Laboratory Predictors of Meningococcal Disease and Vaccination in Children

Studies on the host immune response against
Neisseria meningitidis

Clementien L. Vermont

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Drukwerk
Optima Grafische Communicatie

ISBN 90-77595-721-6

The research described in this thesis was performed at the Laboratory of Vaccine Research, National Vaccine Institute, Bilthoven, The Netherlands and at the Erasmus MC-Sophia, Rotterdam, The Netherlands.

Dit proefschrift werd mede mogelijk gemaakt met financiële steun van het Nederlands Vaccin Instituut te Bilthoven.

Laboratory Predictors of Meningococcal Disease and Vaccination in Children

Studies on the host immune response against *Neisseria meningitidis*

Laboratorium voorspellers van meningokokkenziekte en -vaccinatie in kinderen

Een studie naar de immuunrespons tegen *Neisseria meningitidis*

Proefschrift

ter verkrijging van de graad van doctor aan de Erasmus Unversiteit Rotterdam
op gezag van de
Rector Magnificus Prof.dr. S.W.J. Lamberts
en volgens besluit van het College voor Promoties

De openbare verdediging zal plaatsvinden op woensdag 1 september 2004
om 13.45 uur

door

Clementien Liesbeth Vermont

geboren te Goes

Promotor:	Prof.dr. R. de Groot
Commissie:	Prof.dr. J.J.M. van Dongen Prof.dr. H.A. Verbrugh Prof.dr. T. van der Poll
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“ Geen moed vist ook”
Wim Th. Vermont

Contents

Chapter 1

General introduction

Neisseria meningitidis is a gram-negative diplococcus which was first identified by Anton Weichselbaum in 1887. Strains of *N. meningitidis* can be classified into serogroups based upon the different composition of its capsular polysaccharide. Thirteen serogroups have been identified, of which serogroups A, B, C, W135 and Y are responsible for invasive disease. Additional subclassification is based on the variability of two outer membrane proteins: PorB and PorA. Antigenic variation in PorB is responsible for classification of meningococci into serotypes, whereas PorA is responsible for serosubtyping (www.neisseria.org/nm/typing). The outer membrane of *N. meningitidis* also contains lipopolysaccharide (LPS). The variability of the core structure of LPS is the basis of classification of *N. meningitidis* into at least 12 different immunotypes[1].

N. meningitidis is an exclusively human pathogen and asymptomatic nasopharyngeal carriage among the population is approximately 7-17%[2-4]. The carriage rate increases up to 45% when estimated by the culture of tonsillar tissue harvested on tonsillectomy[5]. Serogroup A is the most commonly found serogroup in meningococcal disease cases in the sub-Saharan countries. Serogroup W135 has caused major disease outbreaks in pilgrims returning from the Hajj in Saudi-Arabia[6]. In the industrialized countries, serogroups B and C are the most common serogroups involved in meningococcal disease. Serogroup Y is frequently cultured in patients in the USA[7]. Unencapsulated meningococci are frequently found in carriers but are never involved in disease.

Meningococcal disease

Despite the ubiquitous presence of *N. meningitidis* in the human pharynx, invasive meningococcal disease, e.g. meningitis and/or septicemia, only occurs in 1,3 per 100000 in the USA up to 14,8 per 100000 inhabitants in Ireland (www.neisseria.org/nm/surveillance). In the Netherlands, meningococcal disease incidence was 4.48 per 100.000 inhabitants in the year 2001[8]. The incidence of invasive meningococcal disease is highest in infants and children below the age of 4 and in teenagers. Risk factors include household crowding and passive or active smoking[9]. Since carriage rates are so high and the disease incidence relatively low,

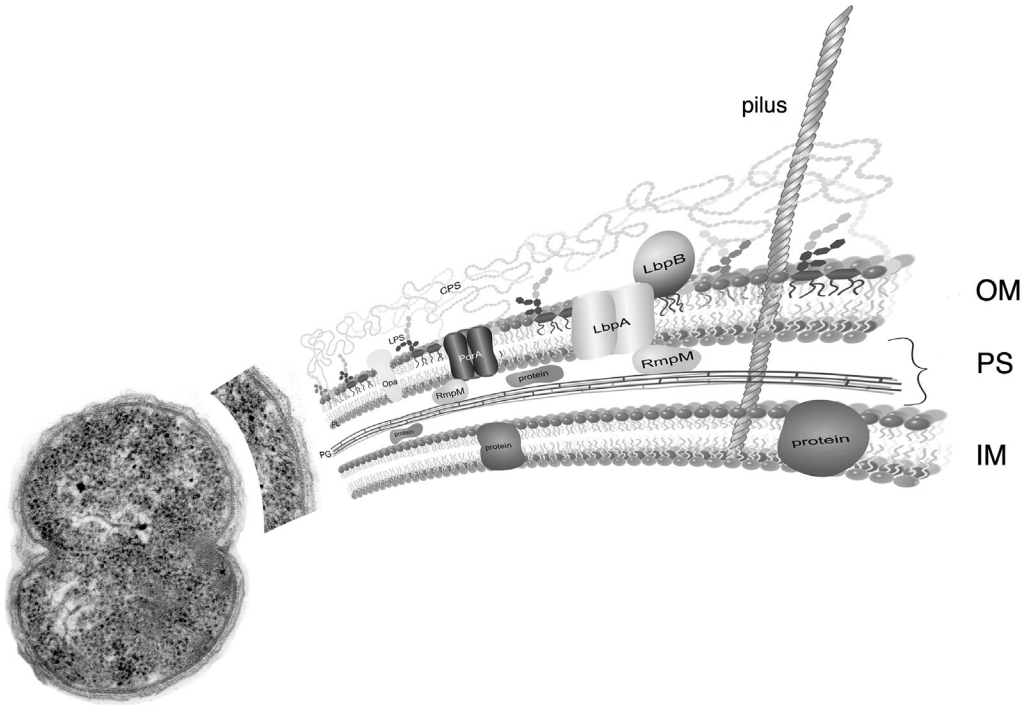

Figure 1. Schematic representation of the N. meningitidis cell surface. The cell envelope is composed of an outer membrane (OM) and an inner membrane (IM), separated by the periplasmic space (PS), and the bacterium is surrounded by a capsular polysaccharide (CPS). Type IV pili protrude through the entire envelope to the environmental medium. The OM is an asymmetric bilayer composed of lipopolysaccharide (LPS) and phospholipids (PL) in the outer leaflet and PL in the inner leaflet. Several integral and cell surface-exposed proteins. LbpA and LbpB represent the integral OMP and the cell surface-exposed protein respectively, which together constitute the lactoferrin receptor. Porin A and B (PorA and PorB) form cation- and anion-selective pores in the outer membrane respectively. The opacity protein (Opa) plays a role in adhesions and invasion of host cells. The reduction-modifiable protein M (RmpM) is constitutively expressed and antigenically invariable. N. meningitidis expresses numerous other, some yet unidentified, OMPs. The PS contains the peptidoglycan layer (PG) and proteins. The IM is a symmetric bilayer composed of PL and contains proteins.

susceptibility for meningooccal disease is thought to be influenced by host genetic polymorphisms in immune response genes or genes associated with the attachment to and invasion of mucosal epithelial cells. The spectrum of meningococcal disease varies from mild upper respiratory tract infections to meningitis, sepsis and ultimately septic shock. The wide range of mild or benign meningococcemia to fulminant septic shock suggests that gene polymorphisms are involved in influencing disease severity as well. The first symptoms of invasive disease are usually non-specific flu-like symptoms like fever, headache, nausea and vomiting, painful legs and malaise. The classical symptom of meningococcal disease is the appearance of petechiae and purpura, although these may also be seen in patients with viral infections or bacterial infections (such as pneumococcal infections). Although meningococci are generally susceptible for antibiotics such as penicillin or cephalosporins, morbidity and mortality rates remain high. In patients with meningococcal sepsis morbidity includes loss of hearing, amputation of limbs and scarring. Mortality rates for meningococcal disease are approximately 10% or higher in case of severe sepsis or septic shock[10]. Treatment preferably occurs on a pediatric intensive care unit and involves antibiotic therapy and circulatory and ventilatory support. Several trials using new therapeutic approaches to treat meningococcal sepsis have been conducted over the last decade. However, recombinant interleukin-1 receptor antagonist, anti-TNF-α-monoclonal antibodies and a human monoclonal antibodies against endotoxin as adjunctive treatment in patients with severe sepsis or septic shock did not significantly reduce mortality[11-13]. The use of recombinant bacterial/permeability-increasing protein (BPI) in a randomized trial in children with severe meningococcal sepsis did also not reduce mortality, although there was a trend towards improved outcome in the patients treated with rBPI[14]. Administration of recombinant human activated protein C in adults with sepsis provided a significant reduction in mortality but was associated with an increased risk of bleeding[15]. Currently, none of these agents is part of standard clinical practice in the treatment of children with meningococcal sepsis.

The role of LPS

Antibiotic treatment effectively kills the bacteria, but meningococcal LPS release in the blood stream is the major cause of damage and can not be specifically inhibited. Once meningococci enter and replicate in the bloodstream, a massive release of outer membrane vesicles containing large amounts of LPS and outer membrane proteins occurs. This process, called “blebbing”, is an essential and exclusive characteristic of meningococcal invasive disease. LPS binds to CD14, a receptor present on monocytes and macrophages[16], and signals through toll like receptor 4 (TLR-4) and the essential protein MD2[17,18]. This process leads to the transcription of pro- and anti-inflammatory cytokine genes and activation of several inflammatory cascades, such as

the coagulation and fibrinolytic system, causing disseminated intravascular coagulation, and the activation of the complement system. High serum levels of TNF-α and IL-6 correlate well with disease severity and outcome. Chemokines, a family of more than 40 different relatively small peptides are mainly involved in chemoattraction and activation of leukocytes to the site of inflammation and the induction of cytokines. Studies in patients with bacterial meningitis caused by *Neisseria meningitidis, Streptococcus pneumoniae* or *Haemophilus influenzae*, showed high levels of chemokines in the cerebrospinal fluid[19-22]. However, the role of these peptides in meningococcal disease, is largely unknown.
In response to the entry of massive amounts of LPS in the blood stream, cells of the immune system, such as neutrophils and peripheral blood mononuclear cells, react with the production and release of cytokines. Exposure to small amounts of LPS can lead to hyporesponsiveness of the immune system to subsequent large amounts of LPS in the blood stream. It is unclear whether this phenomenon of LPS tolerance plays a role in the early stage of meningococcal sepsis in children. Furthermore, cytokine production has been shown to be largely dependent on meningococcal LPS and to a lesser degree on meningococcal outer membrane proteins[23,24]. It is unknown whether the production and release of chemokines is also mainly induced by LPS.
Clearance of LPS from the blood stream by LPS scavengers such as BPI and complement factors has been described. Cholesterol and the lipoproteins HDL and LDL have been implicated to play a role in the innate immune response against LPS by forming complexes with it and neutralize its toxic effects. Little is known about cholesterol and lipoprotein metabolism in children with severe meningococcal disease.

