity and host defense mechanisms

A variety of host defense mechanisms are involved in protection against meningococcal disease. Similarly to other pathogens colonizing the upper respiratory tract, at mucosal sites, non-specific and specific factors inhibiting the attachment, colonization and penetration of bacteria through the respiratory epithelium provide the first line defense. If penetration of bacteria through the respiratory epithelium and entrance to bloodstream occurs, humoral and cellular components of blood and associated tissues provide the last phase of host defense to prevent excessive intra-vascular bacterial multiplication and the development of overwhelming disease. With this respect, specific antibody (IgG or IgM) and the complement system play the most critical role (Goldschneider *et al.* 1969a). Acquired or genetic absence of either of these serum factors predisposes individual to at risk for systemic meningococcal infection (Hobbs *et al.* 1967, Salit 1981, Figueroa *et al.* 1993).

2.3.1 Development of immunity

Natural antibodies against meningococci develop with age and are associated with an increase in serum bactericidal activity (SBA) (Goldschneider *et al.* 1969a, Goldschneider *et al.* 1969b). Infants are thought to be protected from disease through the acquisition of maternally derived antibody during gestation and lactation. With the weaning immunity, the incidence of

meningococcal disease peaks at 6 months to 2 years of age, followed by progressive decrease up to 12 years of age when the attack rate has declined to adult level and natural immunity developed (Goldschneider *et al.* 1969a). Although poorly understood, this natural immunization is thought to occur through prolonged or intermittent colonization at the mucosal surface by non-pathogenic meningococci or commensal *Neisseria* such as *N. lactamica* (Goldschneider *et al.* 1969b, Gold *et al.* 1978, Robinson *et al.* 2002, Troncoso *et al.* 2002). Both capsular and sub-capsular antigens seem to participate in generation of systemic immune response (Goldschneider *et al.* 1969b, Jones and Eldridge 1979, Wedege *et al.* 2003, Jordens *et al.* 2004) thought to be of major importance for host immunity against invasive disease. While beneficial for systemic immunity, the carriage does not, however, always seem to prevent re-colonization with either the homologous or heterologous meningococcal strains (Ala'Aldeen *et al.* 2000).

The contribution of carriage of non-related species to development of immunity is less clear. Nevertheless, serological cross-reactions, also between meningococci and species of other genera (Robbins *et al.* 1972, Kasper *et al.* 1973, Glode *et al.* 1977, Bøvre *et al.* 1983, Devi *et al.* 1991), are quite common and thus likely to be an important mechanism by which majority of humans develop natural immunity against meningococci.

The importance of mucosal immunity to protection against meningococcal disease has been less studied but as the portal of entry is likely to play an important role on host protection. After meningococcal carriage, an increase in specific salivary IgA concentration has been detected (Robinson *et al.* 2002). Whether this carriage-induced mucosal immunity protects against invasive meningococcal disease remains to be determined.

2.3.2 Complement

The complement system appears to have a unique role in the protection of host from meningococcal infection, as highlighted by the high frequency of complement deficiencies in patients with systemic meningococcal disease (Ross and Densen 1984, Figueroa *et al.* 1993) and a number of mechanisms that pathogenic *Neisseria* has evolved to combat this powerful defense mechanism (Ram *et al.* 1999, Jarva *et al.* 2005). It is a part of the innate immune system consisting of over 30 soluble or membrane bound proteins, and acts in defense against

infection directly by its inflammatory, opsonic and lytic activities, and indirectly by enhancing antibody responses against invading pathogens (reviewed in (Erdei *et al.* 1991, Frank and Fries 1991, Kinoshita 1991)). Three pathways of complement cascade activation have been described: the classical pathway, the alternative pathway, and the lectin pathway (Fig. 3). Although initiated by different recognition mechanisms, all these pathways converge at C3 level, leading to deposition of opsonically active C3b and iC3b fragments on bacterial surface and, through the continuation of complement activation through the common terminal pathway (C5-9) and deposition of membrane attack complex (MAC) to outer membranes of gram-negative bacteria, target cell lysis.

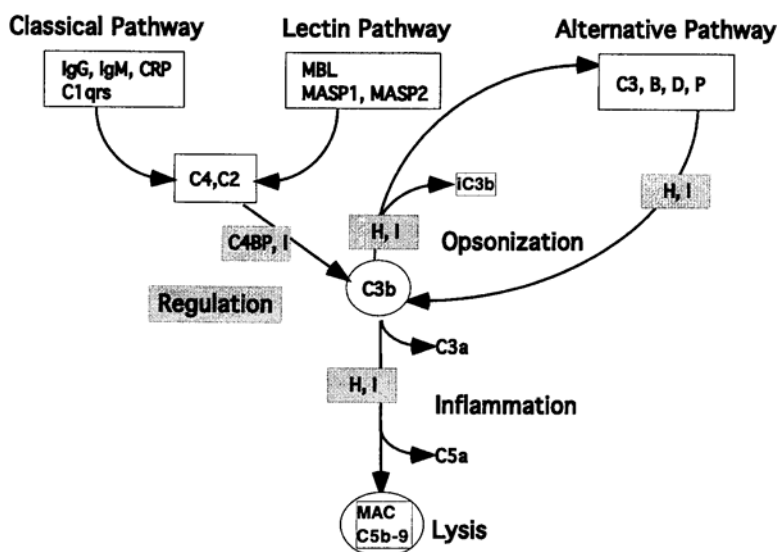

Figure 3. Three pathways of complement cascade activation. Reprinted from Microbes and Infection, Vol 1, Mold C. Role of complement in host defense against bacterial infection. Pages 633-638, Copyright (1999), with permission from Elsevier.

Acquired or genetic deficiency in any of these complement pathways predisposes individual to at increased risk for systemic meningococcal infection, with the preponderance of meningococcal infections among individuals with C3 (C3, factors H and I), alternative pathway (properdin, factor D), and especially, late complement component deficiencies (LCCD; C5 to C9) (Ross and Densen 1984). With the exception of C5 deficiency,

deficiencies in any other components (C6 through C9) of the terminal pathway predispose individual to meningococcal infections solely due to inability to assemble the MAC into bacterial membrane and hence, to express complement mediated serum bactericidal activity.

2.3.3 Serum bactericidal activity (SBA)

Due to inverse relationship between the age-related decrease in the incidence of meningococcal disease with the acquisition of SBA (Goldschneider *et al.* 1969a) and the preponderance of neisserial infections among patients with LCCD (Figueroa *et al.* 1993), SBA has been considered as the functional requirement of utmost importance for protection against meningococcal disease. Sialic acid containing encapsulated meningococci, in contrast to their un-sialylated, un-encapsulated counterparts, activate complement poorly and are therefore relatively resistant to bactericidal activity of normal human serum (Vogel *et al.* 1997, Kahler *et al.* 1998). Thus, specific antibodies and hence, the recruitment of the classical pathway is necessary for lysis of encapsulated organisms. IgG antibodies to serogroup A and C capsular PS (Goldschneider *et al.* 1969b, Williams *et al.* 2003) as well as certain OMPs, especially the PorA and OpcA proteins, seems especially efficient with this functional respect (Saukkonen *et al.* 1987, Rosenqvist *et al.* 1993) and mouse antibodies against these proteins has been further shown to be highly protective *in vivo* in experimental animal models of meningococcal infection ((Saukkonen *et al.* 1987, Saukkonen *et al.* 1989, Toropainen *et al.* 2001) and our unpublished observations). The mechanisms by which specific antibody augments bacterial lysis are not fully understood but for PorA antibody this seems to be due to re-targeting of MAC from non-lytic to lytic loci rather than an increase in the total amount of MAC formed (Drogari-Apiranthitou *et al.* 2002).

2.3.4 Opsonophagocytic activity (OPA)

Besides SBA, several reports suggest that also OPA is an important defense mechanism against meningococcal infections, especially those caused by serogroup B organisms (Ross *et al.* 1987, Schlesinger *et al.* 1994, Platonov *et al.* 1998). While SBA is dependent on the deposition MAC to bacterial membranes through activation of the whole complement cascade, IgG-mediated phagocytosis is not. IgG-mediated phagocytosis is however amplified by complement activation but only requires deposition of opsonically active C3 split products

(C3b, iC3b, C3dg) on the bacterial surface. IgG and deposited C3 fragments can therefore function in concert as opsonins, targeting the invading pathogen for ingestion and killing by professional phagocytes through binding to Fc (FcR) and complement (CR) receptors. Increased OPA by polymorphonuclear leukocytes (PMNLs) has been shown in human sera taken at convalescence and after vaccination with serogroup B outer membrane vesicle (OMV) vaccine (Roberts 1970, Halstensen *et al.* 1984, Sjursen *et al.* 1987, Halstensen *et al.* 1989, Lehmann *et al.* 1999). As for SBA, antibody to both capsular as well as sub-capsular antigens (Roberts 1970, Halstensen *et al.* 1984, Lehmann *et al.* 1999) seems important with this functional respect. The relative contribution of antibody induced complement-mediated bacterial lysis and phagocytosis to host immunity against meningococcal infections remains open.

2.3.5 Effect of antibody specificity on functional activity

To opsonize or initiate complement-mediated bacterial lysis, the first step is the binding of antibody to bacterial surface. As the main and outermost surface component, the capsular PS is the main target for protective antibodies against encapsulated bacteria, including meningococcus.

For serogroup A and C meningococci, the importance antibodies to capsular PS as the mediators of SBA and OPA has been clearly shown (Goldschneider *et al.* 1969b, Roberts 1970, Andreoni *et al.* 1993, Schlesinger *et al.* 1994, Williams *et al.* 2003), and vaccines based on either plain PS or PS conjugated to a protein carrier are highly effective against disease caused by these serogroups (Artenstein *et al.* 1970, Wahdan *et al.* 1973, Peltola *et al.* 1977, Ramsay *et al.* 2001). This does not hold true for serogroup B meningococci against which SBA and OPA has been attributed to be primarily against non-capsular antigens (Zollinger and Mandrell 1983, Williams *et al.* 2003), and the purified capsular PS of which is poorly immunogenic (Wyle *et al.* 1972). Nevertheless, antibodies to B-PS, predominately the IgM isotype, are naturally present in the majority of the adult population (Leinonen and Frasch 1982, Granoff *et al.* 1995). Specific immune responses to B-PS have been observed in the majority of adults and in 30% of children recovering from serogroup B meningococcal disease (Griffiss *et al.* 1984, Granoff *et al.* 1995, Andersen *et al.* 1997). Due to the relatively low avidity (Mandrell and Zollinger 1982) and the poor bactericidal activity of anti-B-PS

antibodies, especially in the presence of human complement (Zollinger and Mandrell 1983), their contribution to protective immunity against serogroup B meningococci has been challenged.

2.3.6 Effect of antibody class and subclass on functional activity

Not all antibody classes and subclasses are equally effective in mediating bacterial lysis or phagocytosis. Of the five immunoglobulin-classes in humans (IgG, IgM, IgD, IgA, IgE), IgG and IgM constitute the bulk of serum antibodies and play the most important role in protection against systemic meningococcal disease.

IgM antibodies constitute the major component of natural antibodies and are the first class of antibodies produced in a primary response to antigen. Due to their pentavalent structure, they are extremely potent activators of complement and hence mediate both efficient SBA and/or OPA by binding to complement receptors on fixed and peripheral phagocytes and possibly also by binding to more recently discovered IgM receptors (Fcα/μR) on macrophages (Shibuya *et al.* 2000).

Of the four human IgG subclasses (IgG1-4) in humans, IgG1 and IgG3 are the predominant subclasses produced against protein antigens such as serogroup B OMV vaccines (Wedege and Michaelsen 1987, Sjursen *et al.* 1990, Naess *et al.* 1999). They are potent complement activators and interact with all known three human FcγRI-III receptors, thereby mediating both efficient SBA and phagocytosis. IgG2, the main subclass produced by B-cells upon polysaccharide vaccination and capable of binding effectively to FcγRII receptor only, has been reported to be effective at high epitope density only while IgG4 is relatively ineffective with both functional respects (Aase and Michaelsen 1994).

To study the effect of antibody isotype on SBA and OPA, a panel of mouse-human chimeric Mabs of all the four human IgG subclasses with identical variable (V) genes against the P1.16 epitope on PorA protein has recently been generated and characterized for their effector functions *in vitro* (Vidarsson *et al.* 2001). While the IgG1-3 subclasses mediated efficient bacterial lysis (relative activity IgG1 = IgG3 > IgG2) and phagocytosis (relative activity IgG3 > IgG1 >> IgG2), IgG4 was unable to do so. How these differences in functional activities *in vitro* are reflected to protection *in vivo* is not known.

2.4 Meningococcal vaccines

Since the recognition of the importance of humoral immunity for protection against meningococcal disease, attempts to prevent this devastating disease through vaccination has been practiced for nearly 100 years.

2.4.1 Early vaccines

With the encouraging results obtained by whole-cell vaccines against typhoid fever, the first efforts to develop vaccines against meningococcal disease using similar approach began as early 1910s (reviewed in (Frasch 1995)). The field trials with these early vaccines based on whole, heat-killed bacteria yielded mixed but generally poor results. Further, side effects, probably caused by high amount of endotoxin (LOS), were common and often severe. Thus, serum therapy (reviewed in (Casadevall and Scharff 1994)) remained as the only choice for treatment to late 1930s until the newly discovered antibiotics sulfonamide and, later on, penicillin replaced it.

2.4.2 Capsular polysaccharide (PS) vaccines

During the Second World War, several outbreaks caused by serogroup A and C meningococci among army recruits prompted early attempts to produce vaccines from purified capsular PS (reviewed in (Frasch 1995)). Three major breakthroughs had accounted the interest in capsular PS as the vaccine candidate. The first was the demonstration (Rake 1933), and later on, the purification and chemical characterization (Scherp and Rake 1935), of the capsular PS from freshly isolated meningococcal isolates. The second was the description of the first reliable and reducible animal model of meningococcal infection model (Miller 1933) that allowed the protective activity of experimentally raised meningococcal anti-sera to be reliably evaluated (Miller and Castles 1936). Finally, using this model, Scherp and Rake demonstrated that the protection afforded by anti-serogroup A meningococcal horse serum was directly proportional to the amount of anti-capsular PS antibody it contained (Scherp and Rake 1945). With the encouragement of these studies and the contemporary success in the development of pneumococcal PS vaccines (MacLeod *et al.* 1945), the first human studies with meningococcal vaccines based on purified capsular PS were conducted in 1940s (Kabat *et al.* 1945). However, the results were not satisfactory, and with the beginning of antibiotic era, the

interest in meningococcal research and vaccine development waned until the first sulfonamide resistant strains emerged in early 1960s.

With the first outbreaks caused by sulfonamide resistant strains among the U.S. army recruits during the Vietnam War (Millar *et al.* 1963), the development of first consistently immunogenic meningococcal vaccines based on capsular PS started at the Walter Reed Army Institute of Research at late 1960s. A major breakthrough in meningococcal vaccines development took place when Goldschneider et al. (Goldschneider *et al.* 1969a, Goldschneider *et al.* 1969b) in a series of seminal studies indicated the importance bactericidal anti-capsular antibody for protection and developed a method for the purification of capsular PS in a high molecular weight form (Gotschlich *et al.* 1969); to be consistently immunogenic in man, PS must have molecular weights > 100 000. The first clinical trials with the new, high molecular weight serogroup C capsular PS vaccine conducted in late 1960s to early 1970s among military recruits showed an excellent efficacy (about 90%) which, as expected, was limited serogroup C meningococci (Artenstein *et al.* 1970, Gold and Artenstein 1971). Later on, using same purification method for serogroup A meningococci, successful efficacy trials with serogroup A PS vaccine were conducted in epidemic situations in Finland (Peltola *et al.* 1977) and Africa (Wahdan *et al.* 1973), leading to international licensing of serogroup A and C capsular PS based vaccines.

Currently, there are two types of PS vaccines, the bivalent serogroup A/C and the tetravalent A/C/Y/W135, in routine clinical use for controlling outbreaks and epidemics caused by serogroups covered by them. A drawback of PS vaccines limiting their utility in routine childhood immunization programs has been their limited efficacy (of especially serogroup C) and the short duration of protection among infants and young children, primarily arising from the T-cell independent nature of the immune response that PS antigens induce. A T-cell dependent immune response, and hence, induction of immunological memory, can be achieved through conjugation of PS antigen to a protein carrier. Thus, new generation glyco-conjugate vaccines have and are currently being developed using the same, successful principal as was used to develop effective childhood vaccines against meningitis caused by *Haemophilus influenzae* type B (reviewed in (Mäkelä and Käyhty 2002)) and which led to nearly complete disappearance of this disease in countries that incorporated it to infant vaccination schedule. In 1999, UK became the first country that introduced meningococcal

group C vaccine into national childhood immunization program, followed by several European countries including Ireland, Iceland, Luxemburg, the Netherlands and Spain (EU-IBIS 2002). More recently, a new quadrivalent serogroup A/C/Y/W135 polysaccharide diphtheria toxoid conjugate vaccine was licensed in the U.S. (Mitka 2005).

No PS based vaccine exists to prevent disease caused by serogroup B meningococci. By contrast to serogroup A and C PS, the purified B-PS is only poorly immunogenic (Wyle *et al.* 1972), even when conjugated to carrier proteins such as tetanus toxoid (Jennings and Lugowski 1981). As stated earlier, this has been attributed to antigenic similarity of the B-PS to sialic acid moieties in human tissues (Finne *et al.* 1983b). To overcome the poor immunogenicity and the concerns of safety raised over B-PS based vaccines (Finne *et al.* 1983b), Jennings *et al*. substituted the *N*-acetyl groups of the sialic acid residues with *N*-propionyl groups prior to its conjugation to a carrier protein (Jennings *et al.* 1986). While promising experimental animals (Jennings *et al.* 1986, Ashton *et al.* 1989, Granoff *et al.* 1998), including non-human primates (Fusco *et al.* 1997), such vaccines have failed to induce a functional antibody responses in man (Bruge *et al.* 2004). Further, even when using *N*-propionyl conjugates, a subset of antibodies show auto-reactivity with host polysialic acid (Granoff *et al.* 1998). Considerable attention has therefore been given to non-capsular antigens, mainly OMPs either in the form of outer membrane vesicles (OMVs) (Fredriksen *et al.* 1991, Sierra *et al.* 1991, Peeters *et al.* 1996) or complexes (Boslego *et al.* 1995) or more recently, purified recombinant proteins (Muttilainen *et al.* 1995, Martin *et al.* 1997, West *et al.* 2001), as alternative strategies for serogroup B vaccine development.

2.4.3 Serogroup B outer membrane vesicle (OMV) vaccines

Attempts to develop vaccines against serogroup B disease have been ongoing since the late 1970's; none has yet been fully successful (reviewed by (Frasch 1995)). While the protective efficacy of single component vaccines, such as diphtheria toxoid, tetanus toxoid, pneumococcal and meningococcal serogroup A and C capsular PS, has often been very good, for serogroup B vaccine development this approach was not rational due to lack of knowledge on the specificity of potentially protective sub-capsular antigens. Thus, vaccines based on multiple rather than single bacterial component were considered the most relevant choice.

The first attempts to develop vaccines against serogroup B disease were based on relatively crude OMP preparations from which majority of the LOS responsible for side effects was removed by extraction of bacterial cells with appropriate detergents (Frasch and Robbins 1978). The OMPs were then separated from detergents with ethanol precipitation and subsequently re-suspended in sodium chloride. While promising in animal studies (Frasch and Robbins 1978, Craven and Frasch 1979), the vaccines failed to induce protective immune response in man (Frasch *et al.* 1982); this was later on attributed to the loss of protein tertiary structure due to precipitate nature of the preparations (Frasch and Peppler 1982).

The next step was then to try producing a soluble vaccine in which the proteins would be displayed in more native conformation, and hence, capable of inducing protective immune response. When grown in liquid culture, meningococci release into medium so called blebs, i.e. outer membrane vesicles (OMVs) complete with outer membrane proteins, LOS and lipids in their native form (DeVoe and Gilchrist 1973). The OMVs are relatively simple to purify from liquid cultures or to produce artificially by extracting bacterial cells with suitable detergents. With the subsequent, selective depletion of LOS by appropriate detergent treatment, soluble OMV vaccines with satisfactory safety profile and immunogenicity can be relatively easily gained (Frasch and Peppler 1982, Frasch *et al.* 1982); adsorption to aluminum salts (Frasch *et al.* 1988) or non-covalent complexing to capsular PS (Peppler and Frasch 1982) was further shown to increase OMV vaccine immunogenicity. Since then, a number of efficacy trials with serogroup B OMP vaccines, composed mainly of class 1 (PorA), class 2/3 (PorB), and class 5 major OMPs, have been conducted in response to prolonged serogroup B epidemics, with variable results (Table 1).

2.4.4 Efficacy trials of serogroup B OMP and OMV vaccines

The first efficacy trial with a serogroup B OMP vaccine was conducted in 1981 in Cape Town, South Africa where 4 440 children aged from 4 months to 5 years were enrolled (Frasch *et al.* 1983). The vaccine was based on OMPs prepared from an un-encapsulated mutant of the group B serotype 2a strain M986 complexed to an equal amount of purified high molecular weight group B capsular PS and contained no adjuvant. While immunogenic in all age groups, the number of study participants was too small to draw definite conclusion

about vaccine efficacy (2 cases in OMP vaccine versus 4 cases in serogroup A/C vaccine group) (Frasch *et al.* 1983).

The second vaccine efficacy trial was conducted in Cuba in 1987-1989 (Sierra *et al.* 1991). The study vaccine (VA-MENGOC-BC™), prepared at the Finlay Institute, Havana, Cuba, was composed of LOS depleted OMVs extracted from a clinical disease isolate (Cu385, B:4:P1.19,15) representative of the local epidemic that was subsequently enriched with un-characterized "high molecular weight protein complexes" (Sierra *et al.* 1991). This preparation was subsequently conjugated non-covalently in 1:1 proportion to serogroup C capsular PS, and finally absorbed to aluminum hydroxide. In this double-blind, placebo controlled, cluster randomized efficacy study, about 106 000 10-14 years old students from 197 boarding schools were enrolled. During 16 months of follow-up, 4 cases occurred among the vaccine recipients compared to 21 cases among those given placebo, and a good efficacy of 83% (95% CI 42-95) against serogroup B disease was detected (Sierra *et al.* 1991). In an attempt to control a prolonged serogroup B epidemic in Brazil caused by a variety of serogroup B strains including the B:4:P1.15 phenotype, two large separate case-control studies with Cuban vaccine were subsequently conducted in Sao Paolo (de Moraes *et al.* 1992) and Rio de Janeiro (Noronha *et al.* 1995). With a total of 4 million children aged 3 months to 9 years enrolled, an efficacy of about 70% was detected for children 4 years or older but was much lower for younger children and absent in infants (de Moraes *et al.* 1992, Noronha *et al.* 1995). There was also a trend to better protection during the first than the second half-year observation period, suggesting that protection could be of short duration (Noronha *et al.* 1995).

The next efficacy trial was conducted in Iquique, Chile, from 1987 to 1989 (Boslego *et al.* 1995). The vaccine, developed at the Walter Reed Army Institute of Research (WRAIR), Washington, DC, differed from the Cuban and Norwegian (see below) OMV vaccines in several respects (Boslego *et al.* 1995). First, in the extraction of OMPs from a clinical disease isolate (8257, B:15:P1.3) representative of the Iquique outbreak, a zwitterionic detergent Embigen BB rather than deoxycholate was used. Second, the OMPs were not in the form of vesicles but consisted primarily of multimeric membrane subunits that were essentially free of LOS. Finally, the vaccine contained no class 5 or other low molecular weight OMPs. Similarly to the Cuban vaccine, the OMPs were conjugated non-covalently to serogroup C

capsular PS, and finally absorbed to aluminum hydroxide. In this double-blind, placebo controlled, randomized efficacy trial, approximately 40 000 volunteers of ages of 1-21 years were enrolled (Boslego *et al.* 1995). After 20 months of follow-up a good efficacy of 70% (95% CI 3-93) was detected for older children (≥5 years); in younger children (1-4 years) no protection was observed (Boslego *et al.* 1995).

In a double-blind, placebo controlled, cluster randomized efficacy study conducted in Norway in 1988-1991, around 172,000 secondary school students aged 14-16 years were enrolled (Bjune *et al.* 1991a, Bjune *et al.* 1991b). The study vaccine (MenBvac™), developed at the Norwegian Institute of Public Health (NIPH), Oslo, Norway, was composed of LOS depleted OMVs extracted from a clinical disease isolate (44/76, B:15:P1.7,16) representative of the local epidemic, absorbed aluminum hydroxide (Fredriksen *et al.* 1991). In contrast to the Cuban and WRAIR vaccines it contained no meningococcal capsular PS. After a relatively long follow-up period of 29 months, an efficacy of 57% (lower confidence limit 28%) was observed (Bjune *et al.* 1991a, Bjune *et al.* 1991b). Similar to the efficacy trial with the Cuban vaccine in Rio de Janeiro (Noronha *et al.* 1995), there was evidence of better protection during the first than the second half observation period (Holst *et al.* 2003). In retrospective calculations, an efficacy of 87% was calculated for the first 10-month observation period whereas it was as low as 30% for the last 10 months (Holst *et al.* 2003).

Currently, a "tailor made" OMV vaccine (MeNZB™) (Holst *et al.* 2005, Oster *et al.* 2005) is undergoing a clinical trial in New Zealand (Ameratunga *et al.* 2005) to combat to a prolonged and intense group B meningococcal disease epidemic (Baker *et al.* 2001). The vaccine was produced in collaboration of NIPH with Chiron (Sienna, Italy) from a local epidemic strain B:4:P1.7b,4 (Martin *et al.* 1998).

Table 1. Summary of completed efficacy trials of serogroup B OMP or OMV vaccines.

Country or region (ref.)	Year	Vaccine	Formulation	Study type	Sample	Observation time	Age group	Efficacy (95% CI%)
Cape Town, South-Africa (Frasch *et al*. 1983)	1981		OMPs from strain M986 (B:2a) + B-PS. No adjuvant	Randomized, double-blind, controlled trial	4 440	1981	4 months-5 years	Too few cases
Cuba (Sierra *et al*. 1991)	1987-1989	Cuban (Finlay Institute, VA-MENGOC-BC™)	OMVs from strain Cu385 (B:4:P1.19,15) + C-PS. Aluminum hydroxide adjuvant	Randomized, double-blind, controlled trial	106 251	16 months	10-14 years	83% (42-95%)
Sao Paolo, Brazil (de Moraes *et al*. 1992)	1990-1991	Cuban (Finlay Institute, VA-MENGOC-BC™)	OMVs from strain Cu385 (B:4:P1.19,15) + C-PS. Aluminum hydroxide adjuvant	Case-control study	112 cases 409 controls	1990-1991	<24 months 24-47 months ≥ 4 years	-37% (<-100-73%) 47% (-72-84%) 74% (16-92%)
Rio de Janeiro, Brazil (Noronha *et al*. 1995)	1990	Cuban (Finlay Institute, VA-MENGOC-BC™)	OMVs from strain Cu385 (B:4:P1.19,15) + C-PS. Aluminum hydroxide adjuvant	Case-control study	275 cases 279 controls	12 months	6-23 months 24-47 months ≥ 4 years	41% (-96-82) 14% (-165-72%) 71% (34-87%)
Iquique, Chile (Boslego *et al*. 1995)	1987-1989	WRAIR	OMPs from strain 8257 (B:15:P1.3) Aluminum hydroxide adjuvant	Randomized, double-blind, controlled trial	40 811	20 months	1-4 years 5-21 years	-39% (<-100-77%) 70% (3-93%)
Norway (Bjune *et al*. 1991)	1988-1991	Norwegian (NIPH, MenBvac™)	OMVs from strain 44/76 (B:15:P1.7,16) Aluminum hydroxide adjuvant	Randomized, double-blind, controlled trial	171 800	29 months	14-16 years	57% (lower 95% CI 28%)

While not satisfactory with all respects, the main importance of these efficacy trials conducted with two-dose schedules was the demonstration that vaccines based on non-capsular antigens do provide significant protection against serogroup B disease (50% to 80%), encouraging further vaccine development in this field to improve their immunogenicity and the persistence of protection especially amongst the youngest children who are most vulnerable to serogroup B disease. As the protection seemed relatively short-lived (Noronha *et al.* 1995, Holst *et al.* 2003), it has been suggested that a third dose given 6-8 months after the primary immunization series could be beneficial (Frasch 1995).

2.4.5 Immunogenicity trials of serogroup B OMV vaccines

One important issue hampering the development of serogroup B meningococcal vaccines has been the lack of reliable laboratory correlates or surrogates for protective immunity, necessitating the use of long-lasting, laborious, and expensive efficacy trials. Although the antibody responses in connection with these efficacy trials have been analyzed extensively (Høiby *et al.* 1991, Rosenqvist *et al.* 1991, Sierra *et al.* 1991, Holst *et al.* 2003, Wedege *et al.* 2003), there is still much uncertainty about the specificity and functional mechanisms of antibodies providing protection against serogroup B disease. In particular, despite the good predictive value of SBA for the protective efficacy of serogroup A and C capsular PS based vaccines the importance of SBA for protection against serogroup B disease remains partly open. Thus, additional immunogenicity trials with the Norwegian and the Cuban OMV vaccines have been conducted later in Iceland (Perkins *et al.* 1998) and Chile (Tappero *et al.* 1999), assessing immune responses in different age groups (infants, toddlers, and adults in Chile and teenagers in Iceland).

In the randomized, double-blind, placebo-controlled trial with the Norwegian and the Cuban OMV vaccines carried out among Icelandic teenagers in 1992-93, the main focus was at the evaluation of SBA and antibody concentrations measured by enzyme immunoassay (EIA) as potential correlates for vaccine efficacy (Perkins *et al.* 1998). As described above, both vaccines had been previously shown to be efficacious for older children and adults in large separate efficacy trials (Bjune *et al.* 1991b, Sierra *et al.* 1991). In contrast to results from

these efficacy trials, the proportion of SBA and EIA responders, defined as individuals with a 4-fold rise in bactericidal antibody titer or anti-OMV IgG antibody level compared with pre-vaccination level, was found to be generally lower among the Cuban vaccine recipients than the Norwegian vaccine recipients (Perkins *et al.* 1998). Six weeks after the second dose, 25% and 54% of the Cuban vaccine recipients, and 71% and 74% of the Norwegian vaccine recipients showed a response in SBA and EIA, respectively, against their homologous vaccine strains. Based on these results it was concluded that SBA and EIA activities might not be optimal correlates for serogroup B OMV vaccine efficacy (Perkins *et al.* 1998).

Another issue related to vaccines based on OMPs from a single meningococcal strain that remains to be settled is the specificity of antibodies and hence, the extent of protection they induce. In the Brazilian and Norwegian efficacy trials, no evidence for serosubtype- or strain-restricted protection for the Cuban and the Norwegian OMV vaccines was detected (de Moraes *et al.* 1992, Wedege *et al.* 1999), suggesting that both vaccines can provide some protection against heterologous strains as well. In line with these results, in the Chilean and Icelandic immunogenicity trials, adults, teenagers and children aged 2 to 4 years were able to develop SBA response against both the homologous, i.e. vaccine type, and heterologous strains (Perkins *et al.* 1998, Tappero *et al.* 1999) though against heterologous strains much lower responses were detected. This was in contrast to infants younger than 1 year among whom SBA responses were mainly directed to homologous PorA protein and no post-vaccination SBA response against the heterologous strains was detected (Tappero *et al.* 1999).

To overcome possible limitations of OMV vaccines arising from the variability of PorA protein, yet exploiting its good immunogenicity and ability to induce bactericidal antibody responses, a hexavalent PorA vaccine (HexaMen) has more recently been developed at the Netherlands Vaccine Institute, NVI (formerly National Institute of Public Health and the Environment, RIVM), in the Netherlands. This vaccine is composed of OMVs from two genetically engineered trivalent strains, each expressing three different PorA proteins (Claassen *et al.* 1996), together covering approximately 80% of the prevalent strains in the UK (Cartwright *et al.* 1999). Clinical phase I and II studies have shown this vaccine to be safe and immunogenic in infants, toddlers and school children, and SBA responses against all of the six PorA subtypes included in the vaccine have been detected (Cartwright *et al.* 1999) (de Kleijn *et al.* 2000). However, multiple doses of vaccine were required to induce

significant rise in SBA and differences were found in the magnitudes of SBA responses to different PorAs, suggesting intrinsic differences in their immunogenicity. To provide even broader protection, a nonavalent vaccine, containing the nine most common PorA subtypes in the industrialized countries, is currently being developed in collaboration of NVI with Wyeth (NJ, USA).

More recently, a new experimental approach to broaden immunity of the conventional OMV vaccines has been described. In this approach, sequential immunization with three doses of microvesicles (MVs) or OMVs prepared from three antigenically different strains rather than one meningococcal strain is used (Moe *et al.* 2002). The rationale behind this approach is that sequential immunization with MVs or OMVs prepared from antigenically different meningococcal strains would direct the immune response from immunodominant, serotype or serosubtype specific one to more conserved, broadly protective antigens that normally are poorly immunogenic when repeated injections are given with OMVs prepared from one strain.

2.4.6 Other serogroup B vaccine candidates

In addition to major OMPs in the form of OMVs, a variety of methods have been developed to express a number of major OMPs in heterologous expression systems. After purification and refolding in the presence of artificial membranes (liposomes) or appropriate detergent micelles, good antibody responses in mice against PorA (Muttilainen *et al.* 1995, Idänpään-Heikkilä *et al.* 1996, Ward *et al.* 1996, Christodoulides *et al.* 1998), PorB (Wright *et al.* 2002), and OpcA (Jolley *et al.* 2001) proteins with bactericidal and infant rat protective activity have been gained, indicating the feasibility of these methods. The main advantage of these approaches is that the vaccine antigens are being presented without of irrelevant bacterial components, thus directing the immune response to epitopes thought to induce protective antibodies also in humans.

Besides major OMPs, either in the form of OMVs or recombinantly produced, purified and refolded antigens, also LOS (Plested *et al.* 1999, Plested *et al.* 2003) and a number minor OMPs have been considered as vaccine antigens. These include the constitutively expressed OMP of unknown function, Neisserial surface protein A (NspA) (Martin *et al.* 1997, Moe *et*

al. 2001), and a number environmentally regulated proteins thought to be essential for bacterial survival through their involvement in iron acquisition pathways, such as transferrin-binding proteins TbpA and TbpB (Schryvers and Morris 1988, Ala'Aldeen *et al.* 1994b), a 70 kDa protein FetA (formerly known as FrpB) (Ala'Aldeen *et al.* 1994a) and a 37 kDa ferric binding protein FbpA (Mietzner *et al.* 1984).

More recently, as a part of completed meningococcal serogroup B genome sequence project (Tettelin *et al.* 2000), a large number of novel proteins have been discovered (Pizza *et al.* 2000). Some of these are conserved in sequence, likely to be expressed constitutively, surface exposed on encapsulated meningococcal strains, able to elicit bactericidal, opsonic and/or animal protective antibody responses in mice, and hence, considered promising as vaccine candidates (Comanducci *et al.* 2002, Masignani *et al.* 2003, Welsch *et al.* 2003, Welsch *et al.* 2004).

The utilization of the cross-reactivity between *N. meningitidis* and *N. lactamica* (Troncoso *et al.* 2002) has also been recently explored (Oliver *et al.* 2002). Despite the lack of bactericidal antibodies, antigens from *N. lactamica* have been shown protect mice against experimental meningococcal infection (Oliver *et al.* 2002).

2.5 Search for serological correlates of protection

Besides SBA assay, a variety of other techniques, such as opsonophagocytic (Aase *et al.* 1998, Naess *et al.* 1999) and whole-blood assays for functional antibodies (Morley *et al.* 2001), non-functional assays (Naess *et al.* 1998, Perez *et al.* 2001, Wedege *et al.* 2003), and assessment of active (Sifontes *et al.* 1997) or passive protection in animal models (Infante *et al.* 1994) have been evaluated for providing additional information about the mechanisms of protective immunity or even for providing better correlates for efficacy of serogroup B meningococcal vaccines.

2.5.1 Assays to measure immunity to serogroup B meningococcus

Of the non-functional assays, the enzyme immunoassays (EIA) have been most widely used to determine not only the magnitude and persistence but also the isotype distribution of anti-meningococcal antibodies. Both OMVs containing multiple OM antigens and purified OMPs,

LOS, or capsular PS have been used as the coating antigen. In several studies, relatively good correlations between anti-OMV IgG levels and SBA titers have been found (Rosenqvist *et al.* 1988, Aase *et al.* 1995, Rosenqvist *et al.* 1995, Naess *et al.* 1999) while in others, only poor association between these two assays have been detected (Milagres *et al.* 1994, Boslego *et al.* 1995), probably reflecting the great variety how these methods are performed in different laboratories. A multi-laboratory evaluation of both assays would be needed to establish the reason for these discrepancies.

Besides antibody quantity and isotype, there is strong evidence that also other qualitative characteristics of antibodies, namely their avidity, may be important to protection against meningococcal disease (Harris *et al.* 2003a, Welsch and Granoff 2004). Recently, a modified EIA, using sodium thiocyanate as a chaotropic agent, has been described to measure the avidity of anti-OMV antibodies (Longworth *et al.* 2002, Vermont *et al.* 2002). Due to scarcity of studies measuring avidity indices (Longworth *et al.* 2002, Vermont *et al.* 2002, Vermont *et al.* 2004), its value as a marker of protective activity and immunological memory following serogroup B OMV vaccination remains to be established.

While being of great value for the detection of antibody kinetics following OMV vaccination, the EIAs do not provide information about the specificity of antibodies induced unless well-defined, purified proteins or other surface structures in right conformation are used as the target antigens. Thus, to get a more thorough picture about specificity of antibodies, an immunoblotting method capable of discriminating antibody binding to different OM antigens (OMPs and LOS) is needed (Wedege and Froholm 1986, Wedege *et al.* 1998). Due to denaturizing conditions during antigen preparing, a duplicate sample treated with appropriate detergent to partially refold the proteins and hence, to detect antibody binding also to conformation-dependent epitopes, is generally included (Wedege *et al.* 1988). In sera from patients and vaccinees antibody responses to several OM antigens are generally detected (Wedege and Froholm 1986, Rosenqvist *et al.* 1995, Wedege *et al.* 1998, Wedege *et al.* 2000). Following three doses of the Norwegian serogroup B OMV vaccine, antibody to class 1, class 5, 80kDa protein and LOS all contributed significantly to SBA against the vaccine strain (Rosenqvist *et al.* 1995).

Of the functional assays, the SBA has most widely used to assess antibody responses in large clinical trials. In this assay, meningococci are incubated with human sera in the presence of suitable human or, if applicable, animal complement, and the bactericidal activity of serum expressed as the reciprocal of serum dilution yielding ≥ 50% or 90% killing as compared to the number of target cells present before incubation with serum and complement. As the species of complement source may greatly affect the SBA titers (Zollinger and Mandrell 1983), for serogroup B SBA assay human complement is strongly recommended. By contrast to serogroup A and C SBA assays (Maslanka *et al.* 1997), the serogroup B SBA assay has not been standardized yet and slightly different protocols are used in different laboratories. This makes the inter-laboratory comparisons somewhat complicated. Since the seminal work of Goldschneider et al. (Goldschneider *et al.* 1969a, Goldschneider *et al.* 1969b), a SBA titer of 4 has been generally used as the threshold of protection. Most studies, however, have not relied on absolute SBA titers as a cut off but instead used ≥ 4-fold rise in SBA titer compared to pre-vaccination level to estimate the potential efficacy of serogroup B meningococcal vaccines. There is some evidence that SBA assay correlates with serogroup B OMV vaccine efficacy (Milagres *et al.* 1994, Boslego *et al.* 1995, Holst *et al.* 2003). However, further studies using SBA as a marker of OMV vaccine responses are required to gain a more comprehensive view of the value of this assay as predictor of both natural and serogroup B vaccine induced immunity.

In addition to SBA, it has been suggested that also OPA is an important defense mechanism against meningococcal infections, especially those caused by serogroup B organisms (Ross *et al.* 1987), and a variety of techniques have been developed to measure this activity. In these assays, live or killed bacteria are first opsonized with human sera in the presence or absence of endogenous or exogenous complement source. After incubation with effector cells, usually freshly isolated human PMNLs, the amount of phagocytosis is measured by viable counts (Schlesinger *et al.* 1994), flow cytometry (Sjursen *et al.* 1989, Lehmann *et al.* 1998) or chemiluminescence (Halstensen *et al.* 1989, Sjursen *et al.* 1992). Increased OPA against meningococci of different serogroups has been detected following vaccination and disease (Roberts 1970, Halstensen *et al.* 1984, Sjursen *et al.* 1987, Halstensen *et al.* 1989, Schlesinger *et al.* 1994, Lehmann *et al.* 1999).

A whole blood assay (WBA) has also been developed to measure the total killing capacity of human blood after infection or vaccination (Ison *et al.* 1995, Ison *et al.* 1999). In this assay heparinized venous blood collected from subjects is inoculated with meningococci, and after incubation, the percentage of bacterial killing given relatively to initial bacterial load. It has been suggested that this assay is more sensitive than the SBA, with WBA activity detected in some individuals lacking SBA (Ison *et al.* 1999). However, the WBA is unlikely to be suitable for large scale evaluation of clinical samples because of the requirement for large volumes of fresh blood and difficulties in the standardization of the assay.

2.5.2 Animal models of meningococcal infection

An alternative mean to study the protective efficacy of existing and candidate vaccines would be an animal model, in which the protection would depend on the same effector mechanisms as in human disease. Such a model should cover both the complement-mediated killing of the bacteria and their phagocytosis, as well as complex interactions of multiple humoral and cellular factors of the host.

Since the first isolation of causative agent of meningococcal meningitis at the late 19th century, a variety of animal species from chicken embryos to monkeys and different routes of infection (intranasal, intraperitoneal, and intrathecal) have been tried to establish a reproducible experimental meningococcal disease in animals, with varying success (reviewed in (DeVoe 1982, Arko 1989)). Of the animal models evaluated, the mouse model requiring exogenous iron load (Gorringe *et al.* 2001) and the infant rat model without enhancers (Saukkonen 1988) have proven most useful and used widely for active and/or passive protection studies.

Mouse models

Since its first description in 1933 (Miller 1933), the iron-dependent mouse infection model with intraperitoneal (i.p) bacterial inoculation has survived with only minor modifications to the present time. In this model, log-grown meningococci are mixed with exogenous iron source to enhance bacterial virulence, originally hog gastric mucin (Miller 1933), later iron dextran (Holbein *et al.* 1979) or human transferrin (Holbein 1981a), and 10^5-10^7 colony forming units (cfu) of appropriate organisms injected i.p. to 6-8 weeks old mice (Gorringe *et*

al. 2001). This model seems to detect differences in virulence between carrier and disease strains (Holbein *et al.* 1979, Holbein 1981b) and has been used widely in active (Sifontes *et al.* 1997, Oftung *et al.* 1999) and passive (Moreno *et al.* 1983, Brodeur *et al.* 1985, Perez-Ramirez *et al.* 1997) immunization studies. In protection studies, reduction in mortality rates and/or bacteremia levels has been used as the end point.

Mouse colonization models with intranasal (i.n.) inoculation of infant (Salit *et al.* 1984, Mackinnon *et al.* 1992) or adult mice (Yi *et al.* 2003) have also been described. In these models meningococci are mixed with exogenous iron source and 10^7-10^{10} cfu of appropriate organisms given i.n. to mice; an i.p. injection of iron dextran is given prior to and after infection to further enhance infection. While i.n. models appear useful for studying the early pathogenesis of infection and the role of mucosal immunity, they are less suitable for large-scale assessment of protection because of the low and variable rate of invasive disease despite consecutive iron injections. Further, by contrast to human host, a preceding lung colonization was necessary for the development of bacteremia (Mackinnon *et al.* 1992).

More recently, an i.n. mouse model without exogenous iron supplementation has been described. In this sequential, influenzae A virus (IAV)-*N. meningitidis* serogroup C infection model, adult BALB/c mice are infected i.n. with mouse-adapted IAV, followed by co-infection with meningococci seven days later (Alonso *et al.* 2003). Fatal meningococcal pneumonia and bacteremia occurred in mice challenged at seven but not ten days after IAV infection. Susceptibility to lethal infection correlated with IAV induced peak in interferon-γ production, suggesting that transient IAV-induced modulation of host innate immunity predisposed mice to systemic meningococcal infection (Alonso *et al.* 2003).

To more accurately mimic host-pathogen interaction in the human host, a meningococcal infection model in transgenic mice expressing human CD46 with human-like tissue specificity (Mrkic *et al.* 1998) has been recently developed (Johansson *et al.* 2003). As described earlier, in the human host, the expression CD46 is thought to be essential for meningococcal virulence by serving as a receptor for pilus-mediated attachment of encapsulated meningococci to epithelial and endothelial cells of nasopharynx and blood-brain barrier (Källström *et al.* 1997). While clearly enhancing the development of bacteremia and meningitis following i.p. infection, after i.n. infection with piliated serogroup W135

meningococci only a slight although significant increase in mortality rates was observed, with 15% (4/27) of the CD46 transgenic mice compared to none (0/27) of the non-transgenic C57BL/6 mice used as the control succumbing to infection. Expression of pili was necessary for the virulence of bacteria as non-piliated isogenic strain caused no signs of disease in either mouse strain. Though the colonization rates were not reported, the authors suggested that this only relatively modest increase in mortality rates was due to the expression of CD46 mainly on the basolateral rather than apical surface of the respiratory epithelial cells (Johansson *et al.* 2003), and hence, opposing the situation in the human host (Varsano *et al.* 1995, Sinn *et al.* 2002). Pre-treatment of animals with antibiotics to diminish normal bacterial flora prior to i.n. inoculation was also necessary, all un-treated CD46 transgenic mice surviving infection. While having potential as a more natural model for human infection, an increase in infection rate is clearly needed for protection studies.

The major advantage of mouse models is that a number of different inbred strains with deficiencies in innate and adaptive immunity are available to elucidate host-pathogen interactions and the mechanisms protection. However, due to extremely low lytic activity of mouse serum relative to humans and rats (Ong and Mattes 1989), and the multiple effects of both iron overload (Mencacci *et al.* 1997, Hor *et al.* 2000, Walker and Walker 2000) as well as dextran (Sahu *et al.* 1994) on phagocyte and complement function, the relevance of mouse models for assessment of protection remains to be fully elucidated.

Infant rat model

With the success in establishing reproducible infection models for other encapsulated gram-negative bacteria in infant rats, namely *Haemophilus influenzae* type b and *Escherichia coli* K1, efforts to develop such model also for meningococci began at KTL in late 1980's, with successful results (Saukkonen 1988).

In this model, 10^3-10^7 cfu of log grown organisms are injected intraperitoneally to 5-7 day-old infant rats, and the development of bacteremia and meningitis is followed by cultivating blood and cerebrospinal fluid samples taken at appropriate time-points (Saukkonen 1988, Welsch *et al.* 2003). Meningococci of all the major serogroups A, B, and C, are infective (Saukkonen 1988, Welsch and Granoff 2004, Welsch *et al.* 2004). The course of infection is reproducible and predictable: a localized peritoneal inflammation is followed by invasion of the bacteria

into the bloodstream. The outcome of disease varies from transient bacteremia to lethal bacteremia and meningitis depending on the challenge strain and dose used (Saukkonen 1988). Importantly, no artificial enhancing factors such as mucin or iron compounds that may have multiple and poorly definable effects on both the bacteria and the host are needed. Though the short period of susceptibility of infant rats to infection (Salit *et al.* 1984) prevents active immunization studies, a large litter size (8-12 pups/litter) guarantees the availability of a large number of animals for short-term experiments such as the passive immunization studies. Later on, the model has been successfully used to identify antigens involved in protection and also to test the protective activity of antibodies elicited by candidate vaccines (Saukkonen *et al.* 1987, Saukkonen *et al.* 1989, Idänpään-Heikkilä *et al.* 1995, Welsch *et al.* 2003, Welsch *et al.* 2004). Like in mouse protection studies, reduction in mortality rates and/or bacteremia levels has been used as the assay end points.