Immunity against *N. meningitidis*
Natural immunity against *N. meningitidis* mainly depends on the acquisition of protective antibody. This has been demonstrated by Goldschneider *et al.*, who showed an inverse relationship between bactericidal anti-meningococcal antibody and meningococcal disease incidence. Infants are protected by maternal antibodies until approximately 6 to 12 months after birth when antibody levels fall rapidly[25]. Meningococcal disease occurs most frequently in children of this age. The acquisition of protective antibody probably occurs following carriage of non-pathogenic species like *N. lactamica.* Serum antibody protects against meningococcal disease by binding to bacterial antigens and activation of the classical pathway or by direct bactericidal killing. For serogroup A and C, Goldschneider showed that the presence of anti-capsular polysaccharide antibody correlates with protection against disease. The innate immune system has several ways of dealing with meningococci in the absence of specific serum antibody. Activation of the alternative complement pathway or the mannose-binding lectin (MBL) pathway can occur due to binding of C3b or MBL to

bacterial surface antigens. The activation of all pathways eventually leads to formation of membrane attack complexes and killing of the bacteria.
Although serum bactericidal activity is not always present in reconvalescent sera, antibodies induced by invasive meningococcal disease are thought to be protective against recurrent meningococcal infections. Knowledge of the immune response following meningococcal infection may provide important information, which can be of use in vaccine development. Antibodies found after systemic meningococcal infections are mainly directed against PorA, PorB, Opc and LPS[26]. The avidity of these antibodies has been shown to be age-dependent[27]. However, the specificity of PorA-antibodies in children after invasive meningococcal disease has never been studied.

Vaccination against *N. meningitidis*

N. meningitidis serogroups A,C, W135 and Y capsular polysaccharide are immunogenic in humans and are used for the production of polysaccharide vaccines. However, these vaccines are less effective in children under the age of 2 and duration of protection is only short. The technique of conjugation of the polysaccharide to a carrier protein has been very succesful in vaccines against *Haemophilus influenzae* and has led to application of this technique to the meningococcal polysaccharide capsules. An effective meningococcal conjugate vaccine to prevent serogroup A disease in the 'meningitis belt' of Africa could safe thousands of lives. Currently, such a vaccine is under development by the Meningitis Vaccine Project[28]. In the late nineties, the incidence of meningococcal disease caused by serogroup C increased enormously in some Western European countries, causing an increase of deaths and commotion in many communities. Therefore, high priority was given to the development and production of a group C conjugate vaccine. The United Kingdom successfully introduced this vaccine, solely on the basis of immunogenicity data instead of waiting for time-consuming large efficacy trials[29]. Immunogenicity was tested by measuring bactericidal activity (SBA), immunoglobulin G titers in enzyme-linked immunosorbent assay (ELISA) and antibody avidity. In several other European countries including Spain, Ireland, Belgium and the Netherlands, group C conjugate vaccines are now introduced into routine infant vaccination programs and have resulted in a dramatic decrease of serogroup C disease. Serogroup B polysaccharide is poorly immunogenic, probably because it has strong similarities with structures on human fetal neural cells. Therefore, several other approaches in the search for an effective vaccine against group B or a universal vaccine against all serogroups are being studied. The completion of the meningococcal genome sequence and the different available techniques to exploit the genome has provided great advances in the search for new possible vaccine candidates. However, thus far no data on efficacy of any of the genome-derived antigens have been published yet. The only vaccines against

serogroup B that are studied in large efficacy trials are OMV vaccines, based on a single PorA subtype. Efficacy of these vaccines was variable and lowest in infants, who are at greatest risk of acquiring meningococcal disease[30-32]. In The Netherlands, the sector Vaccines of the National Institute of Public Health and the Environment (now Netherlands Vaccine Institute, NVI) has produced experimental meningococcal OMV vaccines mainly containing PorA subtypes. These vaccines proved to be safe and immunogenic in young children[33-35]. In order to evaluate the quality of the immune response induced by these vaccines, additional laboratory correlates of protection are urgently needed. Furthermore, the extent of protection offered by these vaccines depends on the cross-reactivity of the antibodies against the PorA subtypes present in the vaccine and needs to be investigated.

Aims of the thesis

In this thesis several aspects of the innate and acquired immune response of children with meningococcal sepsis or septic shock against *N. meningitidis* are described, as well as the immune response induced by experimental outer membrane vesicle vaccines. Specific aims were:

-analysis of the role of cholesterol, lipoproteins and chemokines in the innate immune response of children with severe meningococcal disease
-study of the function of peripheral blood mononuclear cells in response to meningococcal OMVs and the influence of LPS
-evaluation of the avidity of antibodies induced by invasive meningococcal disease
-evaluation of antibody avidity maturation and IgG isotype distribution after vaccination with monovalent as well as multivalent OMV vaccines
-analysis of the cross-reactivity of antibodies against PorA induced by a multivalent OMV vaccine.

Outline of the thesis

The first part of this thesis (chapter 2) contains studies on several aspects of the immune response against *N. meningitidis* in children with meningococcal sepsis. **Chapter 2.1** reviews the role of polymorphisms in genes involved in the host response on the susceptibility for and severity of meningococcal disease. **Chapter 2.2** describes the role of cholesterol and lipoproteins as pathophysiological and prognostic factors in meningococcal sepsis. In **chapter 2.3** levels of chemokines in serum of children in the initial phase of meningococcal sepsis are described. In **chapter 2.4** the response of PBMCs of patients with meningococcal sepsis to meningococcal outer membrane vesicles is described, and in **chapter 2.5** the influence of LPS in meningococcal OMVs

on the cytokine and chemokine production of PBMCs is described.
The second part (chapter 3) of this thesis involves several studies on the immune response after vaccination with an experimental OMV vaccine. First, the recent developments in vaccines for prevention of serogroup B disease are reviewed in **chapter 3.1**. Then, in **chapter 3.2**, the laboratory assays for evaluation of immunogenicity of new vaccines are discussed. In **chapters 3.3 and 3.4** the avidity ELISA as a new method for evaluation of OMV vaccine immunogenicity is described and related to SBA and IgG isotype distribution. In **chapter 3.5** the cross-reactivity of PorA-antibodies after vaccination with a multivalent OMV vaccine is studied.
Finally, in **chapter 4**, the previous studies and future research perspectives are discussed. In **chapter 5** a short English and Dutch summary of the thesis is presented.

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Chapter 2

Meningococcal sepsis in children: genetics and pathogenesis

Chapter 2.1

Genetic influences on meningococcal disease

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Crit Care 2002, 6: 60-65

Abstract

This review discusses the possible involvement of a variety of genetic polymorphisms on the course of meningococcal disease. It has been shown that several common genetic polymorphisms can either influence the susceptibility to meningococcal disease or can account for a higher mortality rate in patients. Gene polymorphisms concerning antibody receptors, LPS binding receptors or proteins, innate complement proteins as well as cytokines and hemostatic proteins are described. The study of genetic polymorphisms might provide important insights in the pathogenesis of meningococcal disease and could make it possible to identify individuals who are at risk of either contracting or dying from meningococcal disease.

Introduction

Neisseria meningitidis is an intracellular, gram-negative diplococcus, which can cause serious illnesses in children as well as in adults. The spectrum of disease varies from a common cold to life-threatening disorders including meningitis and/or a fulminant septic shock. Meningococcal sepsis is characterized by a sudden onset of fever and a petechial or purpuric rash, which may be followed by hypotension and multiple organ failure. Mortality rates are up to 40%[1]. Meningococcal sepsis is characterized by an exceptionally high level of lipopolysaccharide (LPS) found in blood or cerebrospinal fluid. Additional features of meningococcal sepsis include a severe capillary leak syndrome caused by endothelial damage due to circulating mediators, neutrophils and platelets and disseminated intravascular coagulation leading to microthrombi. Late sequelae include skin necrosis and occasionally amputation of limbs.
Rates of carriage are estimated to be 10% in the general population when cultured by nasopharyngeal swabbing and up to 45% when cultured from tonsillar tissue[2]. In contrast, typical annual incidences of meningococcal disease are 5.9 per 100 000 in Ireland and 3.5 per 100,000 in the Netherlands to 0.9-1.5 cases per 100,000 population per year in the United States[1,3,4]. Thus, despite a high carriage rate progression to invasive disease occurs rarely. The clinical picture of patients with meningococcal infections admitted to the hospital varies from mild bacteremia or chronic meningococcemia to a lethal septic shock syndrome. This raises the questions why some patients die within hours despite intensive treatment and why meningococcal bacteremia in other patients is a self-limiting disorder? We propose that this may be explained by variability in host genetic factors. Convincing evidence for the importance of the genetic background in relation to susceptibility to infectious disease was provided by Sørensen et al[5]. They showed that adult adoptees, of whom a biologic parent had died from an infection before the age of 50, had a 5-fold higher risk of dying from an infectious cause than control subjects. In general, host genetic factors include gene mutations, resulting in an absent or deficient protein, which predisposes for disease. Gene mutations are very rare and are estimated to play a role in less than 1% of meningococcal disease cases. Genetic polymorphisms however, are stable gene variants that occur rather frequently in the general population and do not follow simple patterns of heritability. Usually they have minor effects on the regulation or function of proteins, but these subtle changes may very well have important consequences on susceptibility to disease. Furthermore, it has been shown that some genetic polymorphisms influence the severity of the course of a disease and therefore may account for higher mortality rates[6]. Thus, studying these polymorphisms may answer some of the questions about meningococcal disease. This review attempts to describe the current knowledge of the role of genetic influences in severe meningococcal infections (Fig.1).

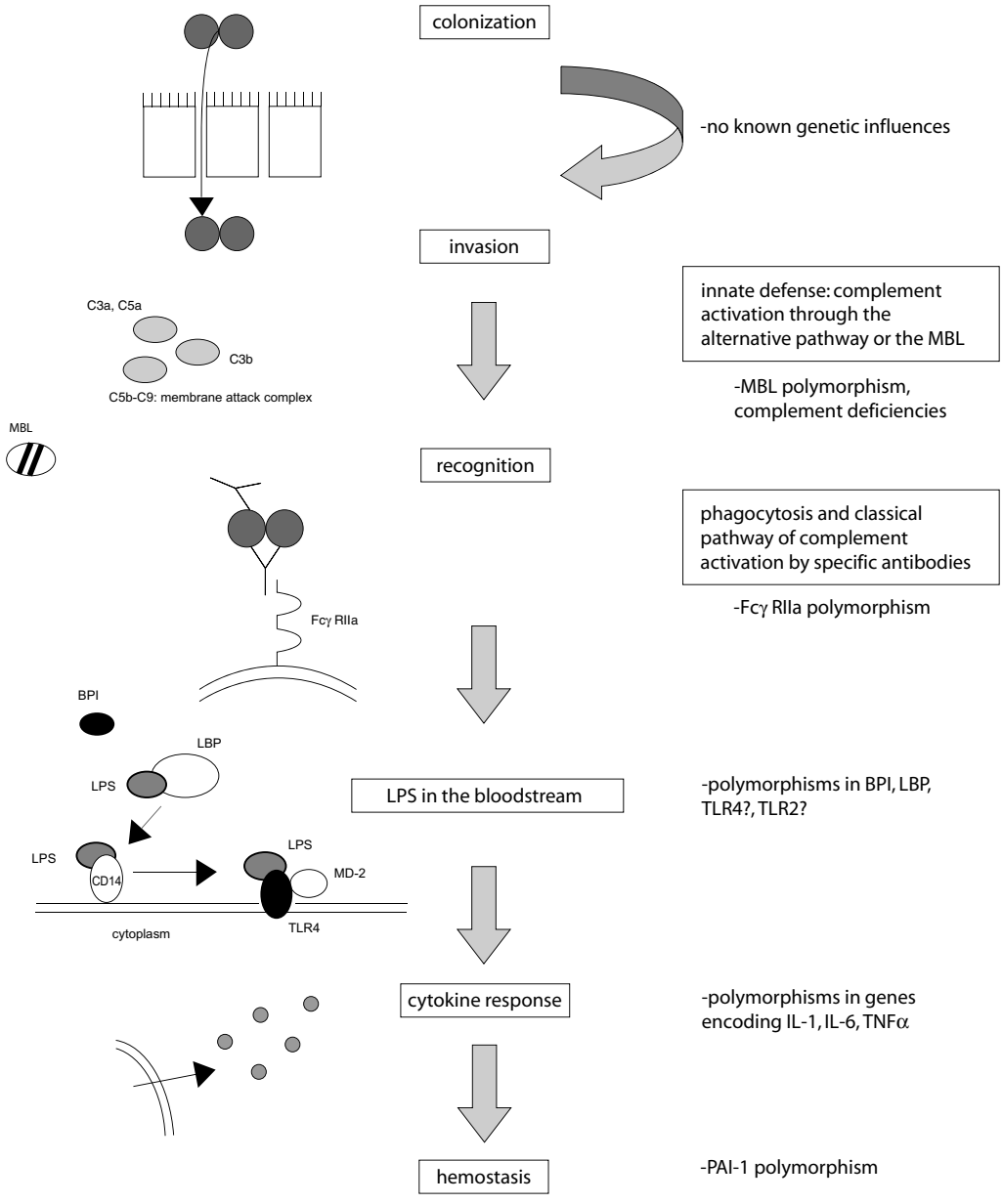

Figure 1. The possible involvement of genetic polymorphisms on the course of meningococcal disease.

Colonization and invasion

Colonization and invasion of the nasopharyngeal mucosa are the first steps in the pathogenesis of meningococcal disease. In short, the filamentous pili proteins on the bacterial surface bind to the CD46 receptor after which Opa and Opc proteins can bind to their respective receptors: CD66 and a heparan sulfate proteoglycan receptor[7-10]. Invasion of the mucosal cells by meningococci then occurs by endocytosis[11]. Active or passive smoking is a well-known risk factor for colonization, but no genetic risk factors in the host for colonization or invasion have been established[12].

Recognition, phagocytosis and complement

Fc-gammareceptor polymorphisms

Antibodies against *Neisseria meningitidis* are important for complement-mediated killing through the classical pathway as well as for phagocytosis of the bacteria. Polymorphonuclear leukocytes possess three major classes of receptors for the constant regions of IgG (FcγR): FcγRI (CD64), FcγRIIa (CD 32) and FcγRIIIb (CD16). FcγRIIa is the only receptor capable of binding IgG1, IgG2 as well as IgG3[13]. Two allotypic forms of this receptor are known: FcγRIIa-H131 and FcγRIIa-R131, based on a single amino acid difference: a histidine (H) or an arginine (R) at position 131[13]. Homozygous FcγRIIa-R/R131 are much less effective in binding IgG2, and therefore less effective in phagocytosis of *Neisseria meningitidis, Haemophilus influenzae* type b and *Staphylococcus aureus*[14,15]*.* This allotype was present in 44% of children surviving a meningococcal septic shock as compared to 23% in a healthy population[14]. Another study showed an association between the FcγRIIa-R/R131 allotype and susceptibility to meningococcal disease in individuals older than 5 years[16]. However, this accounts for less than 30% of all patients, since the majority of pediatric patients suffering from meningococcal disease is younger than 4 years. In all age groups, a correlation was found between FcγRIIa allotype and severity of meningococcal disease, indicated by a longer duration of hospitalization and a higher percentage of complications for patients with the FcγRIIa-R/R131 allotype. Neutrophils with the heterozygous allotype FcγRIIa-R/H131 showed intermediate levels of phagocytosis resulting in intermediate associations with susceptibility to and severity of meningococcal disease[16]. The role of Fc-gamma receptor polymorphisms in complement-deficient individuals has been extensively studied, because these people are highly depending on phagocytosis in their defense against meningococci. The distribution of the different allotypes was not different in late complement component-deficient (LCCD) individuals from the general population but again severity of disease was positively correlated with the FcγRIIa-R/R131 allotype[17]. Thus, LCCD patients with

this allotype are not more susceptible to meningococcal disease but due to ineffective phagocytosis their course of disease is significantly more severe. However, the combination of FcγRIIa-R/R131 and FcγRIIIb-NA2/NA2, which is another genotype associated with a reduced capacity of phagocytosis by neutrophils in vitro, was associated with a higher susceptibility to meningococcal disease of LCCD patients[18].

Complement deficiency

Activation of the complement cascade will eventually lead to the formation of a membrane attack complex, which results in lysis and cell death of the bacterium. Especially deficiencies of the alternative pathway and terminal component deficiencies appear to have a large effect on susceptibility to as well as severity of meningococcal disease, emphasizing the importance of the complement-dependent defense against this particular pathogen. Individuals suffering from LCCD for example have a 7000 to 10 000 higher risk of symptomatic meningococcal infections. However, mortality rates of meningococcal disease in these patients are much lower: 1.5% vs.19% in the general population. Furthermore, the first episode of meningococcal disease occurs at a median age of 17 years, whereas in the general population the median age of occurrence is 3 years[19]. Other complement component deficiencies associated with an increased susceptibility to meningococcal disease are properdin and factor D deficiencies, both components of the alternative pathway. Since no genetic polymorphisms are known with respect to these complement components, detailed discussions about these diseases will be left out in this review.

MBL polymorphisms

Specific antibodies as well as proteins of the innate immune system can activate the complement system. One of these innate pathways is activated by mannose-binding lectin (MBL). This protein binds to sugars on microbial cellwalls and activates two mannose-binding-lectin-associated serine proteases: MASP-1 and MASP-2[20]. Defects in MBL can result in impaired complement activation through this pathway. The *MBL* gene determines the amount of MBL present in plasma. Three variant alleles of exon 1 of this gene have been identified[21-23]. Individuals who are homozygous for one of these variants or carry two different variant alleles have plasma concentrations of less than 1% of wild-type concentrations, whereas heterozygotes have plasma concentrations of about 10%[24]. A large study in children with meningococcal disease revealed that the rates of homozygotes as well as heterozygotes *MBL* variant genotypes were much higher in patients than in controls (7.7 vs 1.5%, 29.9 vs 22.1% respectively). Strikingly, the results of the same study suggested a milder course of disease in individuals with variant genotypes, as is also seen in individuals with LCCD[25]. *MBL* variant genotypes however, are much more common than LCCD in the

general population (especially in African and Oriental populations) and could therefore account for a larger number of meningococcal disease cases[21].