3 AIMS OF THE STUDY

An important question for vaccine development was whether the infant rat infection model could also be used to test the protective activity conferred by human immune sera collected after OMV vaccination, and thus, as a surrogate assay for serogroup B OMV vaccine efficacy. Such a use would need careful validation of the assay conditions for sensitivity and repeatability. In the search for vaccine candidates against serogroup B disease, the specificity and the functional activity of natural antibodies has been less studied. Relatively little was also known about the *in vivo* functional activity of antibodies of different specificity or isotype. To this end, the aims of this thesis were:

- to adapt and standardize the infant rat protection assay for the evaluation of human sera for protective immunity to group B meningococci (I, II)
- to assess the reproducibility of the infant rat protective activity (IRPA) assay (I, II)
- to assess IRPA of sera taken before and after vaccination of Icelandic teenagers with the Norwegian and the Cuban serogroup B meningococcal OMV vaccines, and to compare the results of the IRPA assay to those obtained by SBA assay and anti-OMV EIA (II)
- to assess the specificity and functional activity of natural antibodies responsible for IRPA against 44/76-SL strain (III)
- to assess the influence of antibody specificity (capsular versus sub-capsular antibody), isotype, and the importance of complement-mediated bacterial lysis on protection in the IRPA assay (III, IV)

4 MATERIALS AND METHODS

4.1 Serum samples and monoclonal antibodies used in IRPA assays

In study I, three post-vaccination sera collected from adult volunteers vaccinated twice with the Norwegian meningococcal serogroup B OMV vaccine (Bjune *et al.* 1991b) and one normal human serum from an unvaccinated person with no history of meningococcal disease were used. These sera were selected from a large panel of sera available at the Norwegian (formerly National) Institute of Public Health (Einar Rosenqvist, NIPH, Oslo, Norway).

In study II, pre- and post-vaccination serum samples from 92 teenagers who had received two doses of either the Norwegian OMV vaccine (B:15:P1.7,16), the Cuban OMV vaccine (B:4:P1.19,15), or the serogroup A/C capsular PS control vaccine (Aventis Pasteur, Lyon, France) during a previous immunogenicity trial in Iceland (Perkins *et al.* 1998) were used. Of the 92 study participants, 20 (22%) had received the control (A/C), 37 (40%) the Norwegian, and 35 (38%) the Cuban vaccine. The sera were selected at the Centers for Disease Control and Prevention (Brian Plikaytis, CDC, Atlanta, GA) by criteria described (II) to represent a 25% sample of vaccine responders and non-responders in the original study (Perkins *et al.* 1998). Serum samples were sent frozen to National Public Health Institute (KTL, Helsinki, Finland) from CDC and stored at -20°C until analyzed.

In study III, a representative set (26/92; 28%) of pre-vaccination sera from Icelandic teenagers (Perkins *et al.* 1998) with convergent or divergent IRPA and SBA data (II) against strain 44/76-SL was used. The sera were selected on the basis of the availability of > 0.2-ml volumes and analyzed for functional and specific antibodies as described. In addition, in study III, normal human sera (NHS; n=20) collected from healthy Finnish children of four different age groups (7, 14 and 24 months, and 10 years) were used. These sera had been collected in connection with previous immunogenicity studies (Mäkelä *et al.* 2003) and stored frozen at – 20°C until used. Informed consent and approvals from the ethics committees covered the use of these sera in this study.

In study IV, a panel of mouse-human chimeric Mabs of all the four human IgG subclasses (IgG_{1-4}) with identical variable genes against the P1.16 epitope on the PorA protein

(Vidarsson *et al.* 2001) were used. The production and *in vitro* characteristics of these antibodies have been described elsewhere (Vidarsson *et al.* 2001). The parental mouse P1.16 specific IgG2a antibody MN12H2 (Jiskoot *et al.* 1991) was received from Betsy Kuipers (NVI, Bilthoven, The Netherlands).

The B-PS specific IgG2a antibody Nmb735 (formerly Mab735) (Frosch *et al.* 1985) was a gift from H-P Harthus (Dade Behring, Marburg) and was used as a positive control for protection (dose of 2 µg/pup) throughout the IRPA studies (I-IV).

4.2 Depletion of B-PS specific antibodies

NHS collected from healthy Finnish children were absorbed with aluminum hydroxide gel (Alhydrogel 2%, Brenntag Biosector, Denmark) bound B-PS to deplete B-PS specific antibodies as described (III).

4.3 Bacterial strains and growth conditions

Three *N. meningitidis* strains were used. Strain IH5341 (I) was a cerebrospinal fluid isolate of a human patient and had the phenotype of B:15:P1.7,16 (Käyhty *et al.* 1989). The mother strain 44/76 (II, III, IV) for the Norwegian OMV vaccine (Fredriksen *et al.* 1991) (44/76-SL) was first isolated by Holten from a fatal case of meningococcal disease (Holten 1979) and had a phenotype of B:15:P1.7,16. The Cuban vaccine strain Cu385 (II, III) was their local clinical isolate and had a phenotype of B:4:P1.15 (Sierra *et al.* 1991).

For IRPA studies (I-V), the strains were rat-passaged (Saukkonen 1988) 3-5 times and stored in skimmed milk at -70°C. In SBA assays performed at KTL (III, IV), rat-passaged 44/76-SL strain was used. In other assays, including SBA (I, II, III) and OPA (I, III) assays performed elsewhere, un-passaged strains were used. The expression of different surface antigens in un-passaged and rat-passaged 44/76-SL and Cu385 strains was verified by SDS-gels performed at NIPH and whole-cell EIAs preformed at KTL (Toropainen *et al.* 2001) with monoclonal antibodies described below.

Inoculum for IRPA and SBA assays performed at KTL was prepared from broth-grown, early log phase bacteria essentially as previously described (Saukkonen 1988). Inoculum SBA and

OPA assays performed elsewhere was prepared from plate-grown, log-phase bacteria as described (I (Høiby *et al.* 1991), II, III (Perkins *et al.* 1998), III (Aase *et al.* 1998).

4.4 Monoclonal antibodies used for strain characterization

Monoclonal antibodies used for bacterial strain characterization are listed in Table 2.

Table 2. Monoclonal antibodies (Mab) used for bacterial strain characterization.

Mab	Specificity	Source
Mab 95/750	Group B PS	NIBSC
8B55G9	Serotype P3.15	NIBSC
MN14G21	Serotype P3.4	NIBSC
MN14C11.6	Serosubtype P1.7	NIBSC
MN3C5C	Serosubtype P1.15	NIBSC
MN5C11G	Serosubtype P1.16	NIBSC
MN4A8B2	L3,7,9	NVI
MN43F8.10	L8	NVI
154,D-11	OpcA	NIPH

NIBSC, National Institute for Biological Standards and Control, Potters Bar, UK
NVI, Netherlands Vaccine Institute, Bilthoven, The Netherlands
NIPH, Norwegian Institute of Public Health, Oslo, Norway

4.5 Whole-cell EIA

Whole-cell EIA was performed at KTL as described (Toropainen *et al.* 2001) using heat inactivated (56 ºC, 30 min) bacteria as coating antigen and peroxidase-conjugated rabbit antibodies to mouse immunoglobulins as the second antibody (Dako A/S, Denmark).

4.6 OMV EIA

IgG antibodies to strain IH5341 (I) were measured by EIA as described (Idänpään-Heikkilä *et al.* 1995) using a meningococcal capsular PS-depleted whole-cell preparation of strain IH5341 as the coating antigen and peroxidase-conjugated rabbit antibodies to human immunoglobulin G (IgG) as the second antibody (Dako A/S, Denmark). The results were expressed as titers, i.e. reciprocals of serum dilutions giving an optical density (O.D.) of 0.3 measured at 450 nm.

Anti-OMV IgG antibody concentrations for the Icelandic study sera (II, III) were obtained from B.D. Plikaytis. As reported previously (Perkins *et al.* 1998), these data were obtained at the NIPH and the Finlay Institute, respectively, using OMVs from their respective vaccine preparations as solid-phase antigen in their OMV EIA (Rosenqvist *et al.* 1991, Ferriol Marchena *et al.* 1999). The results were expressed as arbitrary units (U/ml). A vaccine responder was defined as an individual with a ≥ 4-fold and a non-responder as an individual with a < 4-fold rise in anti-OMV IgG level.

4.7 B-PS EIA

Antibodies to meningococcal group B capsular polysaccharide (B-PS) (III) were measured by EIA essentially as described previously (Arakere and Frasch 1991), using B-PS non-covalently complexed to methylated human serum albumin as coating antigen and peroxidase-conjugated rabbit antibodies to human immunoglobulin IgG or IgM (Dako A/S, Denmark) as the second antibody. The results were expressed as titers, i.e. reciprocals of serum dilutions giving an O.D. of 0.3 measured at 450 nm. The sera were assayed starting at 1:50 dilution. Negative samples were assigned a titer of 1:10.

4.8 SBA assay

SBA data for the sera received from the NIPH (I) were obtained from E. Rosenqvist (NIPH) and for the Icelandic sera (II and III) from Dr. B.D. Plikaytis (CDC). SBA was measured at the NIPH (Høiby *et al.* 1991) and CDC (Perkins *et al.* 1998) according to their SBA assay protocols, respectively, using 25% human plasma as the exogenous complement source.

SBA of sera from Finnish children (III) or monoclonal antibodies (IV) were determined at KTL using human serum from an individual without bactericidal antibodies to strain 44/76-SL (III, IV) or serum from 5-6 day-old HsdCpb:WU (III), HsdBrlHan:WIST (IV) or PVG/OlaHsd (IV) rat pups (Harlan, The Netherlands) as exogenous complement source at a final concentration of 20%.

The results were expressed as the reciprocal of the highest serum dilution giving 50% killing of the inoculum (I, II, III) or the lowest antibody concentration giving 90% killing of the inoculum (IV). Sera with titer of <4 were considered SBA negative and those with titer of ≥4

SBA positive (I-III). A vaccine responder was defined as an individual with a ≥ 4-fold and a non-responder as an individual with a < 4-fold rise in SBA titer.

4.9 OPA assay

OPA (I, III) was measured at NIPH as respiratory burst (RB) by flow cytometry with polymorphonuclear leukocytes (PMNLs) as the effector cells and ethanol fixed (I) (Aase and Michaelsen 1994) or live 44/76-SL cells grown on plates to log phase (III) (Aase *et al.* 1998) as the target cells. Human serum without any antibody activity against the target strain was used as complement source at a final concentration of 10%. The percentage of effector cells that had undergone RB was determined, and the results expressed as the reciprocal of the highest serum dilution giving RB above the cut-off line of 15% cells. Sera with titer of < 2 were considered OPA negative and those with titer of ≥ 2 OPA positive.

4.10 Quantification of IgG by flow cytometry

Anti-meningococcal IgG antibodies were quantified (III) at NIPH with live, log grown 44/76-SL cells by flow cytometry as described (Aase *et al.* 1998). Two-fold dilutions of a reference plasma (quantified by EIA against OMVs) was used as an internal standard to create a standard curve with concentration (range 0.07-9.0 mg/ml) on the abscissa and median fluorescence intensity (MFI) on the ordinate to which the MFI of test samples were interpolated.

4.11 Immunoblotting

Immunoblotting (I and III) was carried out at NIPH as described (Wedege *et al.* 1998) with deoxycholate extracted OMVs (Fredriksen *et al.* 1991) from strain 44/76-SL as antigen. Strips loaded with OMV antigens were incubated with 1:200 dilutions of human sera in the absence and presence of 0.15% Empigen BB to detect antibody binding to conformation-dependent epitopes (Wedege *et al.* 1988). The intensities of IgG binding to different OMV antigens [Omp85, FetA (FrpB of mol.wt. 70 kDa), P1.7,16 PorA, P3.15 PorB, Rmp (class 4 protein), OpcA, OpaJ (class 5.5 protein), LOS of immunotypes L3 and L8, and unidentified antigens with higher mol. wt. around 50 kDa (HMW) or in the lower range 20-25 kDa (LMW)] were determined visually. By visual determination, the immunoreactive bands were scored on a

scale from 0 to 4, where scores between 0 and 1.5, between 2 and 2.5, and between 3 and 4 represented no or weak binding, medium binding, and strong binding, respectively.

4.12 Experimental animals

In passive protection studies, 4-7 days old, outbred albino Wistar rat pups (HsdCpb:WU or HsdBrlHan:WIST) and inbred PVG (PVG/OlaHsd) rat pups with normal (designated PVG/c+) or C6 deficient complement (designated PVG/c-) were used.

HsdCpb:WU rats (I, II, III) were purchased as specific pathogen-free from Harlan Nederland (Horst, The Netherlands), bred locally at the Helsinki University Animal Centre (Helsinki, Finland) up to 3 generations until timed-mated to get the litters, and allowed to deliver in the animal facilities of KTL.

For study IV, timed-pregnant female HsdBrlHan:WIST and PVG/OlaHsd rats were purchased from Harlan Nederland (Horst, The Netherlands) and allowed to deliver in the animal facilities of KTL. Conventionally bred complement component C6 deficient PVG rats (designated PVG/c-) (Leenaerts *et al.* 1994) were received from M.R. Daha (Department of Nephrology, Leiden University Medical Center, Leiden, The Netherlands). They were subsequently purified by hysterectomy, propagated up to 5 generations until timed-mated to get the litters and allowed to deliver in the animal facilities of KTL.

As the effect of C6 deficiency on bacterial virulence in non-immune infant rats was not known, in preliminary studies several challenge doses (10^2 to 10^6 cfu/pup) of strain 44/76-SL were tested and the resulting bacteremia levels (cfu/ml) in C6 deficient PVG/c- rats were compared to those of complement sufficient HsdBrlHan:WIST and PVG/c+ rats. No significant differences between complement sufficient and C6 deficient rats were found.

4.13 Infant rat protection assay

The passive protection experiments (I-IV) were done as described previously (Saukkonen 1988). In brief, 4-7-day-old rat pups (average weight 12 g) were randomly redistributed in groups of 5-6 animals and injected intraperitoneally (i.p.) with appropriately diluted, heat-inactivated (56ºC, 30 min) human serum or monoclonal antibodies, in a final volume of 0.1 ml. Saline served as a negative and a monoclonal mouse antibody Nmb735 to serogroup B PS

(Frosch *et al.* 1985) as the positive control for protection. One to two hours later a bacterial challenge of 10^4 to 10^7 cfu/pup was injected i.p. in a final volume of 0.1 ml. Development of meningitis (I) and/or bacteremia (I-IV) was assessed by culturing blood and cerebrospinal fluid (CSF) samples taken at appropriate time-points after bacterial challenge. The limit of detection was 1×10^2 and 1×10^3 cfu/ml for CSF and blood cultures, respectively. Animals with sterile cultures were assigned a value of 0.3× the detection limit, i.e. 30 and 3×10^2 cfu/ml for CSF and blood cultures, respectively.

In all studies (I-IV), reduction in bacteremia levels was used as the main protection assay end-point. In studies II and III, a protection index (PI) was generated based on the reduction in geometric mean concentration (GMC) of bacteria in blood (cfu/ml). PI was equivalent to fold decrease in GM cfu/ml in each experimental group of animals relative to the control group of the same day. For each serum PI was calculated as follows: GM cfu/ml for control animals / GM cfu/ml for serum treated animals. Sera with PI < 1 were assigned a value of 1. A PI of 10 (i.e. a 10-fold reduction in GM cfu/ml blood in the experimental group compared to the control group) was used as cut-off value of protective activity. Thus sera with PI <10 were considered IRPA negative and those with PI ≥10 IRPA positive. A vaccine responder was defined as an individual with at least 10-fold rise in PI (post-vaccination serum PI/pre-vaccination serum PI).

All experimental protocols were reviewed by the Institutional Laboratory Animal Committee and finally approved by the Provincial Board.

4.14 Statistical methods

In statistical analysis, log transformed data was used. For inter- and intra-assay comparisons, the data were subjected to one-way analysis of variance (SPSS Inc., Chicago, Illinois). Statistical differences between GMs (or fold-increases in GMs) in different groups were calculated with two-tailed t test assuming equal variances, and differences between pre- and post-vaccination GMs with paired-samples t test. Pearson correlation coefficients were calculated with SPSS software. Fisher's exact test was used to analyze differences in vaccine responder rates (SPSS). For all comparisons, a P value of ≤0.05 was considered significant.

5 RESULTS

5.1 Adaptation of IRPA assay for use with human sera (I)

For a large-scale evaluation of clinical samples, a sensitive and repeatable assay is needed. Thus, our first aim was the adaptation and standardization of the infant rat protective activity (IRPA) assay for use with human sera (I), paying special attention to the choice of challenge dose and serum dilution used.

5.1.1 Choice of challenge dose

In preliminary studies, several challenge doses (10^4-10^7 cfu/pup) of strain IH5341 (B:15:P1.7,16) were tested, and the development of bacteremia and meningitis was monitored in rat pups (6 animals/group) pre-injected with either saline (control group) or with a monoclonal antibody to the group B capsular polysaccharide (Nmb735) used as a positive control for protection.

The dose of 10^4 cfu/pup did not result in a satisfactory development of either bacteremia or meningitis. Only half of the animals in the control group (3/6) were bacteremic at six hours. The GMC of bacteria in the blood was 9.2×10^3 cfu/ml; none of the animals developed meningitis (I; figure 1). Prolonging the experiment overnight did not increase the infection rate: three of the six animals were bacteremic and only one had bacteria in the CSF. All subsequent experiments were terminated at six hours, like in previous studies (Nurminen *et al.* 1992, Idänpään-Heikkilä *et al.* 1995).

With the higher challenge doses of 10^5 to 10^7 cfu/pup, all saline treated animals were bacteremic at six hours (I; figure 1). The bacterial counts in the blood increased with increasing challenge dose from a GMC of 5.7×10^5 cfu/ml with the dose of 10^5 to 2.3×10^7 cfu/ml with the dose of 10^7. These higher bacterial loads also resulted in high rates of meningitis, varying from 67 to 100% (I; figure 1). However, with the highest dose the sensitivity of the detection of protection decreased. Although highly protective against challenge doses of 10^4-10^6 bacteria, the Nmb735 failed to show any IRPA against challenge with 10^7 meningococci. On the basis of these results, doses of 10^5 and 10^6 cfu/pup were selected for subsequent studies with human sera.

5.1.2 Choice of serum dilution

Four sera, three collected from adults immunized with the Norwegian serogroup B meningococcal OMV vaccine and one from a non-vaccinated person with no history of meningococcal disease, were used for adaptation of the IRPA assay for use with human sera. The sera were first tested at the dilution of 1:10, and then, if protective, at dilutions of 1:30 and 1:100. Most assays were repeated once or twice in separate experiments with a different batch of animals to assess the repeatability of the assay.

As in preliminary experiments, the development of bacteremia and meningitis in saline treated control animals was highly constant with both challenge doses. The non-immune serum had low activity in EIA measuring IgG antibodies to sub-capsular antigens and negligible reaction in immunoblots; it showed no functional activity *in vitro* in SBA assay or OPA assay. When tested for protective activity against a challenge dose of 10^5 in the IRPA assay, the rates of bacteremia were the same as in the control animals in both experiments at the serum dilution 1:10 and the one experiment at dilution 1:30. In one experiment, at dilution of 1:10, the bacterial counts in the blood were reduced to 6.5% (P = .03) of the value in the control group, and there was also a reduction in the rate of meningitis. However, in the other experiments at serum dilutions 1:10 and 1:30 there was no indication of protective activity (I; Table 2). Against the higher dose of 10^6, no protective activity was detected (I; Table 3).

The post-vaccination sera were, as a rule, protective in the IRPA assay and their IRPA could be further quantified by testing different serum dilutions and by varying the challenge dose. With the challenge dose of 10^5, all the three post-vaccination sera were IRPA positive at 1:10 and 1:30 dilutions, whereas the dilution 1:100 gave no protection (I; Table 2). With the higher dose of 10^6 cfu/pup, two sera were IRPA positive at 1:10 dilution; one was still protective at the 1:30 dilution (I; Table 3). These two sera also had the highest SBA titers (1:32 and 1:64) and one of them high but the other one moderate OPA (1:128 and 1:32). By contrast, the serum with low SBA (1:4) but high OPA (1:64) failed to reproducibly show IRPA against the higher challenge dose of 10^6 cfu/pup. In one experiment, at dilution of 1:10, the bacterial counts in the blood were reduced to 3.8% ($P < 0.05$) of the value in the control group, and there was also a reduction in the rate of meningitis. However, in the other two experiments at dilution 1:10 and in the three experiments at dilution 1:30 there was no indication of IRPA (I;

Table 2). Thus, similar to results obtained with group B capsular specific Mab Nmb735, with the higher challenge dose of 10^6 cfu/pup the sensitivity of IRPA assay to detect protection decreased.

The sensitivity of IRPA assay varied also depending on the criterion used. With the challenge dose of 10^6 (I; table 3), a statistically significant reduction in the rate of bacteremia was seen in only 63% (5/8) of cases in which the GMC of bacteria in the blood was significantly reduced. For the dose of 10^5 (I; Table 2), this proportion was even lower (54%, 7/13). When the rate of meningitis was compared with GMC of bacteria in the blood, highly consistent results were obtained in terms of protection: when the serum prevented the development of bacteremia, it also inhibited meningitis. However, because of small number of animals per experimental groups (n=6) and, for the challenge dose of 10^5, a relatively low rate of meningitis even in the saline treated control animals (3/6-4/6), the reductions in the rates of meningitis rarely reached statistical significance ($P < .05$). Therefore, for sensitivity, the most relevant parameter of protection appeared to be the reduction in the bacteremia level.

A strong positive correlation between blood and CSF bacterial counts in both saline and serum treated animals was observed, suggesting that the bacterial concentration in the blood was the direct determinant of their penetration to the CSF. If there were $\geq 10^7$ bacteria in the blood, all animals had bacteria in the CSF. *Vice versa*: when the bacteremia level was $<10^4$ cfu/ml, the CSF was always culture-negative (I; Fig. 2).

We concluded that IRPA assay was entirely feasible for use with human sera, warranting further studies with a larger serum set from a clinical trial. Using bacteremia level as the IRPA assay endpoint, a serum dilution of 1:10 combined with a bacterial dose of 10^5 cfu/pup seemed the most optimal choice in terms of assay sensitivity.

5.2 Assay reproducibility (I, II)

The reproducibility of the IRPA assay is of major importance for large-scale studies. This was assessed by analyzing the repeatability of bacteremia in saline treated control group animals in each of the total of 42 passive protection experiments conducted with the Norwegian vaccine strain 44/76-SL and 29 experiments conducted with the Cuban vaccine strain Cu385 (II).

For both strains, bacteremia levels remained similar throughout the whole one-year and nine-month study period (II; Fig. 1). All (n=252) control animals were bacteremic 6 hrs after i.p. challenge of approximately 10^6 cfu/pup with strain 44/76-SL (GM 5.6×10^6, range 1.7-8.2×10^6). The bacteremia levels ranged from $8.7\text{x}10^4$ to $1.1\text{x}10^7$ cfu/ml with a GM of 1.1×10^6 cfu/ml (95% CI 0.8-1.6×10^6) and coefficients of variation (CV) of 15% (range 5-27%) for intra-assay and 21% for inter-assay comparisons. After a similar challenge dose (GM 7.2×10^6, range 5.8-9.3×10^6) with strain Cu385, 83-100% of the control animals were bacteremic after 6 hrs. The bacteremia levels ranged from 3.8×10^4 to 3.0×10^6 cfu/ml with a GM of 2.3×10^5 cfu/ml (95% CI 1.6-3.3×10^5) and CV of 15% (range 2-30%) for intra-assay and 19% for inter-assay comparisons. Thus, similar to the smaller study (I), the reproducibility of IRPA assessed by the development of bacteremia in saline-treated control animals, was found satisfactory. This held generally true also for reproducibility of the protection assays but in 2 cases out of a total of 12, the results of the protection assays, using at least 10-fold reduction in GM bacteremia level as the criterion of protection, were contradictory (I).

5.3 Serogroup B OMV vaccine responses in IRPA assay (II)

To assess the value of IRPA assay as a correlate of OMV vaccine induced protection in humans, a large-scale evaluation of clinical samples was needed. To this end, pre- and post-vaccination serum samples taken from 92 teenagers who had received two doses of either the Norwegian OMV vaccine, the Cuban OMV vaccine, or the serogroup A/C capsular PS control vaccine during a previous immunogenicity trial in Iceland (Perkins *et al.* 1998) were analyzed for IRPA and the results compared to SBA and anti-OMV IgG data obtained earlier elsewhere (Perkins *et al.* 1998).