The role of LPS and signal transduction

Meningococcal lipopolysaccharide, which is released in enormous amounts during meningococcal sepsis, consists of a polysaccharide part and lipid A, which is responsible for the toxicity of the molecule. LPS can bind to lipopolysaccharide binding protein (LBP) which is produced by hepatocytes and forms a complex[26]. This complex is recognized by the LPS-receptor CD14 on polymorphic nuclear neutrophils and macrophages[27]. The CD14 receptor itself does not have a trans-membrane part, and therefore cannot directly transduce the LPS signal[28]. It has been shown more recently that the toll-like receptor 4 (TLR4) and its co-factor MD2 are essential in the process of LPS signal transduction[29-31]. TLR4/MD-2 initiates the LPS-induced cell response by signaling through the cell membrane, via several signal pathways including activation of NF kappa γ, leading to gene replication and production of pro-inflammatory mediators in the cell nucleus[32]. The response of TLR4/MD-2 on LPS is enhanced by the presence of CD14[28], but much is still unknown about this process.
Theoretically, polymorphisms of the LBP gene could affect the structure of LBP and therefore influence the host defense against LPS. Two LBP genotypes have been studied, both in which a single nucleotide is exchanged for another, resulting , resulting in a different amino acid: T_{292}→G(Cys_{98}→Gly) and C_{1306}→T (Pro_{436}→Leu). No significant differences in the distribution of these genotypes were found between a group of patients with sepsis and healthy controls. However, there was a gender-related relationship between the Cys_{98}→Gly polymorphism and sepsis: a significantly higher proportion of male patients had at least one Gly_{98} allele compared to the control group. It was suggested that this unfavorable association might account for the worse prognosis for male patients with sepsis compared with female patients[33]. There is some evidence that common mutations in the *tlr4* gene are associated with LPS hyporesponsiveness in humans[34]. Also, an excess of rare amino acid polymorphisms in the TLR4 have been shown to have a deleterious effect in humans and are present at significantly higher frequencies in patients with meningococcal sepsis compared to healthy individuals[35]. (B. Beutler, personal communication). Furthermore, it has been shown that an LPS deficient mutant of *Neisseria meningitidis* is capable of inducing the release of pro-inflammatory cytokines by macrophages due to signaling via TLR2[36]. Therefore, genetic alterations in the *tlr2* gene may also prove to be important in influencing the response to LPS in humans.
Another protein with a high affinity for LPS is bactericidal/permeability increasing protein (BPI) which is stored within neutrophilic granules and recently studied as an

adjunctive treatment drug for severe meningococcal sepsis[37]. Three different polymorphisms of the BPI gene were studied as well, but no significant difference in genotype distribution was found between patients and controls[33]. It has to be noted that this study was undertaken in a general sepsis population and therefore the results may not be representative for meningococcal sepsis.

Cytokine response

TNF-α

Mononuclear phagocytes release TNF-α upon stimulation with LPS. The level of TNF-α greatly varies among patients with meningococcal disease. The genetic influence on TNF-α release has been shown clearly by several studies. For example, Westendorp et al found that 60% of the variation in TNF-α production appears to be genetically determined[38]. It has been assumed that a high level of circulating TNF-α is associated with a severe course of disease[39]. However, Westendorp et al also found that family members of non-survivors show a low TNF and high IL-10 production upon whole blood stimulation with LPS[38]. This suggests that an anti-inflammatory cytokine genotype is unfavorable for the outcome of meningococcal disease, which is in contrast with the association of high TNF levels and adverse outcome shown by other studies[39,40]. A biallelic gene polymorphism in the restriction site of NcpI within the TNF-ß gene was associated with a high level of circulating TNF-α , but the influence of this polymorphism on outcome in meningococcal sepsis is unclear[41]. Furthermore, a genetic polymorphism has been found in the promotor region of TNF-α, consisting of a single base replacement at position – 308 (guanosine vs. adenosine), but there is a lack of consensus about the relevance of this polymorphism[42-44]. In summary, it is clear that TNF-α production during meningococcal sepsis is greatly influenced by genetic factors, but the impact of these factors is still under discussion.

IL-1

The IL-1 gene family consists of several genes: *IL1A* and *IL1B* encode the pro-inflammatory mediators IL-1α and IL-1β respectively, whereas *IL1RN* encodes the anti-inflammatory IL-1 receptor antagonist. Single nucleotide polymorphisms occur in all these genes resulting in biallelic genotypes, and have been studied by Read *et al*. The allelic distribution within the *IL1A* gene had no significant consequences but homozygous individuals at position –511 within the *IL1B* gene were more likely to die from meningococcal disease. Furthermore, individuals carrying the rare allele (either homo- or heterozygous) of the *IL1RN* gene were also more likely to die. A combination of these two genotypes leads to an even higher mortality rate up to 42%[43]. Another polymorphism within *IL1RN,* studied in a general sepsis population, is located within

intron 2, which contains a variable number of basepair repeats. Five different alleles can be identified based upon the size of this repeat. The allele frequency of IL-1raA2 was increased in patients with severe sepsis compared with healthy individual[45]. Thus, this gene polymorphism might influence the susceptibility to meningococcal sepsis.

IL-6

IL-6 is a pro-inflammatory cytokine that usually is present in high amounts in meningococcal sepsis patients. A well-known polymorphism in the IL-6 promotor region is a G to C transition at position –174. The G/G genotype is associated with higher levels of IL-6 and this is correlated with high mortality in sepsis or septic shock patients in general[46-48]. As for meningococcal disease, patients with a G/G genotype had a three-fold higher risk of death compared with all other patients' genotypes[49]. This corresponds well with the observation that higher levels of IL-6 are associated with fatal outcome of meningococcal disease[50]. No differences in allele frequencies were found between patients and healthy controls[49].

Hemostasis

PAI-1

An important fibrinolysis gene polymorphism associated with meningococcal disease concerns plasminogen-activator-inhibitor-1 (PAI-1), a protease secreted by endothelial cells, thrombocytes and hepatocytes. It forms a complex with plasminogen activator which is a proteolytic enzyme involved in fibrinolysis. Meningococcal endotoxin induces extremely high levels of PAI-1 in plasma, which can eventually lead to massive coagulation. An intensively studied polymorphism within the promotor region of the *PAI-1* gene is a biallelic single basepair insertion/deletion polymorphism, resulting in either 4 or 5 guanosines at position –675. The homozygous 4G/4G genotype predisposes to higher PAI-1 concentrations upon stimulation with IL-1ß and it was shown that the level of plasma PAI-1 is significantly higher in non-survivors of meningococcal septic shock patients[51,52]. A study by Hermans *et al.* showed that a 4G/4G genotype in patients was associated with a 2-fold higher risk of death from meningococcal disease compared with those with the 4G/5G or 5G/5G genotype[53]. Susceptibility to disease was not affected by the genotype[54]. The same association with poor prognosis was found in severely injured patients after trauma[55]. It appears that 4G/4G patients respond to various stimuli with high PAI-1 levels resulting in an impaired fibrinolysis and microcirculation and therefore have a higher mortality risk. Many other polymorphisms concerning coagulation and fibrinolysis are described in other reviews[56].

Conclusions and future perspectives

It is clear that host genetic factors can play an important role in the various stages of meningococcal infections, but much is still unknown about the genetic background of contracting meningococcal disease. Genetic polymorphisms are commonly prevalent and newly found polymorphisms will have to be tested on their clinical relevance to susceptibility to and severity of meningococcal disease. Possibly, gene polymorphisms also play a role at the important level of colonization and invasion of meningococci, which would make the identification of individuals at high risk possible. Also, it is most likely that individuals with certain combinations of several polymorphisms within the above-described genes have the highest overall risk of dying from meningococcal disease. Therefore, it is of great importance that molecular-genetic techniques that can be easily and rapidly used in the clinical practice are implemented to study the interaction of multiple polymorphisms in severe meningococcal infection.

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Chapter 2.2

Serum lipids and disease severity in children with severe meningococcal sepsis

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Submitted

Abstract

Cholesterol and lipoproteins are important mediators in the innate host response against gram-negative infections. The aim of this study was to evaluate their role in children with severe meningococcal sepsis. Total cholesterol, high-density lipoproteins (HDL) and low-density lipoproteins (LDL) concentrations were measured in serum samples of 57 patients and standard deviation scores (SDS) of these parameters were calculated to correct for age-related differences. To assess disease severity, disease severity scores and selected laboratory parameters were determined. Extremely low levels of total serum cholesterol, HDL and LDL were found in the initial phase of severe meningococcal disease. Total cholesterol levels were significantly lower in nonsurvivors than in survivors, but not their SD scores. Levels of LDL and HDL did not significantly differ between both groups. Total cholesterol, HDL and LDL levels correlated with clinical and laboratory parameters of disease severity and normalized rapidly in survivors of meningococcal sepsis.

Introduction

Meningococcal sepsis is an important cause of mortality and morbidity in children and adults. The disease is characterized by an abrupt onset of fever and petechiae and may progress to purpura fulminans, septic shock and death[1, 2]. Patients with meningococcal sepsis have high levels of circulating lipopolysaccharide (LPS), a constituent of the outer membrane of *Neisseria meningitidis,* which acts as an endotoxin. Brandtzaeg *et al*. demonstrated that the level of endotoxin in the bloodstream of meningococcal sepsis patients correlates with the severity of disease and outcome[3]. In response to endotoxin high levels of tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) are produced, which are associated with septic shock and death as well[4, 5].

Serum total cholesterol as well as high-density lipoproteins (HDL) and low-density lipoproteins (LDL) are thought to be important mediators in the host response against gram-negative infections[6, 7]. Cholesterol and lipoproteins mediate LPS clearance by detoxification forming complexes and neutralizing its toxic effects[8-12]. Low levels of cholesterol and lipoproteins may therefore impair the innate immune response against gram-negative infections.

Levels *et al*. demonstrated that HDL has the highest binding capacity for LPS[13]. It has been reported that HDL-cholesterol is significantly lower in ICU-patients with an infection[4]. Extremely low concentrations of cholesterol are associated with a high risk of death in adult critically ill patients who have been admitted to the Intensive Care Unit (ICU)[4, 14, 15].

Little is known about lipid profiles in critically ill children and particularly in children with severe gram-negative infections such as meningococcal sepsis. Therefore we measured cholesterol and lipoproteins in acute phase sera of children admitted to our pediatric intensive care unit with a clinical diagnosis of meningococcal sepsis or meningococcal septic shock. The aim of this study was to evaluate the role of cholesterol and other lipoproteins in children with meningococcal sepsis or septic shock.

Patients and methods

Patients

For this retrospective study we included children admitted to the Pediatric Intensive Care Unit (PICU) of the Erasmus MC-Sophia Children's Hospital in Rotterdam between July 1997 and March 2000 and between December 2001 and July 2002 with a clinical picture of meningococcal sepsis with or without septic shock. All children fulfilled the following inclusion criteria: age between 1 month and 18 years, presence of petechiae

and/or purpura fulminans, tachycardia, tachypnea and a body temperature of < 36 or > 38.5 ˚C. Septic shock was defined as presence of sepsis with either persistent hypotension despite adequate volume suppletion or two or more features of poor end-organ perfusion (pH <= 7.3, base deficit <-5 or plasma lactate > 2.0 mmol/l; arterial hypoxia defined as pO_2 <75 mm Hg, a pO_2/FiO_2 ratio < 250 or TcO_2 saturation <96% in patients without obvious pulmonary disease, acute renal failure defined as urine output < 0.5 ml/kg/hr for at least 1 hour despite adequate fluid volume loading and without renal disease, or a sudden deterioration of baseline mental status not resulting from meningitis)[16].
Fifty-seven patients met the inclusion criteria, 38 of them participated in a randomized, double-blinded, placebo-controlled study. In this study children received either placebo or one of three dosages of protein C concentrate every 6 hours for the first three days followed by every 12 hours with a maximum of 7 days[17]. The other 19 patients participated in a prospective cohort study.

Disease severity scores
Disease severity was measured using the Pediatric Risk of Mortality (PRISM) score[18], the Sepsis-related Organ Failure Assessment (SOFA) score[19], and the Disseminated Intravascular Coagulation (DIC) score[20].

Blood sampling and assays
Arterial blood samples were drawn as soon as possible but at least within 6 hours after admission. When the arterial line was still present, again blood was drawn 12, 24, 48, 72, and 168 hours after inclusion. Samples were centrifuged and stored at -80°C. Convalescent sera were obtained from all 38 patients participating in the dose-finding study for protein C concentrate one to three months after PICU admission.
Levels of interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) were analyzed using enzyme linked immunosorbent assay (ELISA) (Sanquin, Amsterdam, the Netherlands). Values of TNF-α below the assay's detection limit (<5 pg/ml) were set at 3 pg/ml. Serum cholesterol, HDL-cholesterol and LDL-cholesterol were all determined by a homogeneous enzymatic colorimatic assay (HDL-C plus; LDL-C plus, 2nd generation, Roche Diagnostics) on the Hitachi 917 analyzer. Values of LDL-cholesterol below the assay's detection limit (<0.01 mmol/l) were set at 0.01 mmol/l. Laboratory parameters including white blood cell counts, lactate concentrations and serum C-reactive protein (CRP) were measured on admission. White blood cell count was determined by haemocytometry (Advia 120, Bayer BV). Blood lactate concentrations were determined on a blood gas analyzer (ABL 625, Radiometer Copenhagen). CRP was measured on a Hitachi 912 analyzer by an immunoturbidimetric assay (Roche Diagnostics).

Statistics

Data were analyzed using SPSS (SPSS 11.0 for Windows; SPSS, Inc., Chicago, IL.). Standard deviation (SD) scores were calculated to correct for age-related differences in cholesterol and lipoprotein concentrations. The results are expressed as medians and ranges, unless indicated otherwise. The non-parametric tests were used for comparison between survivors and nonsurvivors and for comparison of clinical and laboratory parameters within or between survivors of sepsis, survivors of septic shock and nonsurvivors of septic shock. Pearson correlation coefficients were obtained for comparison of cholesterol parameters, disease severity scores and cytokines that were log-transformed for analysis. Comparison of non-survival between patients with total cholesterol concentrations below or above the median cholesterol concentration was carried out by Fisher's exact test. Two-tailed P-values of 0.05 or less were considered statistically significant.

Results

Patient characteristics

Fifty-seven patients admitted to the PICU fulfilled the inclusion criteria and were included in the study. The group consisted of 36 boys and 21 girls. Their median age was 2.8 years (range, 0.1-16.1 years). Five children were diagnosed with sepsis without shock; they all survived (sepsis-survivors). Fifty-two children suffered from septic shock, ten of them died (18%) (shock-nonsurvivors) and 42 survived (shock-survivors). All patients showed the clinical picture of meningococcal sepsis and blood-cultures revealed *N. meningitidis* in 51 of them.

Nonsurvivors were significantly younger than sepsis-survivors (1.1 versus 6.0 years, $p<0.05$), and than shock-survivors (1.1 versus 4.3 years, $p<0.05$). The median interval between appearance of petechiae and admission differed significantly between nonsurvivors and survivors (3.4 vs. 5.0 hours, $p=0.04$). Parameters of disease severity differed significantly between nonsurvivors and survivors: PRISM score (31 vs. 20, $p=0.001$), CRP (34 vs. 88 mg/l, $p=0.001$), lactate (6.8 vs. 3.6 mmol/l, $p=0.001$), and IL-6 (1165075 vs. 41041 pg/ml, $p<0.001$). Within survivors parameters of disease severity differed significantly between shock-survivors and sepsis-survivors: PRISM score (22 vs. 9, $p=0.001$), lactate (4.1 vs. 2.2 mmol/l, $p=0.002$), and IL-6 (48085 vs. 376 pg/ml, $p=0.001$). CRP levels did not significantly differ between survivors of sepsis or shock (90 vs. 65 mg/l, $p=0.319$).

Table 1. Total serum cholesterol, HDL and LDL levels and their SDS scores in survivors and nonsurvivors of severe meningococcal disease. Values are expressed as medians (min-max). ns = not significant.

	Nonsurvivors (n=10)	Survivors (n=46)	*P- value*
Total cholesterol (mmol/l)	0.97 (0.63-1.95)	1.60 (0.51-2.90)	0.013
Total cholesterol SDS score	-3.6 (-4.3 to -2.1)	-3.1 (-5.1 to -1.4)	ns
HDL (mmol/l)	0.50 (0.21-0.98)	0.70 (0.20-1.19)	ns
HDL SDS score	-1.8 (-3.0 – 0.1)	-1.8 (-3.6 – 0.1)	ns
LDL (mmol/l)	0.27 (0.01-0.49)	0.38 (0.01-1.47)	ns
LDL SDS score	-2.5 (-3.2 to -2.1)	-2.7 (-5.1 to -1.3)	ns

Table 2. Total cholesterol and lipoproteins levels in patients with meningococcal disease. Patients are categorized into 3 different groups. Values are medians (min.-max.).

	Sepsis survivors (n=5)	Shock survivors (n=41)	Shock nonsurvivors (n=10)
Cholesterol (mmol/l)	2.3 (1.8 - 2.9)∗	1.46 (0.51-2.68)#	0.97 (0.63-1.95) †
Cholesterol SDS	-2.31 (-2.92 to -1.85)∗	-3.15 (-5.11 to -1.43)	-3.57 (-4.28 to -2.07)
HDL (mmol/l)	0.97 (0.64-1.15)∗	0.67 (0.2-1.19)	0.50 (0.21-0.98) †
HDL SDS	-1.11 (-1.89 to -0.71)	-1.84 (-3.64 to 0.14)	-1.82 (-3.04 to 0.11)
LDL (mmol/l)	0.62 (0.5-1.17)∗	0.28 (0.01-1.47)	0.27 (0.01-0.49) †
LDL SDS	-2.60 (-4.15 to -1.84)	-2.65 (-5.17 to -1.33)	-2.51 (-3.29 to -2.09)

∗ significantly different between sepsis survivors and shock survivors

significantly different between shock survivors and shock nonsurvivors

† significantly different between sepsis survivors and shock nonsurvivors p-levels < 0.05.

Cholesterol and lipoprotein levels

In general, total serum cholesterol on admission was very low in all patients with a median of 1.43 (0.51-2.90). Median cholesterol SDS was –3.14 (-5.11 to -1.43). The hypocholesterolemia was caused by low HDL concentrations but in particular LDL cholesterol concentrations. Median HDL SDS was –1.81 (-3.64 to 0.14) whereas median LDL SDS was –2.63 (-5.17 to -1.33) (Wilcoxon Signed Rank test, $p<0.001$).

Cholesterol and lipoproteins in survivors and nonsurvivors

The cholesterol levels of survivors and nonsurvivors are shown in table 1. Total cholesterol levels were significantly lower in nonsurvivors than in survivors (0.97 vs. 1.60, $p=0.013$), while levels of LDL and HDL did not significantly differ between both groups. After correction for age-matched reference values, total cholesterol-SDS, HDL-SDS, and LDL-SDS did not significantly differ between nonsurvivors and survivors. Seven out of ten non-survivors had total cholesterol SDS lower than the median SDS of –3.14. The survival rate was not significantly different between patients with total cholesterol SDS below or above the median SDS.

Cholesterol and lipoproteins in sepsis-survivors and shock-survivors

Table 2 and figure 1 summarize the cholesterol and lipoprotein levels of the patients categorized into three groups. Sepsis-survivors had significantly higher median cholesterol SD scores than shock-survivors (-2.31 vs. –3.15, $p=0.018$). A similar trend was seen between sepsis-survivors and shock-nonsurvivors, although this difference did not reach statistical significance (-2.31 vs –3.57, $p=0.055$). HDL as well as LDL SD scores were not significantly different between the three groups.
Surprisingly, eight patients had an LDL value below the detection limit of the assay (0.01 mmol/l). We analyzed whether these patients differed in age, survival and disease severity indicated by PRISM and laboratory parameters such as lactate, IL-6 and CRP levels from the other patients. None of these parameters were significantly different. Patients with LDL concentrations lower than 0.01 mmol/l had significantly lower total serum cholesterol but not HDL concentrations.

Correlations

The PRISM, SOFA and DIC score were all inversely correlated with total cholesterol, HDL and LDL with rho's between –0.3 and –0.5 (table 3). Total cholesterol levels correlated stronger than HDL and LDL with the disease severity scores. Also IL-6 was inversely correlated with total cholesterol, HDL and LDL, whereas TNF-α was only inversely correlated with total cholesterol and HDL levels. Total cholesterol was positively correlated with cortisol levels. None of the cholesterol parameters correlated with the interval between appearance of petechiae and PICU admission.