In each of the total of 71 passive protection experiments, 1 to 5 paired samples, i.e. sera taken before immunization and six weeks after the second immunization, were assayed for IRPA in an experimental unit of six animals/serum. Based on our earlier study (I), 1:10 serum dilution was used. IRPA was defined as fold decrease in geometric mean (GM) cfu/ml blood in each experimental group of animals relative to the control group of the same day, expressed by protection index (PI). A PI of 10 was used as a cut-off value of protective activity, and a vaccine responder defined as an individual with at least 10-fold rise in PI compared to pre-vaccination level. All sera were tested against both the Norwegian (44/76-SL, B:15:P1.7,16)

and the Cuban (Cu385, B:4:P1.19,15) vaccine strain as in previous SBA and EIA studies (Perkins *et al.* 1998).

5.3.1 Selection of challenge dose

As the adaptation of the IRPA assay for human sera had been done using another strain (I), a series of preliminary experiments was carried out to adjust the assay conditions for 44/76-SL strain and Cu385 strain that had been previously used in SBA assay (Perkins *et al.* 1998). To this end, both strains were first rat-passaged three times and the reproducibility of bacteremia assessed in rat pups (6 animals / group) at six-hour post-infection with 10^5 to 10^7 meningococci. As in the previous study, saline was used as the negative and Nmb735 as the positive control for protection. Most assays were repeated three to five times in separate experiments to assess the repeatability of the assay.

By contrast to 44/76-SL strain, the Cu385 strain proved to be poorly virulent in infant rats. Even with the highest challenge dose of 8×10^7 cfu/pup tested, no reproducible infection could be obtained. Thus modified growth methods (early-log, mid-log, late-log, and stationary phase, broth-grown bacteria) were tested to increase its virulence. As the results were not satisfactory, several additional invasive disease isolates of the same phenotype (B:4:P1.19,15) and a Cuban vaccine strain, previously received from another source (FDA), were tested. Finally, the four-times rat-passaged Cu385 received from FDA was found adequately virulent and chosen as the challenge strain. For a bacterial challenge a dose of approximately 10^6 cfu/pup was chosen as the lowest dose giving satisfactory development of bacteremia with the Cu385 strain. A similar challenge dose was selected for strain 44/76-SL.

5.3.2 Strain characterization

The expression of different OM components and capsular PS in un-passaged strains used in SBA assays and rat-passaged strains used in IRPA assays was studied by SDS-gels and whole-cell EIAs. In strain 44/76-SL, no effect of rat-passage on expression profiles was detected (III; Table 2). The Cu385 strain used in IRPA assays differed from that used in SBA assays (Perkins *et al.* 1998) in two ways. First, it expressed more OpcA protein. Second, it had lost L8 after rat-passage.

5.3.3 Vaccine responses against strain 44/76-SL

As compared to the results from 4-fold rises in anti-OMV IgG levels and to a lesser extent also in SBA titers, the numbers of vaccine responders in IRPA assay were only modest (II; Table 4).

Altogether 46% (42/92) of the pre-vaccination sera were IRPA positive (PI ≥10) with strain 44/76-SL (II; Table 3), compared with 50% (46/92) in the SBA assay (titer ≥4). Six weeks after the second dose, 51% (19/37) of the sera from the Norwegian and 40% (14/35) from the Cuban vaccine recipients were IRPA positive (II; Table 3). Twenty-two percent (8/37) of the Norwegian and 11% (4/35) of the Cuban vaccine recipients had responded in IRPA assay, compared with 65% (24/37; $P<0.05$) and 37% (13/35; $P<0.05$) in SBA assay, and 81% (30/37; $P<0.05$) and 11% (4/35) in EIA measuring anti-OMV IgG antibodies (II; Table 4). In neither of the two OMV vaccine groups was the proportion of IRPA responders significantly higher compared to the controls (II; Table 3). GM PI increased by 1.9-fold in the Norwegian and by 1.4-fold in the Cuban vaccine recipients (II; Table 3); in neither group was the difference between pre- and post-vaccination PIs statistically significant. In the control group, 15% (3/20) of the vaccinees showed an IRPA response compared to none in SBA assay or EIA, but the number of IRPA positive sera and the GM PI showed a slight, non-significant decrease.

5.3.4 Vaccine responses against strain Cu385

Altogether 12% (11/92) of the pre-vaccination sera were IRPA positive with strain Cu385 (II; Table 3), compared 34% (31/92; $P<0.05$) in SBA assay. Six weeks after the second dose, 49% (18/37) of the sera from the Norwegian and 26% (9/35) from the Cuban vaccine group were IRPA positive (II; Table 3). Thirty percent (11/37) of the Norwegian and 14% (5/35) of the Cuban vaccine recipients had responded in IRPA assay, compared to 35% (13/37) and 29% (10/35) in SBA assay, and 51% (19/37; $P<0.05$) and 59% (20/34; $P<0.05$) in EIA. In the Norwegian OMV vaccine group the proportion of IRPA responders was significantly higher compared to the control group (II; Table 3). GM PI increased 3.5 and 2.2-fold in the recipients of the Norwegian and Cuban vaccines, respectively (II; Table 3); in both groups the difference between pre- and post-vaccination PIs was statistically significant ($P<0.05$). None of the control vaccinees showed a response in IRPA or SBA assay.

5.3.5 Effect of pre-vaccination IRPA on detection of responses

To study whether PI of the pre-vaccination sera affected the detection of vaccine responders in general, the IRPA data were analyzed also separately for the recipients of the Norwegian and Cuban vaccines who were IRPA negative (PI < 10) at the time of enrollment. With both strains, the results were similar to those for all vaccines, implying that pre-vaccination IRPA activity did not measurably hinder the detection of vaccine responses.

5.3.6 Correlation of IRPA, SBA, and anti-OMV IgG levels: pre-vaccination sera

For both strains, there was a statistically significant positive correlation between SBA titers and anti-OMV IgG levels (II; Fig. 2A and 2B). The correlation coefficients between PI and anti-OMV IgG levels (II; Fig. 2C and 2D) or PI and SBA titers (II; Figures 2E and 2F), were noticeably lower and significant only for the strain Cu385.

On the average, SBA titers were significantly higher in IRPA-positive (PI ≥10) than in IRPA-negative (PI < 10) sera (II; Fig. 2E and 2F). However, 43% (18/42) of the sera positive with strain 44/76-SL had SBA titers <4 and one third (14/42) had titers < 2 (II; Fig. 2E). Only 11 pre-vaccination sera were IRPA positive against strain Cu385, but the respective proportions were similar: 45% (5/11) of the IRPA positive sera had SBA tires <4 and 27% (3/11) had titers <2 (II; Fig. 2F). On the other hand, 48% (22/46) and 81% (25/31) of the sera with SBA titer of ≥4 against 44/76-SL and Cu385 strains, respectively, were IRPA negative (II; Fig. 2E and 2F). Thus, while the GM SBA titers were significantly higher among the IRPA positive than the IRPA negative pre-vaccination sera, many individual samples were IRPA positive with strain 44/76-SL without having bactericidal activity (SBA titer <4), and *vice versa*.

5.3.7 Correlations of IRPA, SBA, and anti-OMV IgG levels: post-vaccination sera

Similar to the pre-vaccination sera, for both OMV vaccine groups there was a statistically significant positive correlation between SBA titers and anti-OMV IgG levels with strain 44/76-SL (II; Fig. 3A and 3B), and a moderate correlation with strain Cu385 (II; Fig. 4A and 4B). The correlation coefficients between PIs and anti-OMV IgG levels (II; Fig. 3C, 3D, 4C and 4D) or PIs and SBA titers (II; Fig. 3E, 3F, 4E and 4F) were lower but in most cases statistically significant and with the homologous strains, higher than the corresponding pre-

vaccination values, indicating a vaccine response. Despite the vaccine-induced increase in correlation coefficients with the homologous target strains, a significant portion of the SBA positive sera (SBA titer ≥4) remained IRPA negative (PI<10).

To conclude, as compared to 4-four fold rises in anti-OMV IgG and also in SBA titers, the sensitivity of IRPA assay to detect vaccine responders was generally only modest. With pre-vaccination sera, similar numbers of positive sera were detected in IRPA and SBA assays with strain 44/76-SL (46% versus 50%), while with strain Cu385 the sensitivity of the IRPA assay was less than the SBA assay (12% positive in IRPA assay compared to 34% in SBA assay). The correlation between IRPA PIs and SBA titers (or anti-OMV IgG concentrations) with strain 44/76-SL was only modest. A significant portion of IRPA positive pre-vaccination lacked SBA (43%; 18/42), and *vice versa* (48%; 22/46).

5.4 Specificity of natural antibodies conferring IRPA (III)

A significant proportion of IRPA positive pre-vaccination sera with strain 44/76-SL lacked SBA and *vice versa* (II). Thus, our next aim was to assess the specificity and functional activity of natural antibodies responsible for IRPA with 44/76-SL strain, especially among IRPA positive but SBA negative sera. To this end, four subsets of pre-vaccination sera (6-7 sera/subset) of Icelandic teenagers with convergent or discrepant SBA and IRPA results (Table 3) were analyzed for total, specific and functional antibodies by EIA, immunoblotting, IgG quantitation against live meningococcal cells by flow cytometry and OPA assay. Normal human sera (NHS), collected from healthy Finnish children of different ages, were used to verify the findings obtained with Icelandic study sera and a rat strain C6 deficient complement to assess the importance of complement-mediated bacterial lysis for protection.

Table 3. Antibody levels for the pre-vaccination sera from Icelandic teenagers evaluated for functionality and quantity of meningococcal specific antibodies.

	Geometric mean (95% CI)						
Serum subset	**IRPA (PI)**	**SBA (titer)**	**OPA (titer)**	**IgG to OMVs (U/ml)**	**IgG to live bacteria (µg/ml)**	**IgG to B-PS (titer)**	**IgM to B-PS (titer)**
I) IRPA pos, SBA pos (n=6)	**452** (154-1321)	**102** (23-444)	**18** (6-53)	**301** (169-537)	**64** (21-202)	**104** (60-180)	**3662** (2397-5594)
II) IRPA neg, SBA pos (n=6)	**2** (1-4)	**51** (24-108)	**7** (3-19)	**182** (115-288)	**41** (20-86)	**127** (23-694)	**1064** (612-1850)
III) IRPA pos, SBA neg (n=7)	**1007** (337-3013)	**1** (1-2)	**1** (1-2)	**46** (38-55)	**4** (2-6)	**101** (32-317)	**3868** (2380-6287)
IV) IRPA neg, SBA neg (n=7)	**1** (1-2)	**1**	**2** (1-4)	**50** (33-76)	**5** (3-9)	**36** (14-91)	**1378** (907-2094)

Strain 44/76-SL (B:15:P1.7,16) was used in IRPA, SBA, OPA, for preparation of the OMVs and measurement of IgG binding to live bacteria.

5.4.1 IgG to OMVs

The OMV IgG EIA data of the Icelandic pre-vaccination sera resembled closely the data obtained by SBA assay: no significant differences in anti-OMV IgG concentrations between IRPA positive, SBA positive category I and IRPA negative, SBA positive category II sera, or IRPA positive, SBA negative category III and IRPA negative, SBA negative category IV sera were found (Table 3). Highest IgG levels were found for category I. Category II sera showed similar, albeit approximately 1.7-fold lower levels than category I sera while SBA negative category III and IV sera showed negligible IgG levels.

5.4.2 OPA

To find out whether other mechanisms than SBA might be responsible for protection of Icelandic sera in IRPA, OPA of the sera was evaluated with PMNL as effector cells and live 44/76-SL cells as the target. Again, the results were similar to those obtained with SBA assay (Table 3, III; Fig. 1c) with no significant differences between categories I and II, or III and IV, respectively.

5.4.3 IgG to viable meningococci

IgG antibody binding to live 44/76-SL bacteria was determined by flow cytometry. Again, the results were similar to those obtained with the SBA assay (Table 3, III; Fig. 1d). No significant differences in antibody levels between IRPA positive and IRPA negative category I and II sera, or category III and IV sera, were found.

5.4.4 Antibody specificity on immunoblots

The possible association between antibody specificity and IRPA was analyzed by blotting the sera against OMV from strain 44/76-SL followed by evaluation of the IgG binding intensity to several outer membrane components. Most sera (19/26, 73%) showed medium or strong signals to one or more of the antigens. Although large individual variations in the antibody specificities were observed, all sera in category I and II (n=12) demonstrated such signals, thus roughly resembling the findings of SBA and IgG levels. Among the 14 SBA negative

sera in category III and IV, only one serum showed strong signals, six gave medium signals and the remaining seven sera no or negligible signals. No differences in the antigen binding patterns between IRPA positive and IRPA negative category I and II sera, or category III and IV sera were detected that could account for a specific protective activity.

5.4.5 IgG and IgM antibody to serogroup B capsular polysaccharide

As antibodies against capsular polysaccharide cannot be detected by immunoblots, B-PS specific IgG and IgM antibodies were measured by EIA. With the exception of three individual sera with titers > 1,000 (one individual in category I and 2 individuals in category II), the IgG titers to B-PS were very low in all categories (GM IgG titer range 36-127), with 15/26 (58%) of the sera having a titer ≤100 and 22/26 (85%) a titer ≤ 200. No significant differences in titers between IRPA positive and IRPA negative category I and II sera, or category III and IV sera that could explain protection were found (Table 3, III; Fig. 1e). In contrast, IgM titers to B-PS were significantly (P=0.006) higher among the IRPA positive, SBA positive than the IRPA negative, SBA positive category, and among the IRPA positive, SBA negative category than the IRPA negative, SBA negative category (P=0.013) (Table 3, III; Fig. 1f). No significant differences in IgM titers between SBA positive and negative sera were found.

5.4.6 Correlation of IRPA and in vitro antibody measurements against strain 44/76

To confirm the importance B-PS IgM antibodies for protection, the results of functional and quantitative antibody measurements were subjected to correlation analysis. Only IgM antibody to group B capsular polysaccharide (B-PS) correlated positively (r=0.76, P<0.001) with IRPA against strain 44/76-SL (III; Fig. 2f). Studies with NHS collected from Finnish children of different ages (7, 14, and 20 months, and 10 years) confirmed this finding (III; Fig. 4) and showed a clear, age-related increase in B-PS specific IgM antibody that coincided with age-related increase in PI (III; Fig. 3).

5.4.7 Assessment of IRPA in complement component C6-deficient rats

To confirm further the importance of B-PS specific IgM antibody to protection against strain 44/76-SL, two IRPA positive NHS from 10 year-old Finnish children were absorbed with

aluminum hydroxide gel-bound B-PS (Al-B-PS) and the non-absorbed and absorbed sera assayed for IRPA after verification of anti-B-PS IgM depletion. To assess whether protection afforded by these sera was independent on direct bacterial lysis, as suggested by the lack of SBA using human or infant rat serum as the complement source, a rat strain with a deficiency in the terminal part of the complement pathway (C6-deficiency), i.e. in the generation of the membrane attack complex, was used.

The non-absorbed sera were equally protective in both rat strains. As expected, absorption with Al-C-PS did not reduce protective activity. This was in contrast to absorption with Al-B-PS that completely abolished protective activity. (III; Fig. 5).

To conclude, these results strongly suggested that the IRPA conferred by non-immune sera against serogroup B 44/76-SL meningococci was mainly mediated by anti-B-PS IgM antibody. The protection seemed independent of direct bacterial lysis as evident from the lack of SBA *in vitro* and equal protective activity of NHS in complement sufficient and C6 deficient animals.

5.5 Importance of antibody specificity, isotype and complement-mediated bacterial lysis on protection (IV)

As stated above, in sera from un-immunized individuals IRPA against 44/76-SL strain was mainly mediated anti-capsular IgM antibodies. To verify that antibodies of human origin to non-capsular antigens are in general able to confer passive protection in the infant rats, and to study the effect of antibody specificity and isotype to protection in more detail, the parental mouse P1.16 PorA specific mIgG2a Mab (Jiskoot *et al.* 1991), the human IgG1-4 isotypes derived from it (Vidarsson *et al.* 2001), and a monoclonal mouse IgG2a Mab with specificity to B-PS (Nmb735 (Frosch *et al.* 1985)) were studied for IRPA. Like in the previous study (III), C6 deficient rats were used to assess the importance of complement mediated lysis on protection.

5.5.1 Bactericidal activity (BA)

The BA of the Mabs was first reassessed using human and infant rat serum collected from HsdBrlHan:WIST rats as the exogenous complement source. As previously, the B-PS specific

Nmb735 and the parental PorA specific mouse IgG2a were the most potent antibodies in this assay, followed by human IgG isotypes in the order of activity of IgG1=IgG3>IgG2; the IgG4 failed to show any bactericidal activity at the concentrations up to 20 µg/ml tested (Table 4, IV; Table). Approximately 3-fold higher Mab concentrations were needed for BA (≥ 90% killing of the inoculum) with human compared rat complement but this difference did not reach statistical significance (P=0.06, paired one-sided t-test).

5.5.2 IRPA assays with complement sufficient rats

Similar to our previous studies (I and (Toropainen *et al.* 2001)), the B-PS specific Mab Nmb735 (IV; Fig 1a) and the parental PorA specific mouse IgG2a (mIgG2a, IV; Fig. 1b) were highly protective in complement sufficient rats. The lowest dose of 0.02µg/pup of the former and 0.5µg/pup of the latter reduced blood bacterial counts in HsdBrlHan:WIST rats significantly as compared to control animals receiving saline (Table 4). As compared parental PorA specific mIgG2a, the human IgG1 (Table 4, IV; Fig. 1c) and IgG3 (Table 4, IV; Fig. 1e) exhibited approximately similar activity while a 4-fold higher antibody dose (2 compared to 0.5 µg/pup) of IgG2 was needed for equal protective activity (Table 4, IV; Fig 1d). The non-bactericidal human IgG4 (Table 4, IV; Fig. 1f) failed to show significant protective activity at any antibody doses up to 20 µg/pup tested.

Table 4. Bactericidal and infant rat protective activities of monoclonal antibodies (Mab).

	90% killing*		**Passive protection****		
Mab	**Human complement**	**Infant rat complement**	**HsdBrlHan:WIST**	**PVG/c+**	**PVG/c-**
Nmb735	0.08	0.027	0.02	0.02	0.02
mIgG2a	0.08	0.027	0.5	0.5	5.0
hIgG1	0.74	0.25	0.5	5.0	>20
hIgG2	2.2	0.74	2.0	ND	>20
hIgG3	0.74	0.25	0.5	ND	>20
hIgG4	>20	>20	>20	ND	>20

* Lowest antibody concentration (µg/ml) needed for ≥90% killing of the 44/76-SL (B:15:P1.7,16) bacteria in the presence of human or infant rat (HsdBrlHan:WIST) complement.
** Lowest antibody dose needed for passive protection in the infant rats against challenge with approximately 10^6 cfu of 44/76-SL bacteria. Protection was defined as a significant (P<0.05) reduction in geometric mean bacteremia level (cfu/ml blood) compared to control animals given saline.
ND, not determined.

Due to difficulties of the breeder to supply us with complement sufficient partner (PVG/c+) for the C6 deficient rat strain, only the B-PS specific Mab Nmb735, the parental PorA specific mIgG2a, and the human IgG1 isotype could be tested in this rat strain. With the B-PS and PorA specific mouse IgG2a Mabs, similar results were obtained in both complement sufficient rat strains (Tabel 4, IV; Fig. 1a and 1b). This was in contrast to the PorA specific human IgG1 isotype of which a 10-fold higher antibody dose (5 versus 0.5 µg/pup) was needed for equal protection in PVG/c+ compared to HsdBrlHan:WIST rats (Table 4, IV; Fig. 1c). To study the possibility that rat strain specific differences in complement activity might have explained the lower protective activity of the human IgG1 in PVG/c+ compared to HsdBrlHan:WIST rats, the *in vitro* BAs of the parental PorA specific mIgG2a and the human IgG1 isotype were reassessed using pooled serum from either rat strain as the exogenous complement source. No rat strain specific differences in the lytic activity were detected.

5.5.3 IRPA assays with complement component C6 deficient rats

Of the PorA specific antibodies (Table 4, IV;Figs 1b-f), only the parental mIgG2a (Fig 1b) conferred protection in C6 deficient PVG/c- animals, requiring a 10-fold higher antibody dose (5 versus 0.5 µg/pup) for protection as compared to complement sufficient HsdBrlHan:WIST or PVG/c+ animals. This was in contrast to anti-B-PS antibody NmB735 was equally protective in all rat strains (Table 4, IV; Fig 1a).

To conclude, the PorA specific parental mouse IgG2a and the human IgG1-3 isotypes all induced efficient bactericidal activity *in vitro* in the presence of human or infant rat complement and augmented bacterial clearance in complement sufficient animals while the IgG4 was unable to. In C6 deficient animals augmentation of bacterial clearance by anti-PorA antibodies was severely impaired, suggesting that protection afforded anti-PorA antibody was mainly dependent on the activation of the whole complement pathway and subsequent bacterial lysis. This was in contrast to anti-B-PS antibody NmB735 that was equally protective in all rat strains, confirming our previous findings with natural IgM anti-capsular antibodies of human origin (III).

6 DISCUSSION

6.1 Methodological and study design aspects

The development of serogroup B meningococcal vaccines has been hampered by the lack of a reliable animal model for predicting their protective efficacy. Although passive protection in animal models has been widely used to evaluate the protective activity of monoclonal antibodies or immune sera raised in experimental animals, no attempts have been made to study their applicability and validity to assess vaccine responses using human sera.

The purpose of this thesis that includes four consecutive studies (I-IV) was twofold. First, we wanted to evaluate the applicability of the infant rat protection assay to assess natural and serogroup B outer OMV vaccine induced immunity in humans against *N. meningitidis* serogroup B in general. To this end, after careful adaptation of the assay for use with human sera (I), pre- and post-vaccination serum samples from teenagers immunized either of the current two efficacious meningococcal serogroup B OMV vaccines (Bjune *et al.* 1991b, Sierra *et al.* 1991) during a previous immunogenicity trial in Iceland (Perkins *et al.* 1998) were analyzed for IRPA (II), and the results compared to SBA data obtained with the same serum set (Perkins *et al.* 1998). This comparison was necessary, as the SBA assay has been regarded as the "gold standard" surrogate of protection also against disease caused by serogroup B meningococci (Holst *et al.* 2003). Second, we wanted to look more closely the specificity and functional activity of antibodies conferring protection in this model. In these studies, two approaches were used. First, the specificity of antibodies conferring protection in Icelandic study pre-vaccination sera was determined (III). Second, the protective activity well-characterized Mabs of different specificity or antibody isotype was studied (IV). In the last two studies, complement component C6 deficient animals were used to evaluate the importance of complement-mediated bacterial lysis in protection.

In the adaptation of the IRPA assay for use with human sera (I), four human sera were used. Three of them were collected after immunization of adult volunteers with the Norwegian meningococcal serogroup B OMV vaccine (Bjune *et al.* 1991b) and one was normal human serum from an unvaccinated individual with no history of meningococcal disease (representing pre-vaccination serum). These sera were selected from a large panel of sera

available at NIPH on the basis of differences in their antibody profiles measured by immunoblotting and functional activities (SBA and OPA) measured *in vitro*. Because one of the main aims of this study was to examine the reproducibility of the assay by repeating the same assay (same serum dilution, same challenge dose) on several days, for practical and ethical reasons, concerning the use of experimental animals, the number of sera assayed in this study was restricted to four. Thus, the effect of antibody specificity as well functional activity on protection could not be reliably addressed.

To evaluate the applicability of the IRPA assay to detect serogroup B OMV vaccine responses in humans, pre- and post-vaccination serum samples from 92 teenagers, representing a 25% subset of sera from subjects who had received two doses of either the Norwegian OMV vaccine based on strain 44/76-SL (B:15:P1.7,16) (Fredriksen *et al.* 1991), the Cuban OMV vaccine based on strain Cu385 (B:4:P1.19,15) (Sierra *et al.* 1991), or the serogroup A/C capsular PS control vaccine during a previous immunogenicity trial in Iceland (Perkins *et al.* 1998), were used (II). Concerning the use of experimental animals, testing of larger number of sera was considered neither ethically justifiable nor practical. Due to stratified sample selection (25% sample drawn from vaccine-responders as well as non-responders), the percentages of SBA responders reported in the original study (Perkins *et al.* 1998) were roughly retained in the present serum subset (II; Table 1). Samples taken after the second dose were selected for this study because the efficacy trials with these vaccines had been performed with a two-dose schedule (Bjune *et al.* 1991b, Sierra *et al.* 1991) and because in the original study plan comparison of IRPA data with available efficacy data was also included, even though the latter data was obtained from different study populations (Bjune *et al.* 1991b, Sierra *et al.* 1991).