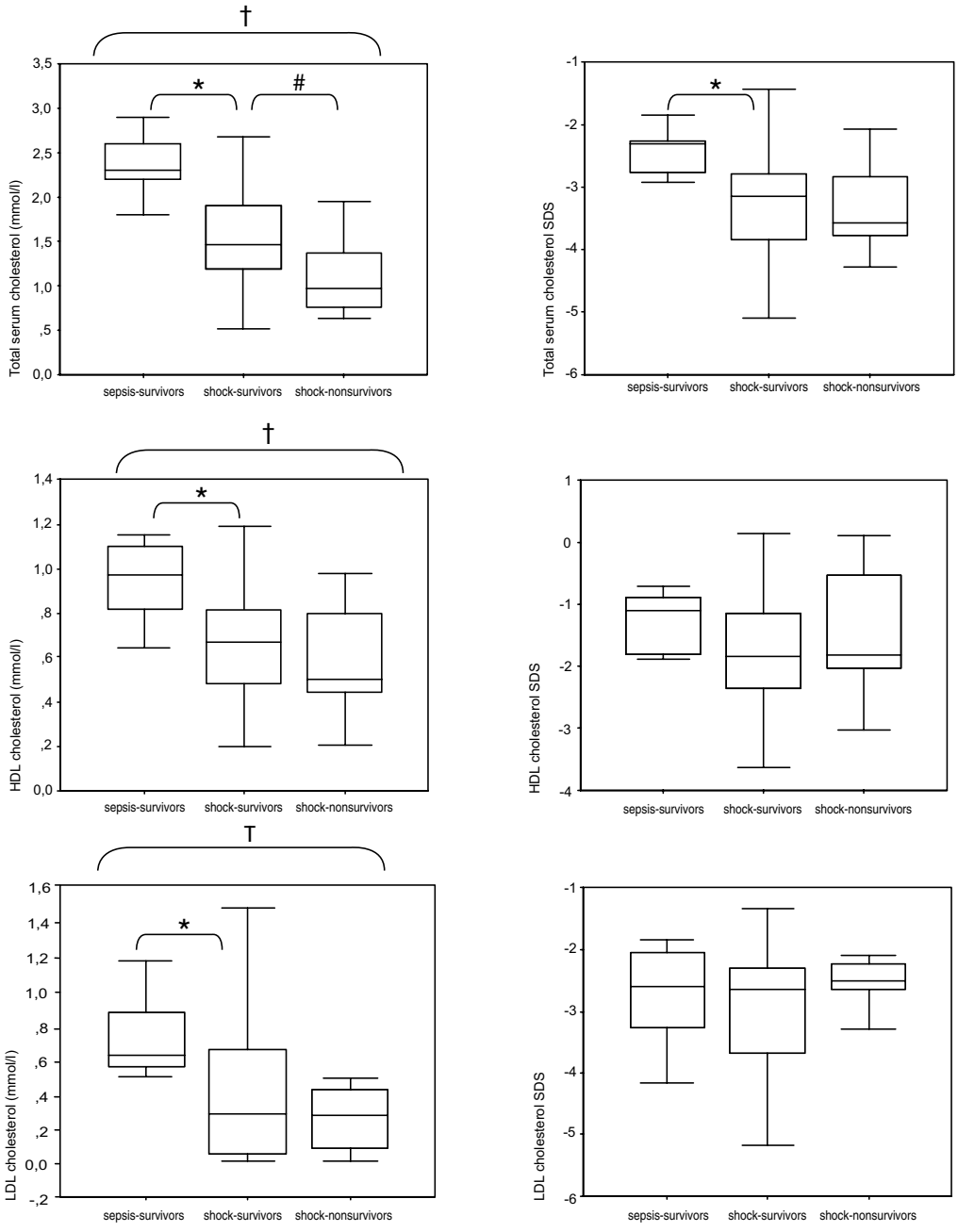

Figure 1. Total cholesterol, HDL and LDL levels and SDS on admission.
⋆ significantly different between sepsis survivors and shock survivors, # between shock survivors and shock nonsurvivors, † between sepsis survivors and shock nonsurvivors.

Table 3. Values are Pearson correlation coefficients; only significant (* P < 0.05, **P < 0.01) values are presented. PRISM, Pediatric Risk of Mortality score; SOFA, Sepsis-related Organ Failure Assessment score; DIC, Disseminated Intravascular Coagulation score.

	PRISM	SOFA	DIC	Log IL6	Log TNF-α	Cortisol	Total chol.	HDL	LDL
Total chol	-0.5**	-0.5**	-0.4**	-0.5**	-0.3*	0.4**	1	0.7**	0.8**
HDL	-0.4**	-0.3*	-0.4**	-0.4**	-0.3*	-	0.7**	1	0.6**
LDL	-0.3*	-0.4**	-0.4**	-0.3**	-	0.3*	0.8**	0.6**	1

Time course

In figure 2 the time course of total cholesterol SDS is depicted for sepsis-survivors and shock-survivors. In both groups total cholesterol SDS steadily increased from 24 hours after admission. Sepsis survivors reached the lower limit of normal after 24 hours, while shock survivors did after 72 hours of PICU admission.

Reconvalescent values

One to three months after PICU admission, reconvalescent levels were assessed in 38 out of 41 shock survivors (table 4). Total cholesterol, HDL and LDL levels and SD scores all were normalized within 3 months.

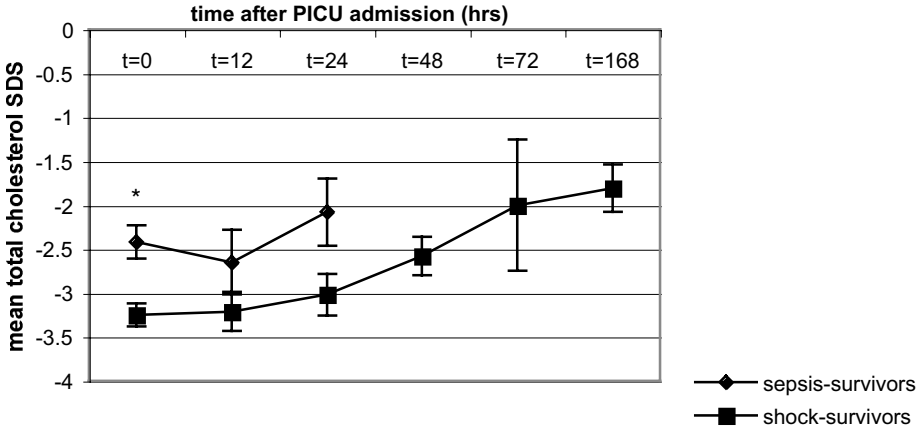

Figure 2. Mean cholesterol SDS ± SEM in patients surviving meningococcal sepsis or septic shock in time after PICU admission. * significant difference between sepsis-survivors and shock-survivors.

	Shock survivors (n=38)
Cholesterol (mmol/l)	3.79 (2.83-5.10)
Cholesterol SDS	0.28 (-1.89 to1.84)
HDL (mmol/l)	1.10 (0.67-1.96)
HDL SDS	-0.18 (-2.21 to 2.18)
LDL (mmol/l)	2.10 (1.16-3.47)
LDL SDS	-0.09 (-2.49 to 2.13)

Table 4. Reconvalescent total cholesterol, HDL and LDL cholesterol levels in children one to three months after meningococcal septic shock.

Discussion

This study is the first to analyze cholesterol and lipoprotein concentrations in children with severe meningococcal sepsis. We found markedly reduced levels of total serum cholesterol, up to –5 SD score. The hypocholesterolemia was associated with low HDL and extremely low LDL concentrations. Patients recovering from meningococcal sepsis showed a steady increase in cholesterol levels already after 24 hours up to subnormal levels. Like other authors, we found lower total cholesterol levels in nonsurvivors than in survivors[21, 22], but neither total cholesterol SDS nor lipoprotein levels and their SD scores were significantly different between these groups[23]. Nonsurvivors were significantly younger than survivors and cholesterol levels increase with age, explaining the similar SD scores. However, the absolute level of total cholesterol might be important in LPS neutralization, while total cholesterol SDS is not.
Moreover, within the group of patients who survived meningococcal disease, we found significantly higher total cholesterol SD scores in sepsis survivors than in shock survivors, indicating that severe hypocholesterolemia in children with meningococcal disease is associated with disease severity and not with disease outcome. This is supported by the inverse correlations between total cholesterol and lipoproteins SD scores, disease severity scores and cytokine levels.
Data on cholesterol and lipoprotein concentrations before the onset of meningococcal disease in these children were not available. The SD scores of cholesterol and lipoproteins one to three months after PICU admission were normalized in all patients, indicating that these children do not suffer from primary but from acquired hypocholesterolemia. This has been described several times in critically ill adults either suffering from infections, other severe diseases or undergoing surgery, but never in children. The cause of this acquired hypocholesterolemia is unknown but probably multifactorial. One possible explanation is that the acute phase response itself is responsible, as was shown in animal studies[24, 25]. Furthermore, cytokines can decrease apolipoprotein synthesis in the liver[26]. High levels of several pro- and anti-

inflammatory cytokines are present in serum of children with meningococcal disease, however, in our study these levels did not correlate with cholesterol or lipoprotein SD scores. Other explanations are the possible upregulation of scavenger receptors removing lipoproteins from the circulation or the redistribution of lipoproteins from the intravascular to the extravascular compartment due to the massive capillary leakage known to be present in meningococcal disease[27]. Finally, several studies have shown that HDL but also LDL are capable of LPS binding and that up to 80% of circulating LPS can be bound by lipoproteins[13, 28].

Surprisingly, we found particularly low LDL concentrations instead of HDL, which has been most commonly associated with the ability of LPS-binding in infectious diseases[28]. Eight patients even had an undetectable LDL concentration which is rarely ever seen. The reason for this finding is unclear.

In our study, total cholesterol concentrations were positively correlated with cortisol levels. Cortisol levels are especially low in children with severe septic shock, which is not yet understood. Relative adrenal insufficiency may be involved in this mechanism. However, cholesterol is obligatory for the production of cortisol and the positive correlation between cholesterol concentrations and cortisol levels suggests that a shortage of cholesterol as a substrate for cortisol may play an additional pathophysiological role in the hypocortisolism seen in these patients. This requires further study.

Reconstituted HDL (rHDL) has been proposed as an adjunctive therapy in severe gram-negative sepsis by increasing the LPS-binding capacity. Recently, rHDL was found to attenuate organ dysfunction in an animal model of endotoxic shock[29]. However, most of these studies are performed by using LPS from *E. coli* or Salmonella species and not from *N. meningitidis*. In our study, hypocholesterolemia in patients with meningococcal disease was mainly associated with extremely low LDL concentrations and to a lesser degree with low HDL concentrations. The total cholesterol, HDL and LDL levels correlated with parameters of severity (scores), and with levels of cytokines, as an indirect reflection of levels of LPS, but not with outcome.

In conclusion, extremely low levels of total serum cholesterol, HDL and LDL were found in the initial phase sera of children with severe meningococcal disease. Only total cholesterol levels were significantly lower in non-survivors than in survivors, but not the SD score. Total cholesterol, HDL and LDL levels on admission were inversely associated with disease severity as depicted by PRISM, SOFA and DIC scores and cytokine levels. Hypocholesterolemia was also associated with hypocortisolism. The concentrations of total cholesterol and lipoproteins steadily increase after 24 hours in recovering patients and were normalized one to three months after PICU admission.

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Chapter 2.3

CC and CXC chemokines accurately predict mortality and disease severity in meningococcal sepsis

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Submitted

Abstract

Background: Chemokines are a superfamily of small peptides, involved in leukocyte chemotaxis and the induction of cytokines in a wide range of infectious diseases. Little is known about their role in meningococcal sepsis in children and their relationship with disease severity and outcome.
Methods: Monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1α, growth related gene product (GRO)-α and interleukin-8 (IL-8) were measured in 58 children with meningococcal sepsis or septic shock on admission and 24 hours thereafter. Nine patients died. Serum chemokine levels of survivors and non-survivors were compared and chemokine levels were correlated with prognostic disease severity scores and various laboratory parameters.
Results: Extremely high levels of all chemokines were measured in the children's acute phase sera. These levels were significantly higher in non-survivors compared to survivors and in patients with septic shock compared to patients with sepsis ($p<0.0001$). Cut-off values for non-survival of 65407 pg/ml, 85427 pg/ml and 460 pg/ml for MCP-1, IL-8 and MIP-1α respectively all had 100% sensitivity and 94-98% specificity. Chemokine levels correlated better with disease outcome and severity than tumor necrosis factor (TNF)-α and similar to interleukin (IL)-6.
In available samples 24 hours after admission, a dramatic decrease of chemokine levels was seen.
Conclusion: initial phase serum levels of chemokines in patients with meningococcal sepsis can predict mortality and correlate strongly with disease severity. Chemokines may play a key role in the pathophysiology of meningococcal disease and are potentially new targets for therapeutic approaches.

Introduction

Neisseria meningitidis is one of the most feared causative agents in childhood infectious diseases, mainly affecting children below the age of 4 and adolescents. It can cause meningitis, sepsis and septic shock, characterized by a rapid development of petechiae or purpura fulminans. Meningococcal lipopolysaccharide (LPS), a constituent of the bacterial outer membrane, plays a central role in the pathophysiology of meningococcal sepsis. The release of large amounts of LPS in the blood stream induces a cascade of reactions by the host immune response, including massive activation of the complement system, the coagulation system and the induction of pro- and anti-inflammatory cytokines. High levels of these inflammatory mediators, such as tumor necrosis factor (TNF)-α and interleukin (IL)-6 are associated with disease fatality.

Chemokines belong to a family of more than 40 relatively small peptides, which are involved in chemoattraction and activation of leukocytes to the site of inflammation and in the induction of cytokine production. Chemokines are thus key determinants of inflammatory reactions and immunity[1-3]. These peptides are secreted by tissue cells, leucocytes and activated epithelial cells[4]. Four different subfamilies can be identified based on the highly conserved presence of the first two cysteine residues, which are either separated or not by other amino acids: the CC, CXC, CX_3C and the C chemokines[5]. Chemokines act through a family of chemokine receptors, which are present on cell types such as leukocytes, dendritic cells and endothelial cells. CXC-chemokines, which include growth related gene product (GRO)-α and IL-8, are potent chemoattractants for neutrophils, whereas the CC-chemokines, including monocyte chemoattractant protein (MCP)-1 and , macrophage inflammatory protein (MIP)-1α, attract monocytes, lymphocytes, basophils, eosinophils and natural killer cells. The C and CX_3C families are represented by only one chemokine each: lymphotactin and fractalkine, respectively. Lymphotactin is thought to be mainly involved in chemoattraction of lympocytes whereas fractalkine, a membrane-bound molecule expressed on endothelial cells, mediates the capturing and adhesion of circulating leucocytes[6, 7]. Chemokines and their receptors play an important role in the innate immunity against infectious diseases such as HIV/Aids and malaria, but also in autoimmune diseases[8, 9]. The role of chemokines in meningococcal sepsis or septic shock, has not been studied intensively so far. In meningococcal disease, lipo-oligosaccharide and outer membrane proteins of the meningococcus induce a strong inflammatory response in patients. Studies in patients with bacterial meningitis, caused by *Neisseria meningitidis, Streptococcus pneumoniae* or *Haemophilus influenzae*, showed high levels of IL-8 and MCP-1 in cerebrospinal fluid and variably increased levels of GRO-α and MIP-1α[10-13]. In studies on chemokines in patients with meningococcal sepsis or septic shock, only serum levels of IL-8 and RANTES

(regulated on activation, normal T cell expressed and secreted) have been reported. IL-8 levels are positively correlated with disease severity and outcome, as opposed to RANTES, which is significantly lower in patients with severe disease and in non-survivors[14-16]. The aim of this study was to measure the serum levels of CXC- and CC-chemokines during the initial phase of meningococcal sepsis in children and determine the role of these peptides in disease severity and mortality.

Materials and Methods

Patients

Children with a clinical diagnosis of meningococcal sepsis or septic shock were included in the study. Inclusion criteria for meningococcal sepsis were: age between 1 month and 18 years, a petechial rash and/or purpura fulminans, tachycardia, tachypnea and a body temperature < 36 or > 38.5˚C. Inclusion criteria for meningococcal septic shock were all of the above and either persistent hypotension despite adequate volume suppletion or two or more features of poor end-organ perfusion (pH <= 7.3, base deficit <-5 or plasma lactate > 2.0 mmol/l; arterial hypoxia defined as pO_2 <75 mmHg, a pO_2/FiO_2 ratio < 250 or TcO_2 saturation <96% in patients without overt pulmonary disease, acute renal failure defined as urine output < 0.5 ml/kg/hr for at least 1 hour despite adequate fluid volume loading and without renal disease, or a sudden deterioration of baseline mental status not resulting from meningitis). As soon as possible but at least within 6 hours after admission on the Pediatric Intensive Care Unit blood was drawn from an arterial line and serum and plasma samples were collected and stored at -80˚C until assays were performed. For this study, either serum or plasma was used to measure chemokines by means of enzyme-linked immunosorbent assay (ELISA). When the arterial line was still present, blood was again drawn 24 hours after inclusion and serum or plasma samples were collected and stored.

Thirty-eight children with a meningococcal septic shock participated in a randomized, placebo-controlled dose-finding study of protein C concentrate[17]. In this study children received either placebo or one of three dosages of protein C concentrate every 6 hours for the first three days followed by every 12 hours with a maximum of 7 days. Serum samples used in the present study were drawn just before infusion of study medication and 24 hours after start of the treatment.

Assays

Serum levels of GRO-α, MIP-1α and MCP-1 were measured by ELISA (Quantikine, R&D Systems) according to the manufacturer's instructions. Samples were first diluted 1:2 in the appropriate buffer and when chemokine concentrations of chemokines were above the upper limit of the standard curve of the assay, additional dilutions up to

1:500 were made. The lower detection limit for GRO-α, MIP-1α and MCP-1 was 20 pg/ml. IL-8 levels were measured by ELISA (Sanquin, Amsterdam, the Netherlands). Clinical data were collected at inclusion and Pediatric Risk of Mortality (PRISM) scores were assessed. Laboratory parameters including white blood cell counts, lactate concentrations and serum C-reactive protein (CRP) were measured on admission.

Statistical analysis

For statistical analysis, samples with chemokine levels below the detection limit were assessed the value of the detection limit of the assays. Values are given as median and ranges. Differences in chemokine levels between survivors and non-survivors were analyzed by Mann-Whitney U tests. ROC curves were calculated for all chemokines to determine the best cut-off values in predicting disease outcome. Correlations between chemokine levels and disease severity parameters were investigated by calculating the Spearman's rho correlation coefficient (r_s). All tests were two-tailed and a p-value< 0.05 was considered significant.

Results

Patients

Fifty-eight patients were included, of which 6 had a meningococcal sepsis and 52 septic shock according to the criteria. The median age of the patients was 4,0 years (range 0,1–16,1). Nine patients died of septic shock (15.5%). Thirty-seven patients needed ventilatory support at the time of first sampling (64%). Mean PRISM score on admission was 22.0 (95% confidence interval (CI) 19.6-24.4), mean SOFA score was 10.2 (95% CI 9.0-11.3) and mean DIC score was 5.1 (95% CI 4.6-5.7). Mean lactate concentration was 4.4 mmol/l (95% CI 3.8-5.0), mean CRP level was 96 mg/l (95% CI 77-114) and mean white blood cell counts were 11.6 x 10^9/l (95% CI 9.4-13.8).