In study III, a representative set (26/92; 28%) of pre-vaccination sera from Icelandic teenagers (Perkins *et al.* 1998) with convergent or divergent IRPA and SBA data (II) against strain 44/76-SL was used to characterize the specificity and functional activity of natural antibodies conferring IRPA with strain 44/76-SL. The sera for this study were selected on the basis of pre-existing IRPA (II) and SBA data (Perkins *et al.* 1998) and the availability of adequate (> 0.2-ml) volumes. NHS (n=20) collected from healthy Finnish children of different ages (7, 14 and 24 months, and 10 years) were used to verify the findings obtained with sera from Icelandic teenagers and to assess the development of IRPA. These sera had been collected in

connection with previous immunogenicity studies (Mäkelä *et al.* 2003) and were randomly selected from samples available at KTL.

In study IV, a mouse P1.16 PorA specific mIgG2a Mab (Jiskoot *et al.* 1991), the human IgG1-4 isotypes derived from it (Vidarsson *et al.* 2001), and a monoclonal mouse IgG2a Mab with specificity to B-PS (Nmb735 (Frosch *et al.* 1985)) were used as model antibodies to assess the effect of antibody specificity (sub-capsular versus capsular antigen), antibody isotype (human IgG1-4), and the importance of complement-mediated bacterial lysis on protection. These Mabs were selected on the basis of their availability, which for the PorA protein well reflects the general interest and importance of this antigen as vaccine candidate against serogroup B meningococcal disease (Muttilainen *et al.* 1995, Claassen *et al.* 1996, Idänpään-Heikkilä *et al.* 1996, Christodoulides *et al.* 1998). In the original study plan, testing of class-switched B-PS specific IgG1 and IgM Mabs of human origin (Raff *et al.* 1988, Raff *et al.* 1991) was also included but due to their poor availability, this plan could not be executed.

For a bacterial challenge strain to adapt the IRPA assay for use with human sera (I) we chose strain IH5341, a CSF isolate from a patient with invasive meningococcal disease (Käyhty *et al.* 1989). This strain, phenotypically homologous (B:15:P1.7,16) to the clone that had in the past decades caused long-drawn epidemics in Northern Europe (Poolman *et al.* 1986), was chosen for a challenge strain largely based on our previous experience on it in this model (Nurminen *et al.* 1992, Idänpään-Heikkilä *et al.* 1995). It was also homologous to the strain 44/76, from which the vaccine that the donors of the human post-vaccination sera received was made.

In studies with the Icelandic study sera (II, III) and Mabs (IV), the Norwegian vaccine (44/76-SL; B:15:P1.7,16) and the Cuban vaccine (Cu385; B:4:P1.19,15) strains were used. As one of our aims was to compare the IRPA data with SBA data (II), a natural choice for the challenge strains used in IRPA studies would have been the exactly same strains that had previously been used to generate the SBA data (Perkins *et al.* 1998). Unfortunately, this was not possible for the Cu385 strain due to poor reproducibility of bacteremia, and the Cu385 received from another source was considered the best choice. As expected, this strain was phenotypically similar to the strain received from CDC and used in their SBA assay except for a higher

expression level of the outer membrane OpcA protein and the loss of L8 expression after rat-passage (II; Table 2). Due to these differences, the results of IRPA and SBA assays were not entirely comparable. Both the Norwegian and the Cuban OMV vaccine lots administered in the Icelandic trial contained the OpcA protein (Griffiths *et al.* 1994).

In passive protection studies rat-passaged strains were used. Criticism has been raised over this procedure (Gorringe *et al.* 2001) as it presumably selects meningococcal variants better adapted to the animals and hence, in theory, moves the animal model one step away from the human disease. We have however found this procedure useful to increase the repeatability of the IRPA assay, which is of utmost importance for large-scale passive protection studies. While mechanisms for increased repeatability of infection after rat-passage are not fully understood, it is probably due to more homogenous inoculum. Interestingly, similar to previous studies in an intranasal mouse model of meningococcal infection (Mackinnon *et al.* 1993), in the Cu385 strain a switch from L3,7,8 immunotype to L3,7 immunotype after rat-passage was detected (II; Table 2), confirming the importance of LOS IT as a virulence determinant (Jones *et al.* 1992). In the 44/76-SL strain, lacking L8 expression already before rat-passages, no change in antigen expression after rat-passage was detected.

For a bacterial challenge dose with the Icelandic sera (II, III), a dose of approximately 10^6 cfu/pup was selected as the lowest dose giving reproducible bacteremia with the Cuban vaccine strain Cu385. For consistency, a similar challenge dose was selected for strain 44/76-SL even though our previous results (I) suggested that this might somewhat decrease IRPA assay sensitivity.

In IRPA studies with Icelandic study sera (II, III), a PI of 10, equivalent to one-log reduction in GM blood bacterial density in an experimental group of animals (n=6), and at least 10-fold rise in PI of paired sera were used as end-points. With the intra- and inter-assay CVs of approximately 20%, a lower threshold was considered inappropriate. The IRPA data were analyzed by several different approaches, including comparison of responder percentages, GM PIs and the proportion of vaccinees achieving a threshold PI of 10. A 10-fold rise in PI was also analyzed separately for study participants without detectable IRPA activity (PI < 10) at study enrolment. In general, all methods gave similar results.

Similarly to previous studies (Goldschneider *et al.* 1969a, Milagres *et al.* 1994, Holst *et al.* 2003, Welsch and Granoff 2004), in SBA assays a titer of 4 was used as the threshold of protection. Although a threshold SBA titer that would predict protection against meningococcal disease at an individual level has not yet been established and will apparently be difficult if not impossible to define, it has been suggested that at population level this threshold correlates well with the Norwegian serogroup B OMV vaccine efficacy (Holst *et al.* 2003).

It is well recognized that the use of complement from heterologous species (especially rabbits) may greatly enhance the SBA of meningococcal anti-PS antibodies compared to their activity with human complement (Griffiss and Goroff 1983, Zollinger and Mandrell 1983, Mandrell *et al.* 1995). Thus, to avoid mistaken conclusions about the protective activity of human antibodies measured *in vivo* in the presence of rat complement (and phagocytes) compared to SBA measured *in vitro* in the presence of human complement (II, III), we used both human and infant rat serum as the exogenous complement source in bactericidal assays *in vitro* with NHS (III) and mouse-human chimeric Mabs (IV). Similar activities were found with both complements, suggesting that the discrepancies between IRPA and SBA data (II, III) were not due to species-specific differences in the efficiency of complement to lyse antibody-targeted bacterial cells.

To find out whether OPA might had explained protection in IRPA positive but SBA negative sera, the OPA of the Icelandic study sera was evaluated as respiratory burst by flow cytometry using human PMNLs as effector cells and live 44/76-SL bacteria as the target cells (III). PMNLs of human origin were used because we wanted to compare IRPA assay with a previously established and standardized OPA assay (Aase *et al.* 1998) and because it would have been very difficult to obtain sufficient amount of PMNLs from infant rats due to their small size.

6.2 Adaptation of IRPA assay for use with human sera (I)

An important question for vaccine development was whether the IRPA assay could be used to test the protective activity conferred by human immune sera and thus possibly as a surrogate assay for vaccine efficacy. Such a use would, however, need careful choice of the assay

conditions for sensitivity and repeatability, with the additional need arising from ethical considerations of the use of experimental animals to keep the number of animals needed small. Therefore, the aim of our first study was the adaptation and standardization of the infant rat infection model for use with human sera.

From previous studies (Saukkonen 1988) we knew that a high challenge dose will lead to a predictable and consistent development of bacteremia and meningitis; on the other hand, in protection assays it is usually easier to prevent an infection caused by a small rather than a large challenge dose. Therefore, in preliminary studies several challenge doses of strain IH5341 were tested, aiming at reproducible infection and sensitive detection of protection with little inter-assay variability. For ethical reasons, early sampling, before the animals get very ill, was desirable. In previous passive protection studies an observation period of six hours after challenge had been used with good results (Saukkonen *et al.* 1989, Nurminen *et al.* 1992). Considering that a longer time of observation might allow the use of a smaller challenge dose and consequently possibly increase the sensitivity of the protection assay (Harris *et al.* 2003a, Harris *et al.* 2003b), we also assessed the outcome 24 hours after challenge with the lowest dose of 10^4 cfu/pup. However, the results were not satisfactory, and six hours was chosen as the standard assay time.

In the six-hour assay, the development of disease with doses of 10^5 to 10^7 cfu/pup was highly constant whether comparing the rates or the levels of bacteremia and meningitis whereas the lower dose of 10^4 was unsatisfactory in terms of consistency and predictability. Although lower challenge doses would probably mimic better the course of natural infection, the doses 10^5 and 10^6 appeared more suitable for protection studies: the development of disease was predictable and reproducible while still allowing a reliable and sensitive detection of protection with a relatively low number of animals per study group (6 pups/group).

For a potential routine test keeping the serum consumption as low as possible is desirable. Thus, it was good to note that 1:10 dilution gave protection; in serological surveys it would not be feasible to use less diluted sera since one assay, with six animals per group, will require a minimum volume of approximately 800 µL of the dilution, e.g. 80 µL of serum. All the post-vaccination sera tested were still protective at the dilution 1:30 against challenge with 10^5 meningococci; the levels but more rarely the rates of bacteremia were reduced as

compared with controls. In contrast, dilution 1:100 gave no protection. On the basis of these results, a 1:10 dilution was chosen for evaluation of OMV vaccine induced IRPA assay responses (II).

As expected, also the challenge dose was an important determinant of the outcome of the IRPA assay. Although highly protective against the lower challenge doses, the Mab Nmb735 showed no protection against challenge with 10^7 meningococci. Similarly, one post-vaccination serum that effectively protected the animals challenged with 10^5 bacteria failed to reproducibly show protection against the higher dose of 10^6. Thus, for passive protection studies the lower bacterial dose of 10^5 seemed more useful, providing that predictable and reproducible infection is obtained.

We found the most consistent and interpretable findings when using a reduction in GM blood bacteremia level (cfu/ml) as the end-point of protection. Moreover, the observation that the rate of meningitis was dependent on the level of bacteremia also in the protection studies suggested that an adequate indicator of protection can be achieved by measuring the bacterial counts in the blood only: when the serum prevented the development of bacteremia, it also inhibited meningitis.

6.3 Assay reproducibility

Due to innate nature of infection studies in experimental animals, they are expectedly subject to considerable biological variability arising form the genetic variability of both the animal host as well as bacterial species in question. In the present studies, the reproducibility of IRPA assay, assessed by the development of bacteremia in saline treated control group animals, was found satisfactory, with coefficients of variation (CV) of approximately 15% (range 5-27%) for intra-assay and of approximately 20% for inter-assay comparisons.

To avoid bias due to uncontrolled (random) variation, a careful allotment of treatments to subjects at random and a strict compliance with standard assay conditions is of major importance. Especially, precautions should be taken to maintain the preparation of the bacterial inoculum (growth phase, method of dilution) and inoculation techniques as closely standardized as possible. To reduce the chance of differences arising due to intrinsic variability of the experimental animals, a high quality of animals used and their uniformity

with respect to age and weight are important. Although the use of genetically defined inbred or F1 hybrid strains of animals would further increase the uniformity of the stock and consequently might reduce intra- as well as inter-experimental variability, we have preferred commercially available outbred Wistar rats (Hsd:WU; I-III or HsdBrlHan:WIST; IV) of specific pathogen free-quality to inbred strains: because of their prolific breeding, large numbers of pups of a suitable age (4-6 days at experiment) are easily available at short notice.

6.4 Serogroup B OMV vaccine responses in IRPA assay (II)

To evaluate the applicability of the IRPA assay to assess serogroup B OMV vaccine responses, paired pre- and post-vaccination serum samples collected from young adults immunized with the Norwegian or the Cuban OMV vaccine during the previous immunogenicity trial in Iceland were used (Perkins *et al.* 1998).

As discussed by Perkins et al. (Perkins *et al.* 1998), in the Icelandic trial the proportions of SBA and EIA responders among the Cuban vaccine recipients were much lower than would be expected from the vaccine efficacy estimate. In the present study, the proportions of IRPA responders were generally lower than those found in SBA and EIA with the homologous strains, and thus even farther from the point estimates of efficacies in the Norwegian (57%) and the Cuban (83%) efficacy trials (Bjune *et al.* 1991b, Sierra *et al.* 1991) (II; Table 4). Thus, the expectation that animal models might shed more light on the poor relationship between SBA activity and protection (Perkins *et al.* 1998) was apparently not fulfilled by our IRPA studies. Further, at best, the correlation between IRPA and SBA or anti-OMV IgG concentrations measured by EIA was only modest. Despite OMV vaccine induced increase in correlation between IRPA and SBA with the homologous strains, a significant portion of the SBA positive sera (SBA titer ≥4) remained IRPA negative (PI<10).

The bacterial challenge dose used to infect the animals is of great importance to the outcome of protection studies and in this study a dose of approximately 10^6 was used combined with 1:10 dilution of human sera. Taking into account the lower number of vaccine responders in IRPA compared to SBA and EIA, the sensitivity of IRPA may not have been optimal. If the serum volume is not a limiting factor, use of less diluted sera, or, alternatively, a lower bacterial dose, as used in other studies (Welsch and Granoff 2004), might have provided

means for better sensitivity. In the present study, the former was not possible because of limited serum volumes and the latter because of the relatively low virulence of the Cu385 strain.

Natural immunity to meningococcal disease develops with age, which, at population level, is associated with the prevalence of SBA (Goldschneider *et al.* 1969a). For serogroup A and C meningococci, the value of SBA as a correlate of protection is well-established. SBA has also been used widely as a surrogate marker for protective immunity and vaccine efficacy against serogroup B disease, although the relevant antigens have been thought to be protein rather than polysaccharide by nature (Zollinger and Mandrell 1983, Williams *et al.* 2003). It was therefore of interest to compare the protection measured by IRPA with SBA. An interesting finding relevant to the recently suggested use of a mean SBA titer of 4, as the threshold predicting protection against invasive serogroup B disease (Holst *et al.* 2003), was that although SBA correlated to some extent with IRPA, many individual samples, especially among the pre-vaccination sera, were strongly protective with strain 44/76-SL without having bactericidal activity (SBA titer <4), and *vice versa*. This is in accordance with the recently published IRPA study with serogroup C meningococci showing that naturally acquired anti-capsular antibodies against serogroup C meningococci can confer protection in the infant rats even in the absence of measurable SBA (Welsch and Granoff 2004).

An important question that remains to be solved is why a significant portion of the SBA positive (titer ≥4) pre-vaccination sera (48% for strain 44/76-SL, 81% for Cu385) compared to only 11% for the serogroup C strain in the study by Welsch and Granoff (Welsch and Granoff 2004) failed to show protection in IRPA. Besides intrinsic differences between the strains, the reason could also lie in the intrinsic differences between these two IRPAs, especially in the serum dilution (1:4 compared 1:10 in our study) and the bacterial challenge dose ($0.8\text{-}1.4\times10^3$ compared $1.7\text{-}9.3\times10^6$ in our study) used. Unfortunately, insufficient volumes of the Icelandic sera prevented further testing of these parameters.

To conclude, although likely to be useful for the pre-clinical evaluation of candidate MenB vaccines (Saukkonen *et al.* 1987, Saukkonen *et al.* 1989, Idänpään-Heikkilä *et al.* 1995, Welsch *et al.* 2003, Welsch *et al.* 2004) and natural immunity (Welsch and Granoff 2004), the

IRPA, as described herein, seemed less suitable for large-scale evaluation of serogroup B OMV vaccine responses in clinical samples.

6.5 Specificity of natural antibodies conferring IRPA (III)

In the search for vaccine candidates, natural immunity to serogroup B meningococcus has been less studied. Thus, we evaluated pre-vaccination sera from Icelandic teenagers with convergent or discrepant SBA and IRPA data with 44/76-SL strain for specific and functional antibodies and could clearly show that natural IgM antibodies to B-PS are able to confer protection against serogroup B disease in an infant rat model of meningococcal infection. Studies with normal human sera from healthy Finnish children of different ages (7, 14 and 24 months, 10 years) confirmed this connection. This finding is in contrast with the general conception of the minor importance of B-PS specific antibodies for protection against serogroup B meningococcal disease. Due to the low avidity, especially of IgG (Mandrell and Zollinger 1982), and the poor bactericidal activity of B-PS specific antibodies in SBA assays with human complement (Zollinger and Mandrell 1983), their role in protection against meningococcal infection has been challenged.

Interestingly, the protection afforded by B-PS specific IgM seemed to be independent of direct lysis of the bacteria, as demonstrated by the inability of the half of the IRPA positive Icelandic sera and the majority (13/14) of the Finnish NHS to promote *in vitro* bacterial killing in the SBA assay (titer <4). This finding was not an artifact arising from intrinsic differences in the ability of rat compared to human complement to augment bactericidal activity of anti-B-PS antibodies as similar, negligible *in vitro* bactericidal activities in NHS were obtained with both complements. In support of these findings, the NHS showed equal protective activity in a complement-sufficient rat strain and in a rat strain with a deficiency (C6 deficiency) in the generation of the membrane attack complex.

Somewhat surprisingly, OPAs with human PMNLs as the effector cells did not, however, explain the protective activity of the IRPA positive but SBA negative Icelandic sera as 5/7 (71%) of these sera were also OPA negative (titers < 2). Thus, the mechanism of protection conferred by B-PS specific antibodies remain to be fully evaluated. Possibly, in the infant rats, the macrophages of the mononuclear phagocyte system (Frasch *et al.* 1976) rather than

PMNLs might have been responsible for bacterial clearance. It is well recognized that Fc and especially C3 receptors on macrophages play a crucial role in the clearance of other well-opsonized encapsulated bacteria from the bloodstream (Rogers 1960, Brown *et al.* 1983a, Bohnsack and Brown 1986, Pelkonen and Pluschke 1989, Noel *et al.* 1990).

With NHS from Finnish children, a clear age-related increase in B-PS specific IgM antibodies was found that closely coincided with the development of IRPA. The origin of anti-B-PS IgM antibody remains open but could be due to carriage of non-virulent serogroup B bacteria or cross-reactive bacteria from other genera, such as *E. coli* K1 or *Moraxella nonliquefaciens*. All these three species share an immunochemically identical polysialic acid capsular polysaccharide (Bøvre *et al.* 1983, Devi *et al.* 1991).

6.6 Importance of antibody specificity, isotype and complement-mediated bacterial lysis on protection (IV)

While there is evidence supporting the importance of both antibody mediated BA (Goldschneider *et al.* 1969a, Goldschneider *et al.* 1969b) and opsonophagocytosis (Ross *et al.* 1987) for protection against serogroup B meningococcal disease, due heterogeneity of antibody responses following disease or serogroup B OMV vaccination with respect to both antibody specificity and subclasses, their relative contribution to protective immunity has been difficult to be delineated. In this study, we addressed this question by assessing the protective activity of the parental mouse P1.16 PorA specific mIgG2a Mab (Jiskoot *et al.* 1991), the four human IgG isotypes (IgG1-4) derived from it (Vidarsson *et al.* 2001), and a monoclonal mouse IgG2a Mab with specificity to B-PS (Nmb735 (Frosch *et al.* 1985)) in complement sufficient and C6 deficient (Leenaerts *et al.* 1994) infant rats.

Our results showed clearly that while the parental PorA specific mouse IgG2a and the human IgG1-3 isotypes derived from it efficiently enhanced bactericidal activity *in vitro* and augmented bacterial clearance *in vivo* in complement sufficient animals, in C6 deficient animals the augmentation of bacterial clearance was severely impaired. This was in contrast to a bactericidal, B-PS specific mouse IgG2a antibody that conferred similar protective activity irrespective of the rat strain used, thus confirming our previous findings with non-bactericidal B-PS specific IgM antibody of human origin (III).

Why antibody to meningococcal capsular compared to sub-capsular antigen is more efficient in conferring protection in C6 deficient animals and also opsonophagocytosis *in vitro* (Andreoni *et al.* 1993) is not clear but has been suggested to arise from the more superficial location of the anti-capsular antibody and/or C3 fragments deposited by it on bacterial surface (Brown *et al.* 1983b), where they are readily recognized by phagocytic cell FcRs and/or CRs.

We found the relative protective activity of human IgG isotypes to be IgG1=IgG3>IgG2>>>IgG4 in complement sufficient HsdBrlHan:WIST rats, a result consistent with the *in vitro* bactericidal activity data in the presence of human (Vidarsson *et al.* 2001) or infant rat complement (IV) but the complete lack of protection by IgG1 and IgG3 in C6 deficient rats being somewhat at variance with the previous phagocytic activity data obtained using human PMNLs as the effector cells and heat-killed 44/76-SL bacteria as the target or measuring respiratory burst with live bacteria (Vidarsson *et al.* 2001). With the parental mouse mIgG2a, a 10-fold higher antibody dose was needed for protection in complement deficient PVG/c- than the isogenic, complement sufficient PVG/c+ rat strain. Possibly, higher antibody doses than the 20μg/pup tested would also have been needed for the human IgG isotypes to confer protection in the PVG/- rat strain.

With the B-PS specific and the parental PorA specific mouse IgG2a Mabs, similar protective activities were detected in both complement sufficient rat strains (HsdBrlHan:WIST and PVG/c+) used. This was in contrast to PorA specific human IgG1 isotype of which a ten-fold higher antibody dose (5.0 compared to 0.5 μg/pup) was needed for passive protection in PVG/c+ compared to HsdBrlHan:WIST animals. As no rat strain specific differences were observed in the BA of the human IgG1 isotype using serum from HsdBrlHan:WIST or PVG/c+ rat as complement source, the reason for the lower protective activity of this Mab in PVG/c+ animals remains to be fully evaluated. Two possibilities exist to explain this difference between *in vitro* BA and *in vivo* protection assays. First, the sensitivity of the BA assay (using 20% infant rat serum as exogenous complement source) may not have been satisfactory to detect rat strain specific differences in complement activity. Second, there may be rat strain differences in the ability of rat phagocyte FcR to bind antibody of human origin.

7 CONCLUSIONS AND FUTURE CONSIDERATIONS

Development of meningococcal serogroup B vaccines has been hampered by the lack of suitable animal models to test their protective efficacy. In the present study, we addressed this question by assessing human sera taken before and after serogroup B OMV vesicle vaccination for protective activity in the IRPA assay, and by comparing the results with data obtained with previously established laboratory assays (SBA assay and EIA) and with vaccine efficacy data obtained earlier in different study populations. Since the specificity and the mechanisms of antibodies providing protective immunity are also far from resolved, we also looked more closely the specificity and functional activity of natural antibodies conferring protection in this animal model. Well-characterized Mabs were used to assess the influence of antibody specificity and isotype on protection, and complement component C6 deficient animals to evaluate the importance of complement-mediated bacterial lysis on protection.

Although likely to be useful for the pre-clinical evaluation of candidate MenB vaccines, natural immunity, and assessment of mechanisms of protection, the IRPA assay, as described herein, is probably less suitable for large-scale evaluation of serogroup B OMV vaccine responses in clinical samples. First, the percentages of IRPA assay responders were much lower than the vaccine efficacies observed in the two large protection trials and also generally lower than the number of responders in the SBA assay that is widely used as "gold standard" assay reflecting protection. Thus, even after careful application of the method for human sera, the assay did not seem to serve as a robust surrogate for vaccine-induced protection. In future studies, efforts to more sensitive animal models are thus needed.

Second, the IRPA assay is very laborious, time- and, most importantly, laboratory animal-consuming. For the new generation mono- and multivalent MenB vaccines developed, designed to confer protection to multiple MenB strains, the number of target strains for the *in vitro* and *in vivo* assays to evaluate their immunogenicity is likely to increase. With a serum set as large as in this study, this would require an enormous effort to be achieved in the IRPA assay.

Third, when using IRPA assay for serogroup B OMV vaccine testing, differences in the virulence of test strains have to be carefully considered. In our hands, the Cuban vaccine strain used in the previous SBA assays was not applicable in IRPA due to poor and variable rate of disease the infant rats. At present, the only tools to overcome the problems of low virulence are to try consecutive rat-passages or, if not successful, replace the non-virulent strain with an acceptable virulent one. Apparently, the prerequisites of a meningococcal strain suitable for *in vivo* assays are more complicated than for *in vitro* SBA assays. Thus, studies assessing meningococcal virulence factors in animal models are desperately needed.

Even with these limitations, the IRPA assay seemed, however, to give some additional value over the SBA assay in that many SBA negative pre-vaccination sera were often IRPA positive. Interestingly, in sera taken before vaccination, IRPA seemed to be mainly mediated by serogroup B capsular PS specific IgM antibodies. A clear connection between the acquisition of natural B-PS specific IgM antibodies and IRPA was also indicated. Even taking the limitations arising from the measurement of the protective activity of human antibodies in the presence of complement and phagocytic cells from heterologous species, our results argue that importance capsular PS specific antibodies on protective immunity against serogroup B disease may have been underestimated. Considering the typically rapid onset of invasive disease following infection, such antibodies could be of critical importance for protection at the early stages infection when antibody production to other bacterial surface structures has not yet efficiently started. In the future, the origin of natural B-PS specific antibodies as well as their mechanism of protection in the IRPA assay would be important to investigate to assess the relevance of these findings to protection in humans.

The IRPA assay seemed also to possess potential to study the mechanisms how antibodies of different specificity afford protection. Using complement sufficient and C6 deficient rat strains, it was clearly shown that basic differences existed in the mechanisms how antibodies to sub-capsular and capsular meningococcal antigens confer protection. For the former, complement-mediated bacterial lysis seemed a prerequisite for protection while for the latter other mechanisms such as opsonophagocytosis were more likely to be involved.

To conclude, in the future more effort should be put to study the relevance meningococcal infection models to assess protection afforded by antibodies of human origin in general.

Although the basic immunology and defense mechanisms operating in human and rodents can be assumed to be quite similar and in our studies no major differences in the lytic activity of infant rat serum compared to human serum were detected, caution should be exercised when interpreting results obtained from animal models to protection in humans. This could be even more important to take into account when dealing with microbes as strict human pathogens as meningococci – due to species-specific differences in innate and adaptive immunity, the interplay between the microbe and host can be profoundly different depending on species in question. Taking into consideration the importance of both complement and phagocytes for immunity against meningococcal disease and the variety of mechanisms that this human pathogen has evolved to counteract these immune mechanisms, a more careful comparison of the rat or mouse and human innate and adaptive immune systems is needed before the relevance of the current and future animal models developed to predict protection in humans can be established.

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9 REFERENCES

Aase A and Michaelsen TE. Opsonophagocytic activity induced by chimeric antibodies of the four human IgG subclasses with or without help from complement. Scand J Immunol. 1994; 39(6): 581-7.

Aase A, Bjune G, Høiby EA *et al.* Comparison among opsonic activity, antimeningococcal immunoglobulin G response, and serum bactericidal activity against meningococci in sera from vaccinees after immunization with a serogroup B outer membrane vesicle vaccine. Infect Immun. 1995; 63(9): 3531-6.

Aase A, Høiby EA and Michaelsen TE. Opsonophagocytic and bactericidal activity mediated by purified IgG subclass antibodies after vaccination with the Norwegian group B meningococcal vaccine. Scand J Immunol. 1998; 47(4): 388-96.

Abdillahi H and Poolman JT. Whole-cell ELISA for typing *Neisseria meningitidis* with monoclonal antibodies. FEMS Microbiology Letters. 1987; 48(367-71.

Abdillahi H and Poolman JT. *Neisseria meningitidis* group B serosubtyping using monoclonal antibodies in whole-cell ELISA. Microbial Pathogenesis. 1988; 4(1): 27-32.

Ala'Aldeen DA, Davies HA and Borriello SP. Vaccine potential of meningococcal FrpB: studies on surface exposure and functional attributes of common epitopes. Vaccine. 1994a; 12(6): 535-41.

Ala'Aldeen DA, Stevenson P, Griffiths E *et al.* Immune responses in humans and animals to meningococcal transferrin-binding proteins: implications for vaccine design. Infect Immun. 1994b; 62(7): 2984-90.

Ala'Aldeen DA, Neal KR, Ait-Tahar K *et al.* Dynamics of meningococcal long-term carriage among university students and their implications for mass vaccination. J Clin Microbiol. 2000; 38(6): 2311-6.

Alcala B, Salcedo C, Arreaza L *et al.* The epidemic wave of meningococcal disease in Spain in 1996-1997: probably a consequence of strain displacement. J Med Microbiol. 2002; 51(12): 1102-6.

Alonso JM, Guiyoule A, Zarantonelli ML *et al.* A model of meningococcal bacteremia after respiratory superinfection in influenza A virus-infected mice. FEMS Microbiol Lett. 2003; 222(1): 99-106.

Ameratunga S, Macmillan A, Stewart J *et al.* Evaluating the post-licensure effectiveness of a group B meningococcal vaccine in New Zealand: a multi-faceted strategy. Vaccine. 2005; 23(17-18): 2231-4.

Andersen J, Berthelsen L and Lind I. Measurement of antibodies against meningococcal capsular polysaccharides B and C in enzyme-linked immunosorbent assays: towards an improved surveillance of meningococcal disease. Clin Diagn Lab Immunol. 1997; 4(3): 345-51.

Andreoni J, Kayhty H and Densen P. Vaccination and the role of capsular polysaccharide antibody in prevention of recurrent meningococcal disease in late complement component-deficient individuals. J Infect Dis. 1993; 168(1): 227-31.

Arakere G and Frasch CE. Specificity of antibodies to *O*-acetyl-positive and *O*-acetyl-negative group C meningococcal polysaccharides in sera from vaccinees and carriers. Infect Immun. 1991; 59(12): 4349-56.

Arko RJ. Animal models for pathogenic *Neisseria* species. Clin Microbiol Rev. 1989; 2 Suppl(S56-9.

Artenstein MS, Gold R, Zimmerly JG *et al.* Prevention of meningococcal disease by group C polysaccharide vaccine. N Engl J Med. 1970; 282(8): 417-20.

Ashton FE, Ryan JA, Michon F *et al.* Protective efficacy of mouse serum to the *N*-propionyl derivative of meningococcal group B polysaccharide. Microb Pathog. 1989; 6(6): 455-8.

Ashton FE, Ryan JA, Borczyk A *et al.* Emergence of a virulent clone of *Neisseria meningitidis* serotype 2a that is associated with meningococcal group C disease in Canada. J Clin Microbiol. 1991; 29(11): 2489-93.

Baker MG, Martin DR, Kieft CE *et al.* A 10-year serogroup B meningococcal disease epidemic in

New Zealand: descriptive epidemiology, 1991-2000. J Paediatr Child Health. 2001; 37(5): S13-9.

Bakir M, Yagci A, Ulger N *et al.* Asymtomatic carriage of *Neisseria meningitidis* and *Neisseria lactamica* in relation to *Streptococcus pneumoniae* and *Haemophilus influenzae* colonization in healthy children: apropos of 1400 children sampled. Eur J Epidemiol. 2001; 17(11): 1015-8.

Barlow AK, Heckels JE and Clarke IN. The class 1 outer membrane protein of *Neisseria meningitidis*: gene sequence and structural and immunological similarities to gonococcal porins. Molecular Microbiology. 1989; 3(2): 131-9.

Bash MC, Lesiak KB, Banks SD *et al.* Analysis of *Neisseria meningitidis* class 3 outer membrane protein gene variable regions and type identification using genetic techniques. Infect Immun. 1995; 63(4): 1484-90.

Berrington AW, Tan YC, Srikhanta Y *et al.* Phase variation in meningococcal lipooligosaccharide biosynthesis genes. FEMS Immunol Med Microbiol. 2002; 34(4): 267-75.

Bjune G, Gronnesby JK, Hoiby EA *et al.* Results of an efficacy trial with an outer membrane vesicle vaccine against systemic serogroup B meningococcal disease in Norway. NIPH Ann. 1991a; 14(2): 125-30; discussion 30-2.

Bjune G, Høiby EA, Grønnesby JK *et al.* Effect of outer membrane vesicle vaccine against group B meningococcal disease in Norway. Lancet. 1991b; 338(8775): 1093-6.

Bohnsack JF and Brown EJ. The role of the spleen in resistance to infection. Annu Rev Med. 1986; 37(49-59.

Borrow R, Longworth E, Gray SJ *et al.* Prevalence of de-*O*-acetylated serogroup C meningococci before the introduction of meningococcal serogroup C conjugate vaccines in the United Kingdom. FEMS Immunol Med Microbiol. 2000; 28(3): 189-91.

Boslego J, Garcia J, Cruz C *et al.* Efficacy, safety, and immunogenicity of a meningococcal group B (15:P1.3) outer membrane protein vaccine in Iquique, Chile. Vaccine. 1995; 13(9): 821-9.

Brandtzaeg P, Bjerre A, Ovstebo R *et al. Neisseria meningitidis* lipopolysaccharides in human pathology. J Endotoxin Res. 2001; 7(6): 401-20.

Brodeur BR, Larose Y, Tsang P *et al.* Protection against infection with *Neisseria meningitidis* group B serotype 2b by passive immunization with serotype-specific monoclonal antibody. Infect Immun. 1985; 50(2): 510-6.

Brown EJ, Hosea SW and Frank MM. The role of antibody and complement in the reticuloendothelial clearance of pneumococci from the bloodstream. Rev Infect Dis. 1983a; 5 Suppl 4(S797-805.

Brown EJ, Joiner KA, Cole RM *et al.* Localization of complement component 3 on *Streptococcus pneumoniae*: anti-capsular antibody causes complement deposition on the pneumococcal capsule. Infect Immun. 1983b; 39(1): 403-9.

Bruge J, Bouveret-Le Cam N, Danve B *et al.* Clinical evaluation of a group B meningococcal *N*-propionylated polysaccharide conjugate vaccine in adult, male volunteers. Vaccine. 2004; 22(9-10): 1087-96.

Bøvre K, Bryn K, Closs O *et al.* Surface polysaccharide of *Moraxella non-liquefaciens* identical to *Neisseria meningitidis* group B capsular polysaccharide. A chemical and immunological investigation. NIPH Ann. 1983; 6(1): 65-73.

Cartwright K, Morris R, Rumke H *et al.* Immunogenicity and reactogenicity in UK infants of a novel meningococcal vesicle vaccine containing multiple class 1 (PorA) outer membrane proteins. Vaccine. 1999; 17(20-21): 2612-9.

Cartwright KA, Stuart JM, Jones DM *et al.* The Stonehouse survey: nasopharyngeal carriage of meningococci and *Neisseria lactamica*. Epidemiol Infect. 1987; 99(3): 591-601.

Cartwright KA and Ala'Aldeen DA. *Neisseria meningitidis*: clinical aspects. J Infect. 1997; 34(1): 15-9.

Casadevall A and Scharff MD. Serum therapy revisited: animal models of infection and development of passive antibody therapy. Antimicrob Agents Chemother. 1994; 38(8): 1695-702.

Caugant DA, Frøholm LO, Bøvre K *et al.* Intercontinental spread of a genetically distinctive complex of clones of *Neisseria meningitidis* causing epidemic disease. Proc Natl Acad Sci U S A. 1986; 83(13): 4927-31.

Caugant DA, Kristiansen BE, Frøholm LO *et al.* Clonal diversity of *Neisseria meningitidis* from a population of asymptomatic carriers. Infect Immun. 1988; 56(8): 2060-8.

Christodoulides M, Brooks JL, Rattue E *et al.* Immunization with recombinant class 1 outer-membrane protein from *Neisseria meningitidis*: influence of liposomes and adjuvants on antibody avidity, recognition of native protein and the induction of a bactericidal immune response against meningococci. Microbiology. 1998; 144 (Pt 11)(3027-37.

Claassen I, Meylis J, van der Ley P *et al.* Production, characterization and control of a *Neisseria meningitidis* hexavalent class 1 outer membrane protein containing vesicle vaccine. Vaccine. 1996; 14(10): 1001-8.

Comanducci M, Bambini S, Brunelli B *et al.* NadA, a novel vaccine candidate of *Neisseria meningitidis*. J Exp Med. 2002; 195(11): 1445-54.

Craven DE and Frasch CE. Protection against group B meningococcal disease: evaluation of serotype 2 protein vaccines in a mouse bacteremia model. Infect Immun. 1979; 26(1): 110-7.

Cruz C, Pavez G, Aguilar E *et al.* Serotype-specific outbreak of group B meningococcal disease in Iquique, Chile. Epidemiol Infect. 1990; 105(1): 119-26.

Danelli MG, Alves CM, Bastos RC *et al.* Human immune response to epitopes on the meningococcal outer membrane class 5 protein following natural infection. FEMS Immunol Med Microbiol. 1996; 15(2-3): 159-68.

de Greeff SC, de Melker HE, Spanjaard L *et al.* [The first effect of the national vaccination campaign against meningococcal-C disease: a rapid and sharp decrease in the number of patients]. Ned Tijdschr Geneeskd. 2003; 147(23): 1132-5.

de Jonge MI, Hamstra HJ, van Alphen L *et al.* Mapping the binding domains on meningococcal Opa proteins for CEACAM1 and CEA receptors. Mol Microbiol. 2003; 50(3): 1005-15.

de Kleijn ED, de Groot R, Labadie J *et al.* Immunogenicity and safety of a hexavalent meningococcal outer-membrane-vesicle vaccine in children of 2-3 and 7-8 years of age. Vaccine. 2000; 18(15): 1456-66.

de Moraes JC, Perkins BA, Camargo MC *et al.* Protective efficacy of a serogroup B meningococcal vaccine in Sao Paulo, Brazil. Lancet. 1992; 340(8827): 1074-8.

De Schrijver K and Maes I. An outbreak of serogroup C meningococcal disease in the province of Antwerp (Belgium) in 2001-2002. Eur J Epidemiol. 2003; 18(11): 1073-7.

de Vries FP, van Der Ende A, van Putten JP *et al.* Invasion of primary nasopharyngeal epithelial cells by *Neisseria meningitidis* is controlled by phase variation of multiple surface antigens. Infect Immun. 1996; 64(8): 2998-3006.

de Vries FP, Cole R, Dankert J *et al. Neisseria meningitidis* producing the Opc adhesin binds epithelial cell proteoglycan receptors. Mol Microbiol. 1998; 27(6): 1203-12.

Deghmane AE, Giorgini D, Larribe M *et al.* Down-regulation of pili and capsule of *Neisseria meningitidis* upon contact with epithelial cells is mediated by CrgA regulatory protein. Mol Microbiol. 2002; 43(6): 1555-64.

Delvig AA, Michaelsen TE, Aase A *et al.* Vaccine-induced IgG antibodies to the linear epitope on the PorB outer membrane protein promote opsonophagocytosis of *Neisseria meningitidis* by human neutrophils. Clin Immunol Immunopathol. 1997; 84(1): 27-35.

Derrick JP, Urwin R, Suker J *et al.* Structural and evolutionary interference from molecular variation in *Neisseria* porins. Infection and Immunity. 1999; 67(5): 2406-13.

Devi SJ, Schneerson R, Egan W *et al.* Identity between polysaccharide antigens of *Moraxella nonliquefaciens*, group B *Neisseria meningitidis*, and *Escherichia coli* K1 (non-*O* acetylated). Infect Immun. 1991; 59(2): 732-6.

DeVoe IW and Gilchrist JE. Release of endotoxin in the form of cell wall blebs during in vitro growth of *Neisseria meningitidis*. J Exp Med. 1973; 138(5): 1156-67.

DeVoe IW. The meningococcus and mechanisms of pathogenicity. Microbiol Rev. 1982; 46(2): 162-90.

Domingo P, Muniz-Diaz E, Baraldes MA *et al.* Associations between Fc gamma receptor IIA polymorphisms and the risk and prognosis of

meningococcal disease. Am J Med. 2002; 112(1): 19-25.

Drogari-Apiranthitou M, Kuijper EJ, Dekker N *et al.* Complement activation and formation of the membrane attack complex on serogroup B *Neisseria meningitidis* in the presence or absence of serum bactericidal activity. Infect Immun. 2002; 70(7): 3752-8.

Edwards EA, Devine LF, Sengbusch GH *et al.* Immunological investigations of meningococcal disease. III. Brevity of group C acquisition prior to disease occurrence. Scand J Infect Dis. 1977; 9(2): 105-10.

Emonts M, Hazelzet JA, de Groot R *et al.* Host genetic determinants of *Neisseria meningitidis* infections. Lancet Infect Dis. 2003; 3(9): 565-77.

Erdei A, Fust G and Gergely J. The role of C3 in the immune response. Immunol Today. 1991; 12(9): 332-7.

Estabrook MM, Christopher NC, Griffiss JM *et al.* Sialylation and human neutrophil killing of group C *Neisseria meningitidis*. J Infect Dis. 1992; 166(5): 1079-88.

Estabrook MM, Griffiss JM and Jarvis GA. Sialylation of *Neisseria meningitidis* lipooligosaccharide inhibits serum bactericidal activity by masking lacto-*N*-neotetraose. Infect Immun. 1997; 65(11): 4436-44.

EU-IBIS. Invasive *Neisseria meningitidis* in Europe -2002. Available at: http://www.euibis.org/ reports.htm.

Feavers IM, Heath AB, Bygraves JA *et al.* Role of horizontal genetic exchange in the antigenic variation of the class 1 outer membrane protein of *Neisseria meningitidis*. Molecular Microbiology. 1992a; 6(4): 489-95.

Feavers IM, Suker J, McKenna AJ *et al.* Molecular analysis of the serotyping antigens of *Neisseria meningitidis*. Infect Immun. 1992b; 60(9): 3620-9.

Feil EJ, Maiden MC, Achtman M *et al.* The relative contributions of recombination and mutation to the divergence of clones of *Neisseria meningitidis*. Mol Biol Evol. 1999; 16(11): 1496-502.

Ferriol Marchena X, Garcia Malberti AM, Ochoa Azze R *et al.* [Validation of an ELISA for the measurement of human IgG against serogroup B Neisseria meningitidis protein]. Rev Cubana Med Trop. 1999; 51(2): 99-105.

Figueroa J, Andreoni J and Densen P. Complement deficiency states and meningococcal disease. Immunol Res. 1993; 12(3): 295-311.

Finne J, Finne U, Deagostini-Bazin H *et al.* Occurrence of alpha 2-8 linked polysialosyl units in a neural cell adhesion molecule. Biochem Biophys Res Commun. 1983a; 112(2): 482-7.

Finne J, Leinonen M and Mäkelä PH. Antigenic similarities between brain components and bacteria causing meningitis. Implications for vaccine development and pathogenesis. Lancet. 1983b; 2(8346): 355-7.

Fischer M, Hedberg K, Cardosi P *et al.* Tobacco smoke as a risk factor for meningococcal disease. Pediatr Infect Dis J. 1997; 16(10): 979-83.

Fogarty J. Trends in serogroup C meningococcal disease in the Republic of Ireland. Euro Surveill. 1997; 2(10): 75-76.

Frank MM and Fries LF. The role of complement in inflammation and phagocytosis. Immunol Today. 1991; 12(9): 322-6.

Frasch CE, Parkes L, McNelis RM *et al.* Protection against group B meningococcal disease. I. Comparison of group-specific and type-specific protection in the chick embryo model. J Exp Med. 1976; 144(2): 319-29.

Frasch CE and Robbins JD. Protection against group B meningococcal disease. III. Immunogenicity of serotype 2 vaccines and specificity of protection in a guinea pig model. J Exp Med. 1978; 147(3): 629-44.

Frasch CE and Peppler MS. Protection against group B *Neisseria meningitidis* disease: preparation of soluble protein and protein-polysaccharide immunogens. Infect Immun. 1982; 37(1): 271-80.

Frasch CE, Peppler MS, Cate TR *et al.* (1982). Immunogenecity and clinical evaluation of group B *Neisseria meningitidis* outer membrane protein vaccines. Seminars in infectious disease. JB Robbins, Hill JC and Sadoff JC, Thieme-Stratton, New York. 4**:** 263-67.

Frasch CE, Coetzee G, Zahradnik JM *et al.* Development and evaluation of group B serotype 2

protein vaccines: report of a group B field trial. Med Trop. 1983; 43(177-80.

Frasch CE, Zollinger WD and Poolman JT. Serotype antigens of *Neisseria meningitidis* and a proposed scheme for designation of serotypes. Reviews of Infectious Diseases. 1985; 7(4): 504-10.

Frasch CE, Zahradnik JM, Wang LY *et al.* Antibody response of adults to an aluminum hydroxide-adsorbed *Neisseria meningitidis* serotype 2b protein-group B polysaccharide vaccine. J Infect Dis. 1988; 158(4): 710-8.

Frasch CE (1995). Meningococcal vaccines: past, present and future. Meningococcal Disease. K Cartwright. Chichester, John Wiley & Sons Ltd**:** 245-83.

Fredriksen JH, Rosenqvist E, Wedege E *et al.* Production, characterization and control of MenB-vaccine "Folkehelsa": an outer membrane vesicle vaccine against group B meningococcal disease. NIPH Ann. 1991; 14(2): 67-79.

Frosch M, Gorgen I, Boulnois GJ *et al.* NZB mouse system for production of monoclonal antibodies to weak bacterial antigens: isolation of an IgG antibody to the polysaccharide capsules of *Escherichia coli* K1 and group B meningococci. Proc Natl Acad Sci U S A. 1985; 82(4): 1194-8.

Fusco PC, Michon F, Tai JY *et al.* Preclinical evaluation of a novel group B meningococcal conjugate vaccine that elicits bactericidal activity in both mice and nonhuman primates. J Infect Dis. 1997; 175(2): 364-72.

Glode MP, Robbins JB, Liu TY *et al.* Cross-antigenicity and immunogenicity between capsular polysaccharides of group C *Neisseria meningitidis* and of *Escherichia coli* K92. J Infect Dis. 1977; 135(1): 94-104.

Gold R and Artenstein MS. Meningococcal infections. 2. Field trial of group C meningococcal polysaccharide vaccine in 1969-70. Bull World Health Organ. 1971; 45(3): 279-82.

Gold R, Goldschneider I, Lepow ML *et al.* Carriage of *Neisseria meningitidis* and *Neisseria lactamica* in infants and children. J Infect Dis. 1978; 137(2): 112-21.

Goldschneider I, Gotschlich EC and Artenstein MS. Human immunity to the meningococcus. I. The role of humoral antibodies. J Exp Med. 1969a; 129(6): 1307-26.

Goldschneider I, Gotschlich EC and Artenstein MS. Human immunity to the meningococcus. II. Development of natural immunity. J Exp Med. 1969b; 129(6): 1327-48.

Gorringe AR, Reddin KM, Voet P *et al.* (2001). Animal models for meningococcal disease. Meningococcal vaccines: methods and protocols. AJ Pollard and Maiden MC. Totowa, Humana Press**:** 241-54.

Gotschlich EC, Goldschneider I and Artenstein MS. Human immunity to the meningococcus. IV. Immunogenicity of group A and group C meningococcal polysaccharides in human volunteers. J Exp Med. 1969; 129(6): 1367-84.

Granoff DM, Kelsey SK, Bijlmer HA *et al.* Antibody responses to the capsular polysaccharide of *Neisseria meningitidis* serogroup B in patients with meningococcal disease. Clin Diagn Lab Immunol. 1995; 2(5): 574-82.

Granoff DM, Bartoloni A, Ricci S *et al.* Bactericidal monoclonal antibodies that define unique meningococcal B polysaccharide epitopes that do not cross-react with human polysialic acid. J Immunol. 1998; 160(10): 5028-36.

Griffiss JM and Goroff DK. IgA blocks IgM and IgG-initiated immune lysis by separate molecular mechanisms. J Immunol. 1983; 130(6): 2882-5.

Griffiss JM, Brandt BL, Broud DD *et al.* Immune response of infants and children to disseminated infections with *Neisseria meningitidis*. J Infect Dis. 1984; 150(1): 71-9.

Griffiss JM, O'Brien JP, Yamasaki R *et al.* Physical heterogeneity of neisserial lipooligosaccharides reflects oligosaccharides that differ in apparent molecular weight, chemical composition, and antigenic expression. Infect Immun. 1987; 55(8): 1792-800.

Griffiths E, Sierra G and Holst J (1994). Quality control of the Cuban and Norwegian serogroup B vaccines used in the Iceland study. Proceedings of the Ninth International Pathogenic *Neisseria* Conference. JS Evans, Yost SE, Maiden MCJ and Feavers IM**:** 437.

Grizot S and Buchanan SK. Structure of the OmpA-like domain of RmpM from *Neisseria meningitidis*. Mol Microbiol. 2004; 51(4): 1027-37.

Guttormsen HK, Wetzler LM and Naess A. Humoral immune response to the class 3 outer membrane protein during the course of meningococcal disease. Infect Immun. 1993; 61(11): 4734-42.

Guttormsen HK, Wetzler LM and Solberg CO. Humoral immune response to class 1 outer membrane protein during the course of meningococcal disease. Infect Immun. 1994; 62(4): 1437-43.

Halstensen A, Haneberg B, Froholm LO *et al.* Human opsonins to meningococci after vaccination. Infect Immun. 1984; 46(3): 673-6.

Halstensen A, Sjursen H, Vollset SE *et al.* Serum opsonins to serogroup B meningococci in meningococcal disease. Scand J Infect Dis. 1989; 21(3): 267-76.

Hammerschmidt S, Hilse R, van Putten JP *et al.* Modulation of cell surface sialic acid expression in *Neisseria meningitidis* via a transposable genetic element. Embo J. 1996a; 15(1): 192-8.

Hammerschmidt S, Muller A, Sillmann H *et al.* Capsule phase variation in *Neisseria meningitidis* serogroup B by slipped-strand mispairing in the polysialyltransferase gene (*siaD*): correlation with bacterial invasion and the outbreak of meningococcal disease. Mol Microbiol. 1996b; 20(6): 1211-20.

Haralambous E, Weiss HA, Radalowicz A *et al.* Sibling familial risk ratio of meningococcal disease in UK Caucasians. Epidemiol Infect. 2003; 130(3): 413-8.

Harris SL, Finn A and Granoff DM. Disparity in functional activity between serum anticapsular antibodies induced in adults by immunization with an investigational group A and C *Neisseria meningitidis*-diphtheria toxoid conjugate vaccine and by a polysaccharide vaccine. Infect Immun. 2003a; 71(6): 3402-8.

Harris SL, King WJ, Ferris W *et al.* Age-related disparity in functional activities of human group C serum anticapsular antibodies elicited by meningococcal polysaccharide vaccine. Infect Immun. 2003b; 71(1): 275-86.

Hibberd ML, Sumiya M, Summerfield JA *et al.* Association of variants of the gene for mannose-binding lectin with susceptibility to meningococcal disease. Meningococcal Research Group. Lancet. 1999; 353(9158): 1049-53.

Hobbs JR, Milner RD and Watt PJ. Gamma-M deficiency predisposing to meningococcal septicaemia. Br Med J. 1967; 4(579): 583-6.

Hobbs MM, Seiler A, Achtman M *et al.* Microevolution within a clonal population of pathogenic bacteria: recombination, gene duplication and horizontal genetic exchange in the *opa* gene family of *Neisseria meningitidis*. Mol Microbiol. 1994; 12(2): 171-80.

Holbein BE, Jericho KW and Likes GC. *Neisseria meningitidis* infection in mice: influence of iron, variations in virulence among strains, and pathology. Infect Immun. 1979; 24(2): 545-51.

Holbein BE. Enhancement of *Neisseria meningitidis* infection in mice by addition of iron bound to transferrin. Infect Immun. 1981a; 34(1): 120-5.

Holbein BE. Differences in virulence for mice between disease and carrier strains of *Neisseria meningitidis*. Can J Microbiol. 1981b; 27(7): 738-41.

Holst J, Feiring B, Fuglesang JE *et al.* Serum bactericidal activity correlates with the vaccine efficacy of outer membrane vesicle vaccines against *Neisseria meningitidis* serogroup B disease. Vaccine. 2003; 21(7-8): 734-7.

Holst J, Feiring B, Naess LM *et al.* The concept of "tailor-made", protein-based, outer membrane vesicle vaccines against meningococcal disease. Vaccine. 2005; 23(17-18): 2202-5.

Holten E. Serotypes of *Neisseria meningitidis* isolated from patients in Norway during the first six months of 1978. Journal of Clinical Microbiology. 1979; 9(2): 186-8.

Hor LI, Chang YK, Chang CC *et al.* Mechanism of high susceptibility of iron-overloaded mouse to *Vibrio vulnificus* infection. Microbiol Immunol. 2000; 44(11): 871-8.

Høiby EA, Rosenqvist E, Frøholm LO *et al.* Bactericidal antibodies after vaccination with the Norwegian meningococcal serogroup B outer

membrane vesicle vaccine: a brief survey. NIPH Ann. 1991; 14(2): 147-55.

Idänpään-Heikkilä I, Muttilainen S, Wahlström E *et al.* The antibody response to a prototype liposome vaccine containing *Neisseria meningitidis* outer membrane protein P1 produced in *Bacillus subtilis*. Vaccine. 1995; 13(16): 1501-8.

Idänpään-Heikkilä I, Wahlström E, Muttilainen S *et al.* Immunization with meningococcal class 1 outer membrane protein produced in *Bacillus subtilis* and reconstituted in the presence of Zwittergent or Triton X-100. Vaccine. 1996; 14(9): 886-91.

Infante JF, Marrero O, Sifontes S *et al.* Evaluation of the efficacy of human antimeningococcal immunoglobulin G in infant rats experimentally infected with *Neisseria meningitidis* group B. Arch Med Res. 1994; 25(4): 455-61.

Ison CA, Heyderman RS, Klein NJ *et al.* Whole blood model of meningococcal bacteraemia--a method for exploring host-bacterial interactions. Microb Pathog. 1995; 18(2): 97-107.

Ison CA, Anwar N, Cole MJ *et al.* Assessment of immune response to meningococcal disease: comparison of a whole-blood assay and the serum bactericidal assay. Microb Pathog. 1999; 27(4): 207-14.

Jack DL, Dodds AW, Anwar N *et al.* Activation of complement by mannose-binding lectin on isogenic mutants of *Neisseria meningitidis* serogroup B. J Immunol. 1998; 160(3): 1346-53.

Jack DL, Jarvis GA, Booth CL *et al.* Mannose-binding lectin accelerates complement activation and increases serum killing of *Neisseria meningitidis* serogroup C. J Infect Dis. 2001; 184(7): 836-45.

Jansen C, Wiese A, Reubsaet L *et al.* Biochemical and biophysical characterization of *in vitro* folded outer membrane porin PorA of *Neisseria meningitidis*. Biochim Biophys Acta. 2000; 1464(2): 284-98.

Jarva H, Ram S, Vogel U *et al.* Binding of the complement inhibitor C4bp to serogroup B *Neisseria meningitidis*. J Immunol. 2005; 174(10): 6299-307.

Jarvis GA and Vedros NA. Sialic acid of group B *Neisseria meningitidis* regulates alternative complement pathway activation. Infect Immun. 1987; 55(1): 174-80.

Jennings HJ, Bhattacharjee AK, Bundle DR *et al.* Strucutres of the capsular polysaccharides of *Neisseria meningitidis* as determined by 13C-nuclear magnetic resonance spectroscopy. J Infect Dis. 1977; 136 Suppl(S78-83.

Jennings HJ and Lugowski C. Immunochemistry of groups A, B, and C meningococcal polysaccharide-tetanus toxoid conjugates. J Immunol. 1981; 127(3): 1011-8.

Jennings HJ, Roy R and Gamian A. Induction of meningococcal group B polysaccharide-specific IgG antibodies in mice by using an N-propionylated B polysaccharide-tetanus toxoid conjugate vaccine. J Immunol. 1986; 137(5): 1708-13.

Jennings MP, Srikhanta YN, Moxon ER *et al.* The genetic basis of the phase variation repertoire of lipopolysaccharide immunotypes in *Neisseria meningitidis*. Microbiology. 1999; 145 (Pt 11)(3013-21.

Jiskoot W, Hoogerhout P, Beuvery EC *et al.* Preparation and application of a fluorescein-labeled peptide for determining the affinity constant of a monoclonal antibody-hapten complex by fluorescence polarization. Analytical Biochemistry. 1991; 196(2): 421-6.

Johansson L, Rytkönen A, Bergman P *et al.* CD46 in meningococcal disease. Science. 2003; 301(5631): 373-5.

Jolley KA, Kalmusova J, Feil EJ *et al.* Carried meningococci in the Czech Republic: a diverse recombining population. J Clin Microbiol. 2000; 38(12): 4492-8.

Jolley KA, Appleby L, Wright JC *et al.* Immunization with recombinant Opc outer membrane protein from *Neisseria meningitidis*: influence of sequence variation and levels of expression on the bactericidal immune response against meningococci. Infect Immun. 2001; 69(6): 3809-16.

Jones DM and Eldridge J. Development of antibodies to meningococcal protein and lipopolysaccharide serotype antigens in healthy-carriers. J Med Microbiol. 1979; 12(1): 107-11.

Jones DM, Borrow R, Fox AJ *et al.* The lipooligosaccharide immunotype as a virulence

determinant in *Neisseria meningitidis*. Microb Pathog. 1992; 13(3): 219-24.

Jordens JZ, Williams JN, Jones GR *et al.* Development of immunity to serogroup B meningococci during carriage of *Neisseria meningitidis* in a cohort of university students. Infect Immun. 2004; 72(11): 6503-10.

Kabat E, Kaiser H and Sikorski H. Preparation of the type-specific polysaccharide of the type I meningococcus and a study of its effectiveness as an antigen in human beings. J Exp Med. 1945; 80(299-307):

Kaczmarski EB. Meningococcal disease in England and Wales: 1995. Commun Dis Rep CDR Rev. 1997; 7(4): R55-9.

Kahler CM, Martin LE, Shih GC *et al.* 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. 1998; 66(12): 5939-47.

Kasper DL, Winkelhake JL, Brandt BL *et al.* Antigenic specificity of bactericidal antibodies in antisera to *Neisseria meningitidis*. J Infect Dis. 1973; 127(4): 378-87.

Kawula TH, Aho EL, Barritt DS *et al.* Reversible phase variation of expression of *Neisseria meningitidis* class 5 outer membrane proteins and their relationship to gonococcal proteins II. Infect Immun. 1988; 56(2): 380-6.

Kinoshita T. Biology of complement: the overture. Immunol Today. 1991; 12(9): 291-5.

Krizova P and Musilek M. Changing epidemiology of meningococcal invasive disease in the Czech republic caused by new clone *Neisseria meningitidis* C:2a:P1.2(P1.5), ET-15/37. Cent Eur J Public Health. 1995; 3(4): 189-94.

Kroll JS, Wilks KE, Farrant JL *et al.* Natural genetic exchange between *Haemophilus* and *Neisseria*: intergeneric transfer of chromosomal genes between major human pathogens. Proc Natl Acad Sci U S A. 1998; 95(21): 12381-5.

Källström H, Liszewski MK, Atkinson JP *et al.* Membrane cofactor protein (MCP or CD46) is a cellular pilus receptor for pathogenic *Neisseria*. Mol Microbiol. 1997; 25(4): 639-47.

Käyhty H, Poolman J, Abdillahi H *et al.* Sero- and subtypes of group B meningococci causing invasive infections in Finland in 1976-87. Scand J Infect Dis. 1989; 21(5): 527-35.

Lawrence J and Handford S. Meningococcal meningitis increase in Moscow associated with serogroup A: advice to travellers. Eurosurveillance Weekly. 2003; 7(46): Available at: http://www.eurosurveillance.org/archives/archives-02.asp.

Leenaerts PL, Stad RK, Hall BM *et al.* Hereditary C6 deficiency in a strain of PVG/c rats. Clin Exp Immunol. 1994; 97(3): 478-82.

Lehmann AK, Halstensen A and Bassoe CF. Flow cytometric quantitation of human opsonin-dependent phagocytosis and oxidative burst responses to meningococcal antigens. Cytometry. 1998; 33(4): 406-13.

Lehmann AK, Halstensen A, Aaberge IS *et al.* Human opsonins induced during meningococcal disease recognize outer membrane proteins PorA and PorB. Infect Immun. 1999; 67(5): 2552-60.

Leinonen M and Frasch CE. Class-specific antibody response to group B *Neisseria meningitidis* capsular polysaccharide: use of polylysine precoating in an enzyme-linked immunosorbent assay. Infect Immun. 1982; 38(3): 1203-7.

Longworth E, Borrow R, Goldblatt D *et al.* Avidity maturation following vaccination with a meningococcal recombinant hexavalent PorA OMV vaccine in UK infants. Vaccine. 2002; 20(19-20): 2592-6.

Lystad A and Aasen S. The epidemiology of meningococcal disease in Norway 1975-91. NIPH Ann. 1991; 14(2): 57-65; discussion 65-6.

Lytton EJ and Blake MS. Isolation and partial characterization of the reduction-modifiable protein of *Neisseria gonorrhoeae*. J Exp Med. 1986; 164(5): 1749-59.

Mackinnon FG, Gorringe AR, Funnell SG *et al.* Intranasal infection of infant mice with *Neisseria meningitidis*. Microb Pathog. 1992; 12(6): 415-20.

Mackinnon FG, Borrow R, Gorringe AR *et al.* Demonstration of lipooligosaccharide immunotype and capsule as virulence factors for *Neisseria meningitidis* using an infant mouse intranasal

infection model. Microb Pathog. 1993; 15(5): 359-66.

MacLeod CM, Hodges RG, Heidelberger M *et al.* Prevention of pneumococcal pneumonia by immunization with specific capsular polysaccharides. J Exp Med. 1945; 82(445-65.

Maiden MC, Suker J, McKenna AJ *et al.* Comparison of the class 1 outer membrane proteins of eight serological reference strains of *Neisseria meningitidis*. Molecular Microbiology. 1991; 5(3): 727-36.

Maiden MC. Population genetics of a transformable bacterium: the influence of horizontal genetic exchange on the biology of *Neisseria meningitidis*. FEMS Microbiology Letters. 1993; 112(3): 243-50.

Maiden MC, Bygraves JA, Feil E *et al.* Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. Proc Natl Acad Sci U S A. 1998; 95(6): 3140-5.

Malorny B, Morelli G, Kusecek B *et al.* Sequence diversity, predicted two-dimensional protein structure, and epitope mapping of neisserial Opa proteins. J Bacteriol. 1998; 180(5): 1323-30.

Mandrell RE and Zollinger WD. Measurement of antibodies to meningococcal group B polysaccharide: low avidity binding and equilibrium binding constants. J Immunol. 1982; 129(5): 2172-8.

Mandrell RE, Griffiss JM and Macher BA. 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. 1988; 168(1): 107-26.

Mandrell RE and Zollinger WD. Human immune response to meningococcal outer membrane protein epitopes after natural infection or vaccination. Infect Immun. 1989; 57(5): 1590-8.

Mandrell RE, Kim JJ, John CM *et al.* Endogenous sialylation of the lipooligosaccharides of *Neisseria meningitidis*. J Bacteriol. 1991; 173(9): 2823-32.

Mandrell RE and Apicella MA. Lipo-oligosaccharides (LOS) of mucosal pathogens: molecular mimicry and host-modification of LOS. Immunobiology. 1993; 187(3-5): 382-402.

Mandrell RE, Azmi FH and Granoff DM. Complement-mediated bactericidal activity of human antibodies to poly alpha2-->8 *N*-acetylneuraminic acid, the capsular polysaccharide of *Neisseria meningitidis* serogroup B. J Infect Dis. 1995; 172(5): 1279-89.

Martin D, Cadieux N, Hamel J *et al.* Highly conserved *Neisseria meningitidis* surface protein confers protection against experimental infection. J Exp Med. 1997; 185(7): 1173-83.

Martin DR, Walker SJ, Baker MG *et al.* New Zealand epidemic of meningococcal disease identified by a strain with phenotype B:4:P1.4. J Infect Dis. 1998; 177(2): 497-500.

Masignani V, Comanducci M, Giuliani MM *et al.* Vaccination against *Neisseria meningitidis* using three variants of the lipoprotein GNA1870. J Exp Med. 2003; 197(6): 789-99.

Maslanka SE, Gheesling LL, Libutti DE *et al.* Standardization and a multilaboratory comparison of *Neisseria meningitidis* serogroup A and C serum bactericidal assays. The Multilaboratory Study Group. Clin Diagn Lab Immunol. 1997; 4(2): 156-67.

McGuinness B, Barlow AK, Clarke IN *et al.* Deduced amino acid sequences of class 1 protein (PorA) from three strains of *Neisseria meningitidis*. Synthetic peptides define the epitopes responsible for serosubtype specificity. Journal of Experimental Medicine. 1990; 171(6): 1871-82.

Mencacci A, Cenci E, Boelaert JR *et al.* Iron overload alters innate and T helper cell responses to Candida albicans in mice. J Infect Dis. 1997; 175(6): 1467-76.

Merker P, Tommassen J, Kusecek B *et al.* Two-dimensional structure of the Opc invasin from *Neisseria meningitidis*. Mol Microbiol. 1997; 23(2): 281-93.

Michaelsen TE, Aase A, Kolberg J *et al.* PorB3 outer membrane protein on *Neisseria meningitidis* is poorly accessible for antibody binding on live bacteria. Vaccine. 2001; 19(11-12): 1526-33.

Mietzner TA, Luginbuhl GH, Sandstrom E *et al.* Identification of an iron-regulated 37,000-dalton

protein in the cell envelope of *Neisseria gonorrhoeae*. Infect Immun. 1984; 45(2): 410-6.

Milagres LG, Ramos SR, Sacchi CT *et al.* Immune response of Brazilian children to a *Neisseria meningitidis* serogroup B outer membrane protein vaccine: comparison with efficacy. Infect Immun. 1994; 62(10): 4419-24.

Milagres LG, Gorla MCA, Sacchi CT *et al.* Specificity of bactericidal antibody response to serogroup B meningococcal strains in brazilian children after immunization with an outer membrane vaccine. Infect Immun. 1998; 66(10): 4755-61.

Millar JW, Siess EE, Feldman HA *et al. In Vivo* and *in Vitro* Resistance to Sulfadiazine in Strains of *Neisseria Meningitidis*. JAMA. 1963; 186(139-41.

Miller CP. Experimental meningococcal infection in mice. Science. 1933; 78(340-41.

Miller CP and Castles R. Experimental meningococcal infection in the mouse. J Infect Dis. 1936; 58(263-79.

Miller E, Salisbury D and Ramsay M. Planning, registration, and implementation of an immunisation campaign against meningococcal serogroup C disease in the UK: a success story. Vaccine. 2001; 20 Suppl 1(S58-67.

Mitka M. New vaccine should ease meningitis fears. JAMA. 2005; 293(12): 1433-4.

Moe GR, Zuno-Mitchell P, Lee SS *et al.* Functional activity of anti-Neisserial surface protein A monoclonal antibodies against strains of *Neisseria meningitidis* serogroup B. Infect Immun. 2001; 69(6): 3762-71.

Moe GR, Zuno-Mitchell P, Hammond SN *et al.* Sequential immunization with vesicles prepared from heterologous Neisseria meningitidis strains elicits broadly protective serum antibodies to group B strains. Infect Immun. 2002; 70(11): 6021-31.

Moore PS, Hierholzer J, DeWitt W *et al.* Respiratory viruses and mycoplasma as cofactors for epidemic group A meningococcal meningitis. Jama. 1990; 264(10): 1271-5.

Moran AP, Prendergast MM and Appelmelk BJ. Molecular mimicry of host structures by bacterial lipopolysaccharides and its contribution to disease. FEMS Immunol Med Microbiol. 1996; 16(2): 105-15.

Moreno C, Hewitt J, Hastings K *et al.* Immunological properties of monoclonal antibodies specific for meningococcal polysaccharides: the protective capacity of IgM antibodies specific for polysaccharide group B. J Gen Microbiol. 1983; 129(2451-6.

Morley SL, Cole MJ, Ison CA *et al.* Immunogenicity of a serogroup B meningococcal vaccine against multiple *Neisseria meningitidis* strains in infants. Pediatr Infect Dis J. 2001; 20(11): 1054-61.

Mrkic B, Pavlovic J, Rulicke T *et al.* Measles virus spread and pathogenesis in genetically modified mice. J Virol. 1998; 72(9): 7420-7.

Munro R. Meningococcal disease: treatable but still terrifying. Intern Med J. 2002; 32(4): 165-9.

Muttilainen S, Idänpään-Heikkilä I, Wahlström E *et al.* The *Neisseria meningitidis* outer membrane protein P1 produced in *Bacillus subtilis* and reconstituted into phospholipid vesicles elicits antibodies to native P1 epitopes. Microb Pathog. 1995; 18(6): 423-36.

Mäkelä PH and Käyhty H. Evolution of conjugate vaccines. Expert Rev Vaccines. 2002; 1(3): 399-410.

Mäkelä PH, Käyhty H, Leino T *et al.* Long-term persistence of immunity after immunisation with *Haemophilus influenzae* type b conjugate vaccine. Vaccine. 2003; 22(2): 287-92.

Naess LM, Oftung F, Aase A *et al.* Human T-cell responses after vaccination with the Norwegian group B meningococcal outer membrane vesicle vaccine. Infect Immun. 1998; 66(3): 959-65.

Naess LM, Aarvak T, Aase A *et al.* Human IgG subclass responses in relation to serum bactericidal and opsonic activities after immunization with three doses of the Norwegian serogroup B meningococcal outer membrane vesicle vaccine. Vaccine. 1999; 17(7-8): 754-64.

Nassif X, Lowy J, Stenberg P *et al.* Antigenic variation of pilin regulates adhesion of *Neisseria meningitidis* to human epithelial cells. Mol Microbiol. 1993; 8(4): 719-25.

Newcombe J, Cartwright K, Dyer S *et al.* Naturally occurring insertional inactivation of the porA gene of *Neisseria meningitidis* by integration of IS1301. Mol Microbiol. 1998; 30(2): 453-4.

Nikaido H. Porins and specific channels of bacterial outer membranes. Mol Microbiol. 1992; 6(4): 435-42.

Noel GJ, Mosser DM and Edelson PJ. Role of complement in mouse macrophage binding of *Haemophilus influenzae* type b. J Clin Invest. 1990; 85(1): 208-18.

Noronha CP, Struchiner CJ and Halloran ME. Assessment of the direct effectiveness of BC meningococcal vaccine in Rio de Janeiro, Brazil: a case-control study. Int J Epidemiol. 1995; 24(5): 1050-7.

Nurminen M, Butcher S, Idänpään-Heikkilä I *et al.* The class 1 outer membrane protein of *Neisseria meningitidis* produced in *Bacillus subtilis* can give rise to protective immunity. Mol Microbiol. 1992; 6(17): 2499-506.

Oftung F, Lovik M, Andersen SR *et al.* A mouse model utilising human transferrin to study protection against *Neisseria meningitidis* serogroup B induced by outer membrane vesicle vaccination. FEMS Immunol Med Microbiol. 1999; 26(1): 75-82.

Oliver KJ, Reddin KM, Bracegirdle P *et al.* Neisseria lactamica protects against experimental meningococcal infection. Infect Immun. 2002; 70(7): 3621-6.

Ong GL and Mattes MJ. Mouse strains with typical mammalian levels of complement activity. J Immunol Methods. 1989; 125(1-2): 147-58.

Oster P, Lennon D, O'Hallahan J *et al.* MeNZB: a safe and highly immunogenic tailor-made vaccine against the New Zealand Neisseria meningitidis serogroup B disease epidemic strain. Vaccine. 2005; 23(17-18): 2191-6.

Peeters CC, Rumke HC, Sundermann LC *et al.* Phase I clinical trial with a hexavalent PorA containing meningococcal outer membrane vesicle vaccine. Vaccine. 1996; 14(10): 1009-15.

Pelkonen S and Pluschke G. Roles of spleen and liver in the clearance of *Escherichia coli* K1 bacteraemia in infant rats. Microb Pathog. 1989; 6(2): 93-102.

Peltola H, Mäkelä H, Käyhty H *et al.* Clinical efficacy of meningococcus group A capsular polysaccharide vaccine in children three months to five years of age. N Engl J Med. 1977; 297(13): 686-91.

Peltola H, Kataja JM and Mäkelä PH. Shift in the age-distribution of meningococcal disease as predictor of an epidemic? Lancet. 1982; 2(8298): 595-7.

Peppler MS and Frasch CE. Protection against group B *Neisseria meningitidis* disease: effect of serogroup B polysaccharide and polymyxin B on immunogenicity of serotype protein preparations. Infect Immun. 1982; 37(1): 264-70.

Perez O, Lastre M, Lapinet J *et al.* Immune response induction and new effector mechanisms possibly involved in protection conferred by the Cuban anti-meningococcal BC vaccine. Infect Immun. 2001; 69(7): 4502-8.

Perez-Ramirez PJ, Infante Bourzac JF, Sifontes Rodriguez S *et al.* Evaluation of the antimeningococcal immunoglobin activity against five strains of *Neisseria meningitidis* group B from Latin America using mice as model. Arch Med Res. 1997; 28(4): 591-5.

Perkins BA, Jonsdottir K, Briem H *et al.* Immunogenicity of two efficacious outer membrane protein-based serogroup B meningococcal vaccines among young adults in Iceland. J Infect Dis. 1998; 177(3): 683-91.

Pizza M, Scarlato V, Masignani V *et al.* Identification of vaccine candidates against serogroup B meningococcus by whole-genome sequencing. Science. 2000; 287(5459): 1816-20.

Platonov AE, Shipulin GA, Vershinina IV *et al.* Association of human Fc gamma RIIa (CD32) polymorphism with susceptibility to and severity of meningococcal disease. Clin Infect Dis. 1998; 27(4): 746-50.

Plested JS, Makepeace K, Jennings MP *et al.* Conservation and accessibility of an inner core lipopolysaccharide epitope of *Neisseria meningitidis*. Infect Immun. 1999; 67(10): 5417-26.

Plested JS, Harris SL, Wright JC *et al.* Highly conserved *Neisseria meningitidis* inner-core lipopolysaccharide epitope confers protection

against experimental meningococcal bacteremia. J Infect Dis. 2003; 187(8): 1223-34.

Poolman JT, Lind I, Jonsdottir K *et al.* Meningococcal serotypes and serogroup B disease in north-west Europe. Lancet. 1986; 2(8506): 555-8.

Prince SM, Achtman M and Derrick JP. Crystal structure of the OpcA integral membrane adhesin from *Neisseria meningitidis*. Proc Natl Acad Sci U S A. 2002; 99(6): 3417-21.

Prinz T and Tommassen J. Association of iron-regulated outer membrane proteins of *Neisseria meningitidis* with the RmpM (class 4) protein. FEMS Microbiol Lett. 2000; 183(1): 49-53.

Pron B, Taha MK, Rambaud C *et al.* Interaction of *Neisseria meningitidis* with the components of the blood-brain barrier correlates with an increased expression of PilC. J Infect Dis. 1997; 176(5): 1285-92.

Raff HV, Devereux D, Shuford W *et al.* Human monoclonal antibody with protective activity for *Escherichia coli* K1 and *Neisseria meningitidis* group B infections. J Infect Dis. 1988; 157(1): 118-26.

Raff HV, Bradley C, Brady W *et al.* Comparison of functional activities between IgG1 and IgM class-switched human monoclonal antibodies reactive with group B streptococci or *Escherichia coli* K1. J Infect Dis. 1991; 163(2): 346-54.

Rake G. Studies on meningococcus infection: I. Biological properties of "fresh" and "stock" strains of the meningococcus. J Exp Med. 1933; 57(549-59.

Ram S, Mackinnon FG, Gulati S *et al.* The contrasting mechanisms of serum resistance of *Neisseria gonorrhoeae* and group B *Neisseria meningitidis*. Mol Immunol. 1999; 36(13-14): 915-28.

Ramsay M, Kaczmarski E, Rush M *et al.* Changing patterns of case ascertainment and trends in meningococcal disease in England and Wales. Commun Dis Rep CDR Rev. 1997; 7(4): R49-54.

Ramsay ME, Andrews N, Kaczmarski EB *et al.* Efficacy of meningococcal serogroup C conjugate vaccine in teenagers and toddlers in England. Lancet. 2001; 357(9251): 195-6.

Richardson AR and Stojiljkovic I. HmbR, a hemoglobin-binding outer membrane protein of *Neisseria meningitidis*, undergoes phase variation. J Bacteriol. 1999; 181(7): 2067-74.

Robbins JB, Myerowitz L, Whisnant JK *et al.* Enteric bacteria cross-reactive with *Neisseria meningitidis* groups A and C and *Diplococcus pneumoniae* types I and 3. Infect Immun. 1972; 6(5): 651-6.

Roberts RB. The relationship between group A and group C meningococcal polysaccharides and serum opsonins in man. J Exp Med. 1970; 131(3): 499-513.

Robinson K, Neal KR, Howard C *et al.* Characterization of humoral and cellular immune responses elicited by meningococcal carriage. Infect Immun. 2002; 70(3): 1301-9.

Rogers DE. Host mechanisms which act to remove bacteria from the blood stream. Bacteriol Rev. 1960; 24(50-66.

Rosenqvist E, Harthug S, Frøholm LO *et al.* Antibody responses to serogroup B meningococcal outer membrane antigens after vaccination and infection. J Clin Microbiol. 1988; 26(8): 1543-8.

Rosenqvist E, Høiby EA, Bjune G *et al.* Human antibody responses after vaccination with the Norwegian group B meningococcal outer membrane vesicle vaccine: results from ELISA studies. NIPH Ann. 1991; 14(2): 169-79.

Rosenqvist E, Høiby EA, Wedege E *et al.* The 5C protein of *Neisseria meningitidis* is highly immunogenic in humans and induces bactericidal antibodies. J Infect Dis. 1993; 167(5): 1065-73.

Rosenqvist E, Høiby EA, Wedege E *et al.* Human antibody responses to meningococcal outer membrane antigens after three doses of the Norwegian group B meningococcal vaccine. Infect Immun. 1995; 63(12): 4642-52.

Rosenqvist E, Musacchio A, Aase A *et al.* Functional activities and epitope specificity of human and murine antibodies against the class 4 outer membrane protein (Rmp) of *Neisseria meningitidis*. Infect Immun. 1999; 67(3): 1267-76.

Ross SC and Densen P. Complement deficiency states and infection: epidemiology, pathogenesis and consequences of neisserial and other infections

in an immune deficiency. Medicine (Baltimore). 1984; 63(5): 243-73.

Ross SC, Rosenthal PJ, Berberich HM *et al.* Killing of *Neisseria meningitidis* by human neutrophils: implications for normal and complement-deficient individuals. J Infect Dis. 1987; 155(6): 1266-75.

Russell JE, Jolley KA, Feavers IM *et al.* PorA variable regions of *Neisseria meningitidis*. Emerg Infect Dis. 2004; 10(4): 674-8.

Rytkönen A, Albiger B, Hansson-Palo P *et al.* *Neisseria meningitidis* undergoes PilC phase variation and PilE sequence variation during invasive disease. J Infect Dis. 2004; 189(3): 402-9.

Sacchi CT, de Lemos AP, Camargo MC *et al.* Meningococcal disease caused by *Neisseria meningitidis* serogroup B serotype 4 in Sao Paulo, Brazil, 1990 to 1996. Rev Inst Med Trop Sao Paulo. 1998a; 40(2): 65-70.

Sacchi CT, Lemos AP, Whitney AM *et al.* Correlation between serological and sequencing analyses of the PorB outer membrane protein in the *Neisseria meningitidis* serotyping system. Clin Diagn Lab Immunol. 1998b; 5(3): 348-54.

Sahu A, Kozel TR and Pangburn MK. Specificity of the thioester-containing reactive site of human C3 and its significance to complement activation. Biochem J. 1994; 302(429-36.

Salit IE. Meningococcemia caused by serogroup W135. Association with hypogammaglobulinemia. Arch Intern Med. 1981; 141(5): 664-5.

Salit IE, Van Melle E and Tomalty L. Experimental meningococcal infection in neonatal animals: models for mucosal invasiveness. Can J Microbiol. 1984; 30(8): 1022-9.

Salleras L, Dominguez A and Cardenosa N. Dramatic decline of serogroup C meningococcal disease in Catalonia (Spain) after a mass vaccination campaign with meningococcal C conjugated vaccine. Vaccine. 2003; 21(7-8): 729-33.

Sarkari J, Pandit N, Moxon ER *et al.* Variable expression of the Opc outer membrane protein in *Neisseria meningitidis* is caused by size variation of a promoter containing poly-cytidine. Mol Microbiol. 1994; 13(2): 207-17.

Saukkonen K, Abdillahi H, Poolman JT *et al.* Protective efficacy of monoclonal antibodies to class 1 and class 3 outer membrane proteins of *Neisseria meningitidis* B:15:P1.16 in infant rat infection model: new prospects for vaccine development. Microb Pathog. 1987; 3(4): 261-7.

Saukkonen K. Experimental meningococcal meningitis in the infant rat. Microb Pathog. 1988; 4(3): 203-11.

Saukkonen K, Leinonen M, Abdillahi H *et al.* Comparative evaluation of potential components for group B meningococcal vaccine by passive protection in the infant rat and in vitro bactericidal assay. Vaccine. 1989; 7(4): 325-8.

Scherp H and Rake G. Studies on meningococcus infection: VIII. The type I specific substance. J Exp Med. 1935; 61(753-69.

Scherp HW and Rake G. Studies on meningococcal infection. XIII. Correlation between antipolysaccharide and the antibody which protects mice against infection with type I meningococci. J Exp Med. 1945; 81(85-92):

Schlesinger M, Greenberg R, Levy J *et al.* Killing of meningococci by neutrophils: effect of vaccination on patients with complement deficiency. J Infect Dis. 1994; 170(2): 449-53.

Scholten RJ, Bijlmer HA, Tobi H *et al.* Upper respiratory tract infection, heterologous immunisation and meningococcal disease. J Med Microbiol. 1999; 48(10): 943-6.

Schryvers AB and Morris LJ. Identification and characterization of the transferrin receptor from *Neisseria meningitidis*. Mol Microbiol. 1988; 2(2): 281-8.

Seiler A, Reinhardt R, Sarkari J *et al.* Allelic polymorphism and site-specific recombination in the opc locus of *Neisseria meningitidis*. Mol Microbiol. 1996; 19(4): 841-56.

Shibuya A, Sakamoto N, Shimizu Y *et al.* Fc alpha/mu receptor mediates endocytosis of IgM-coated microbes. Nat Immunol. 2000; 1(5): 441-6.

Sierra GV, Campa HC, Varcacel NM *et al.* Vaccine against group B *Neisseria meningitidis*: protection trial and mass vaccination results in Cuba. NIPH Ann. 1991; 14(2): 195-207.

Sifontes S, Infante JF, Perez P *et al.* The hyperferremic mouse model for the evaluation of the effectiveness of VA-MENGOC-BC against *Neisseria meningitidis* B clinical isolates. Arch Med Res. 1997; 28(1): 41-5.

Sinn PL, Williams G, Vongpunsawad S *et al.* Measles virus preferentially transduces the basolateral surface of well-differentiated human airway epithelia. J Virol. 2002; 76(5): 2403-9.

Sjursen H, Bjerknes R, Halstensen A *et al.* Serum opsonins to group B meningococci. Acta Pathol Microbiol Immunol Scand [C]. 1987; 95(6): 283-9.

Sjursen H, Bjerknes R, Halstensen A *et al.* Flow cytometric assay for the measurement of serum opsonins to *Neisseria meningitidis* serogroup B, serotype 15. J Immunol Methods. 1989; 116(2): 235-43.

Sjursen H, Wedege E, Rosenqvist E *et al.* IgG subclass antibodies to serogroup B meningococcal outer membrane antigens following infection and vaccination. Apmis. 1990; 98(12): 1061-9.

Sjursen H, Lehmann V, Naess A *et al.* Monocyte phagocytosis of opsonized *Neisseria meningitidis* serogroup B. Apmis. 1992; 100(3): 209-20.

Smirnova I, Mann N, Dols A *et al.* Assay of locus-specific genetic load implicates rare Toll-like receptor 4 mutations in meningococcal susceptibility. Proc Natl Acad Sci U S A. 2003; 100(10): 6075-80.

Snyder LA, Butcher SA and Saunders NJ. Comparative whole-genome analyses reveal over 100 putative phase-variable genes in the pathogenic *Neisseria* spp. Microbiology. 2001; 147(Pt 8): 2321-32.

Spratt BG, Bowler LD, Zhang QY *et al.* Role of interspecies transfer of chromosomal genes in the evolution of penicillin resistance in pathogenic and commensal *Neisseria* species. J Mol Evol. 1992; 34(2): 115-25.

Stephens DS and McGee ZA. Attachment of *Neisseria meningitidis* to human mucosal surfaces: influence of pili and type of receptor cell. J Infect Dis. 1981; 143(4): 525-32.

Stephens DS, Hoffman LH and McGee ZA. Interaction of *Neisseria meningitidis* with human nasopharyngeal mucosa: attachment and entry into columnar epithelial cells. J Infect Dis. 1983; 148(3): 369-76.

Stephens DS, Spellman PA and Swartley JS. Effect of the (alpha 2-->8)-linked polysialic acid capsule on adherence of *Neisseria meningitidis* to human mucosal cells. J Infect Dis. 1993; 167(2): 475-9.

Stern A and Meyer TF. Common mechanism controlling phase and antigenic variation in pathogenic neisseriae. Mol Microbiol. 1987; 1(1): 5-12.

Swartley JS, Marfin AA, Edupuganti S *et al.* Capsule switching of *Neisseria meningitidis*. Proc Natl Acad Sci U S A. 1997; 94(1): 271-6.

Taha MK, Parent Du Chatelet I, Schlumberger M *et al. Neisseria meningitidis* serogroups W135 and A were equally prevalent among meningitis cases occurring at the end of the 2001 epidemics in Burkina Faso and Niger. J Clin Microbiol. 2002; 40(3): 1083-4.

Tappero JW, Lagos R, Ballesteros AM *et al.* Immunogenicity of 2 serogroup B outer-membrane protein meningococcal vaccines: a randomized controlled trial in Chile. JAMA. 1999; 281(16): 1520-7.

Tettelin H, Saunders NJ, Heidelberg J *et al.* Complete genome sequence of *Neisseria meningitidis* serogroup B strain MC58. Science. 2000; 287(5459): 1809-15.

Tinsley CR and Heckels JE. Variation in the expression of pili and outer membrane protein by *Neisseria meningitidis* during the course of meningococcal infection. Journal of General Microbiology. 1986; 132(Pt 9): 2483-90.

Tommassen J, Vermeij P, Struyve M *et al.* Isolation of *Neisseria meningitidis* mutants deficient in class 1 (porA) and class 3 (porB) outer membrane proteins. Infect Immun. 1990; 58(5): 1355-9.

Toropainen M, Saarinen L, van der Ley P *et al.* Murine monoclonal antibodies to PorA of *Neisseria meningitidis* show reduced protective activity *in vivo* against B:15:P1.7,16 subtype variants in an infant rat infection model. Microb Pathog. 2001; 30(3): 139-48.

Troncoso G, Sanchez S, Criado MT *et al.* Analysis of *Neisseria lactamica* antigens putatively implicated in acquisition of natural immunity to

Neisseria meningitidis. FEMS Immunol Med Microbiol. 2002; 34(1): 9-15.

Tsai CM, Frasch CE and Mocca LF. Five structural classes of major outer membrane proteins in *Neisseria meningitidis*. J Bacteriol. 1981; 146(1): 69-78.

Wahdan MH, Rizk F, el-Akkad AM *et al.* A controlled field trial of a serogroup A meningococcal polysaccharide vaccine. Bull World Health Organ. 1973; 48(6): 667-73.

Walker EM, Jr. and Walker SM. Effects of iron overload on the immune system. Ann Clin Lab Sci. 2000; 30(4): 354-65.

van der Ende A, Hopman CT, Zaat S *et al.* Variable expression of class 1 outer membrane protein in *Neisseria meningitidis* is caused by variation in the spacing between the -10 and -35 regions of the promoter. J Bacteriol. 1995; 177(9): 2475-80.

van der Ende A, Hopman CT and Dankert J. Deletion of *porA* by recombination between clusters of repetitive extragenic palindromic sequences in *Neisseria meningitidis*. Infect Immun. 1999; 67(6): 2928-34.

van der Ende A, Hopman CT and Dankert J. Multiple mechanisms of phase variation of PorA in *Neisseria meningitidis*. Infect Immun. 2000; 68(12): 6685-90.

van der Ende A, Hopman CT, Keijzers WC *et al.* Outbreak of meningococcal disease caused by PorA-deficient meningococci. J Infect Dis. 2003; 187(5): 869-71.

van der Ley P, Heckels JE, Virji M *et al.* Topology of outer membrane porins in pathogenic *Neisseria* spp. Infect Immun. 1991; 59(9): 2963-71.

van der Pol WL, Huizinga TW, Vidarsson G *et al.* Relevance of Fcgamma receptor and interleukin-10 polymorphisms for meningococcal disease. J Infect Dis. 2001; 184(12): 1548-55.

Ward SJ, Scopes D, Christodoulides M *et al.* Expression of *Neisseria meningitidis* class 1 porin as a fusion protein in *Escherichia coli*: the influence of liposomes and adjuvants on the production of a bactericidal immune response. Microb Pathog. 1996; 21(6): 499-512.

Varsano S, Frolkis I and Ophir D. Expression and distribution of cell-membrane complement regulatory glycoproteins along the human respiratory tract. Am J Respir Crit Care Med. 1995; 152(3): 1087-93.

Wedege E and Froholm LO. Human antibody response to a group B serotype 2a meningococcal vaccine determined by immunoblotting. Infect Immun. 1986; 51(2): 571-8.

Wedege E and Michaelsen TE. Human immunoglobulin G subclass immune response to outer membrane antigens in meningococcal group B vaccine. J Clin Microbiol. 1987; 25(8): 1349-53.

Wedege E, Bryn K and Frøholm LO. Restoration of antibody binding to blotted meningococcal outer membrane proteins using various detergents. J Immunol Methods. 1988; 113(1): 51-9.

Wedege E, Høiby EA, Rosenqvist E *et al.* Immune responses against major outer membrane antigens of *Neisseria meningitidis* in vaccinees and controls who contracted meningococcal disease during the Norwegian serogroup B protection trial. Infect Immun. 1998; 66(7): 3223-31.

Wedege E, Nøkleby H and Bjune G. No evidence for serosubtype-restricted protection among teenagers vaccinated with the Norwegian group B outer membrane vesicle vaccine. J Infect Dis. 1999; 180(1): 242.

Wedege E, Bolstad K, Wetzler LM *et al.* IgG antibody levels to meningococcal porins in patient sera: comparison of immunoblotting and ELISA measurements. J Immunol Methods. 2000; 244(1-2): 9-15.

Wedege E, Kuipers B, Bolstad K *et al.* Antibody specificities and effect of meningococcal carriage in icelandic teenagers receiving the Norwegian serogroup B outer membrane vesicle vaccine. Infect Immun. 2003; 71(7): 3775-81.

Welsch JA, Moe GR, Rossi R *et al.* Antibody to genome-derived neisserial antigen 2132, a *Neisseria meningitidis* candidate vaccine, confers protection against bacteremia in the absence of complement-mediated bactericidal activity. J Infect Dis. 2003; 188(11): 1730-40.

Welsch JA and Granoff D. Naturally acquired passive protective activity against *Neisseria meningitidis* Group C in the absence of serum bactericidal activity. Infect Immun. 2004; 72(10): 5903-9.

Welsch JA, Rossi R, Comanducci M *et al.* Protective activity of monoclonal antibodies to genome-derived neisserial antigen 1870, a *Neisseria meningitidis* candidate vaccine. J Immunol. 2004; 172(9): 5606-15.

Verheul AF, Snippe H and Poolman JT. Meningococcal lipopolysaccharides: virulence factor and potential vaccine component. Microbiol Rev. 1993; 57(1): 34-49.

Vermont CL, van Dijken HH, van Limpt CJ *et al.* Antibody avidity and immunoglobulin G isotype distribution following immunization with a monovalent meningococcal B outer membrane vesicle vaccine. Infect Immun. 2002; 70(2): 584-90.

Vermont CL, Van Dijken HH, De Groot R *et al.* PorA-specific differences in antibody avidity after vaccination with a hexavalent Men B outer membrane vesicle vaccine in toddlers and school children. Vaccine. 2004; 22(23-24): 3008-13.

West D, Reddin K, Matheson M *et al.* Recombinant *Neisseria meningitidis* transferrin binding protein A protects against experimental meningococcal infection. Infect Immun. 2001; 69(3): 1561-7.

WHO. Control of epidemic meningococcal disease. WHO practical guidelines. 2nd ed. 1998. Geneva, Switzerland, World Health Organization.
Available at: http://www.who.int/emc-documents/meningitis/shoemcbac983c.html. 1998;

Vidarsson G, van Der Pol WL, van Den Elsen JM *et al.* Activity of human IgG and IgA subclasses in immune defense against *Neisseria meningitidis* serogroup B. J Immunol. 2001; 166(10): 6250-6.

Williams JN, Jones GR, Christodoulides M *et al.* Serological correlates of protection against meningococci in a cohort of university students, before and during an outbreak of serogroup C infection. J Infect Dis. 2003; 187(9): 1433-41.

Virji M, Käyhty H, Ferguson DJ *et al.* The role of pili in the interactions of pathogenic *Neisseria* with cultured human endothelial cells. Mol Microbiol. 1991; 5(8): 1831-41.

Virji M, Alexandrescu C, Ferguson DJ *et al.* Variations in the expression of pili: the effect on adherence of *Neisseria meningitidis* to human epithelial and endothelial cells. Mol Microbiol. 1992; 6(10): 1271-9.

Virji M, Makepeace K, Ferguson DJ *et al.* Meningococcal Opa and Opc proteins: their role in colonization and invasion of human epithelial and endothelial cells. Mol Microbiol. 1993a; 10(3): 499-510.

Virji M, Saunders JR, Sims G *et al.* Pilus-facilitated adherence of *Neisseria meningitidis* to human epithelial and endothelial cells: modulation of adherence phenotype occurs concurrently with changes in primary amino acid sequence and the glycosylation status of pilin. Mol Microbiol. 1993b; 10(5): 1013-28.

Virji M, Makepeace K and Moxon ER. Distinct mechanisms of interactions of Opc-expressing meningococci at apical and basolateral surfaces of human endothelial cells; the role of integrins in apical interactions. Mol Microbiol. 1994; 14(1): 173-84.

Virji M, Watt SM, Barker S *et al.* The N-domain of the human CD66a adhesion molecule is a target for Opa proteins of *Neisseria meningitidis* and *Neisseria gonorrhoeae*. Mol Microbiol. 1996; 22(5): 929-39.

Vogel U, Hammerschmidt S and Frosch M. Sialic acids of both the capsule and the sialylated lipooligosaccharide of *Neisseria meningitis* serogroup B are prerequisites for virulence of meningococci in the infant rat. Med Microbiol Immunol. 1996; 185(2): 81-7.

Vogel U, Weinberger A, Frank R *et al.* Complement factor C3 deposition and serum resistance in isogenic capsule and lipooligosaccharide sialic acid mutants of serogroup B *Neisseria meningitidis*. Infect Immun. 1997; 65(10): 4022-9.

Woods JP and Cannon JG. Variation in expression of class 1 and class 5 outer membrane proteins during nasopharyngeal carriage of *Neisseria meningitidis*. Infect Immun. 1990; 58(2): 569-72.

Wright JC, Williams JN, Christodoulides M *et al.* Immunization with the recombinant PorB outer membrane protein induces a bactericidal immune response against *Neisseria meningitidis*. Infect Immun. 2002; 70(8): 4028-34.

Wyle FA, Artenstein MS, Brandt BL *et al.* Immunologic response of man to group B meningococcal polysaccharide vaccines. J Infect Dis. 1972; 126(5): 514-21.

Yazdankhah SP, Kriz P, Tzanakaki G *et al.* Distribution of serogroups and genotypes among disease-associated and carried isolates of *Neisseria meningitidis* from the Czech Republic, Greece, and Norway. J Clin Microbiol. 2004; 42(11): 5146-53.

Yi K, Stephens DS and Stojiljkovic I. Development and evaluation of an improved mouse model of meningococcal colonization. Infect Immun. 2003; 71(4): 1849-55.

Zhu P, Morelli G and Achtman M. The *opcA* and (psi) *opcB* regions in *Neisseria*: genes, pseudogenes, deletions, insertion elements and DNA islands. Mol Microbiol. 1999; 33(3): 635-50.

Zollinger WD and Mandrell RE. Importance of complement source in bactericidal activity of human antibody and murine monoclonal antibody to meningococcal group B polysaccharide. Infect Immun. 1983; 40(1): 257-64.