Chemokines

MCP-1 and IL-8 were detectable in all patient samples with a median value of 5340 (range 91- 445600) and 9541 (28-427500) respectively. MIP-1α was detectable in 33 out of 58 patients (57%) with a median value of 164 (20-9784) and GRO-α levels were detectable in 40 patients (69%) with a median value of 892 (20-101150). MIP-1α, GRO-α and MCP-1 levels were significantly higher in patients with septic shock than those in patients with sepsis (p=0.009, p=0.005 and p=0.006, respectively), whereas there was no significant difference in IL-8 levels between patients with sepsis and septic shock (p=0.066). Chemokine levels on admission strongly correlated with each other, as well as with levels of IL-6 and TNF-α with Spearman correlation coefficients ranging from 0.56 to 0.91 (data not shown). Significant differences were seen between

survivors and non-survivors (Mann Whitney U test, $p < 0.0001$) for all serum chemokine levels, as well as for the cytokines TNF-α and IL-6 (Figure 1). All non-survivors had higher levels of MCP-1, MIP-1α, IL-8 and IL-6 compared to survivors. Using ROC curve analysis cut-off values of 65407 pg/ml for MCP-1, 460 pg/ml for MIP-1α, 85427 pg/ml for IL-8 and 361ng/ml for IL-6 were determined which all had 100% sensitivity and a specificity between 94 and 98% in predicting non-survival.
We found positive correlations between chemokine levels and PRISM scores (IL-8 r_s = 0.72; MIP-1α r_s = 0.67; GRO-αr_s = 0.70 and MCP-1 r_s = 0.62, $p<0.0001$) (Figure 2). Correlation coefficients between MIP-1α, GRO-α and MCP-1 and PRISM scores were higher than between TNF-α levels and PRISM (r_s = 0.56) and slightly lower than between IL-6 and PRISM (r_s = 0.73). High correlations were also found between serum chemokine levels and DIC scores, SOFA scores and laboratory parameters for disease severity and activation of coagulation such as lactate concentration, CRP and white blood cell counts, D-dimers and fibrinogen levels (Table 1). Furthermore, initial serum levels of MCP-1 and MIP-1α, but not of GRO-α and IL-8 were negatively correlated with the interval between the appearance of petechiae and time of blood sampling (both $r_s = –0.28$, $p=0.037$).
Eight out of nine non-survivors died within 24 hours after admission. Chemokine levels in available sera of children 24 hours after PICU admission showed a significant decrease, as shown in Figure 3.

Table 1. Spearman correlation coefficients between serum chemokine levels, laboratory parameters for disease severity an disease severity scores on admission.

	SOFA	DIC	lactate	WBC	CRP	D-dimers	fibrinogen
IL-8	0.62	0.70	0.41	-0.57	-0.35	0.53	-0.69
MIP1α	0.79	0.72	0.59	-0.66	-0.47	0.64	-0.83
GRO-α	0.78	0.72	0.52	-0.70	-0.43	0.66	-0.81
MCP-1	0.68	0.78	0.51	-0.70	-0.50	0.60	-0.79

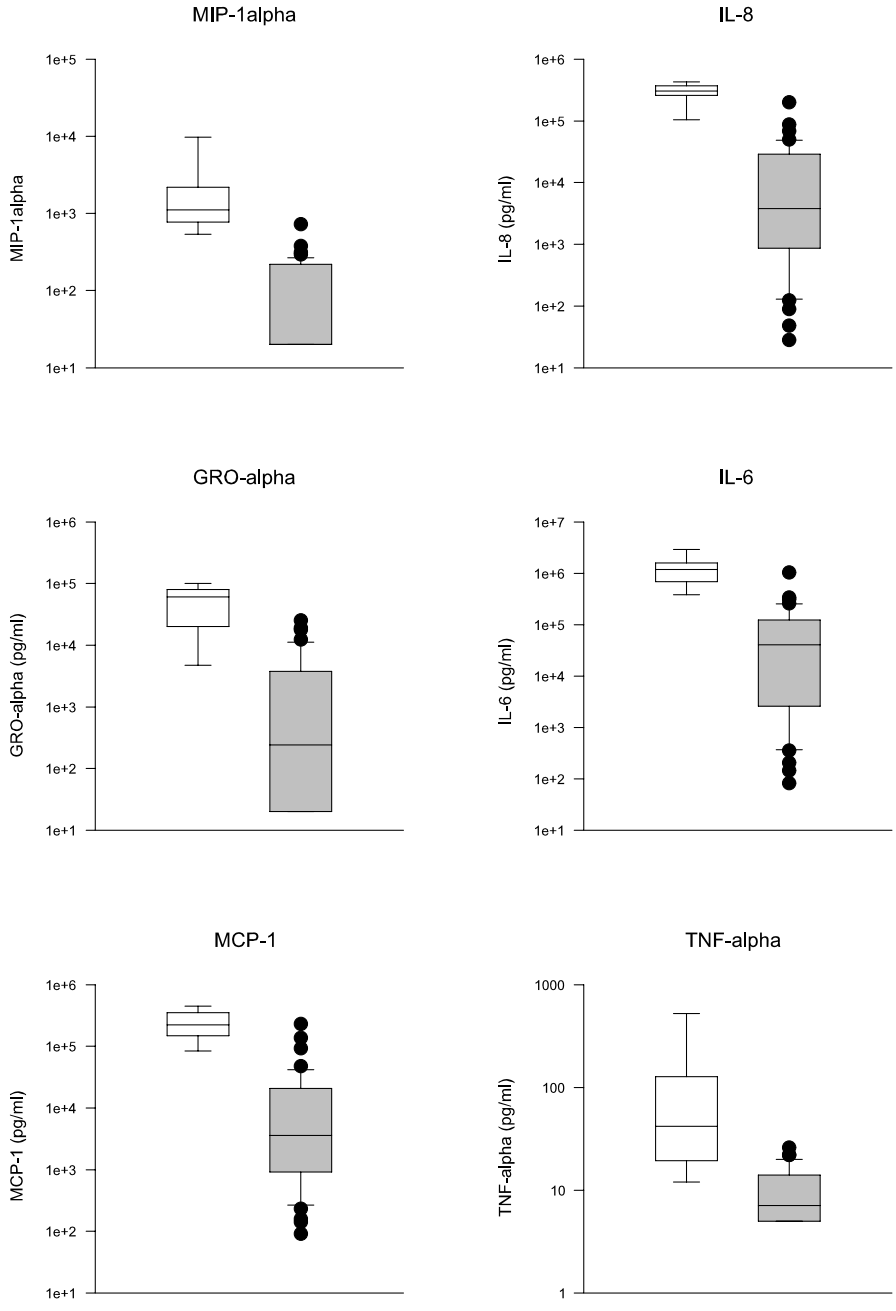

Figure 1. Levels of GRO-α, MCP-1, MIP-1α, IL-8, TNF-α and IL-6 in sera of survivors versus non-survivors of meningococcal sepsis or septic shock. Black lines in boxes represent median values, boxes represent inerquartile ranges and bars represent the range.

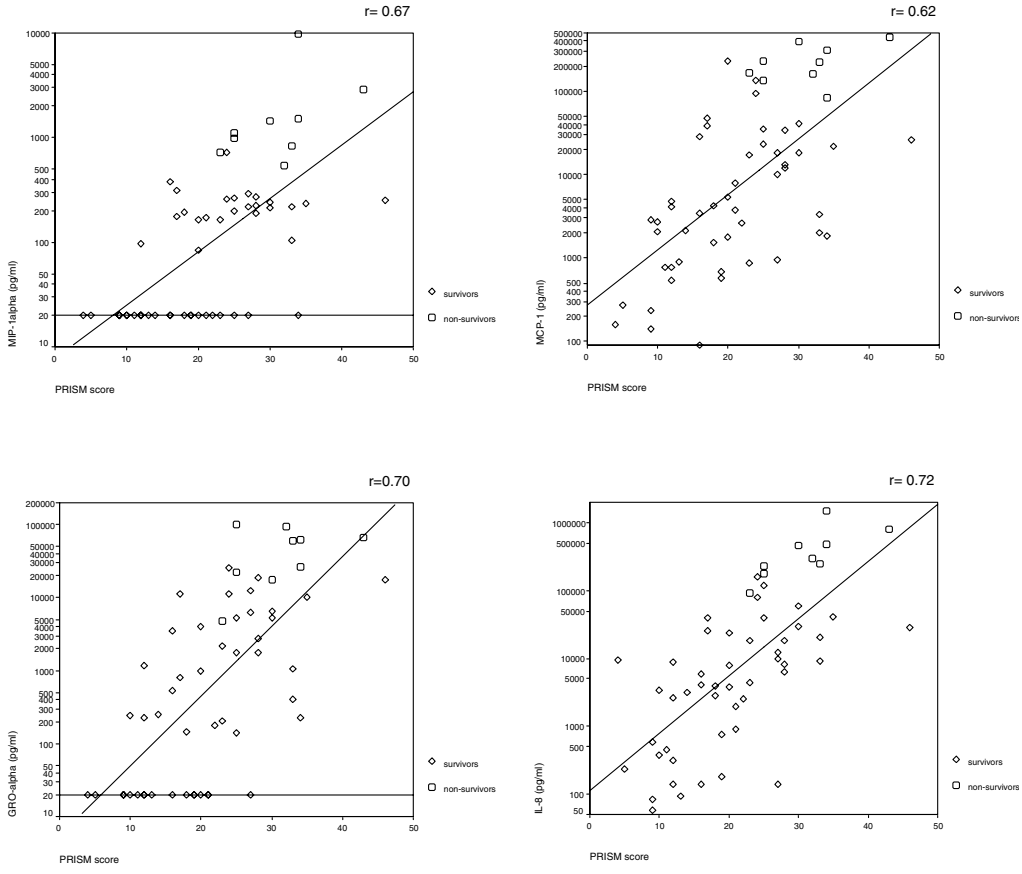

Figure 2. Correlation between chemokine levels in serum sampls of children with meningococcal sepsis or septic shock and PRISM scores on admission. The horizontal lines in the MIP-1α and GRO-α charts indicate the detection limit for the assay.

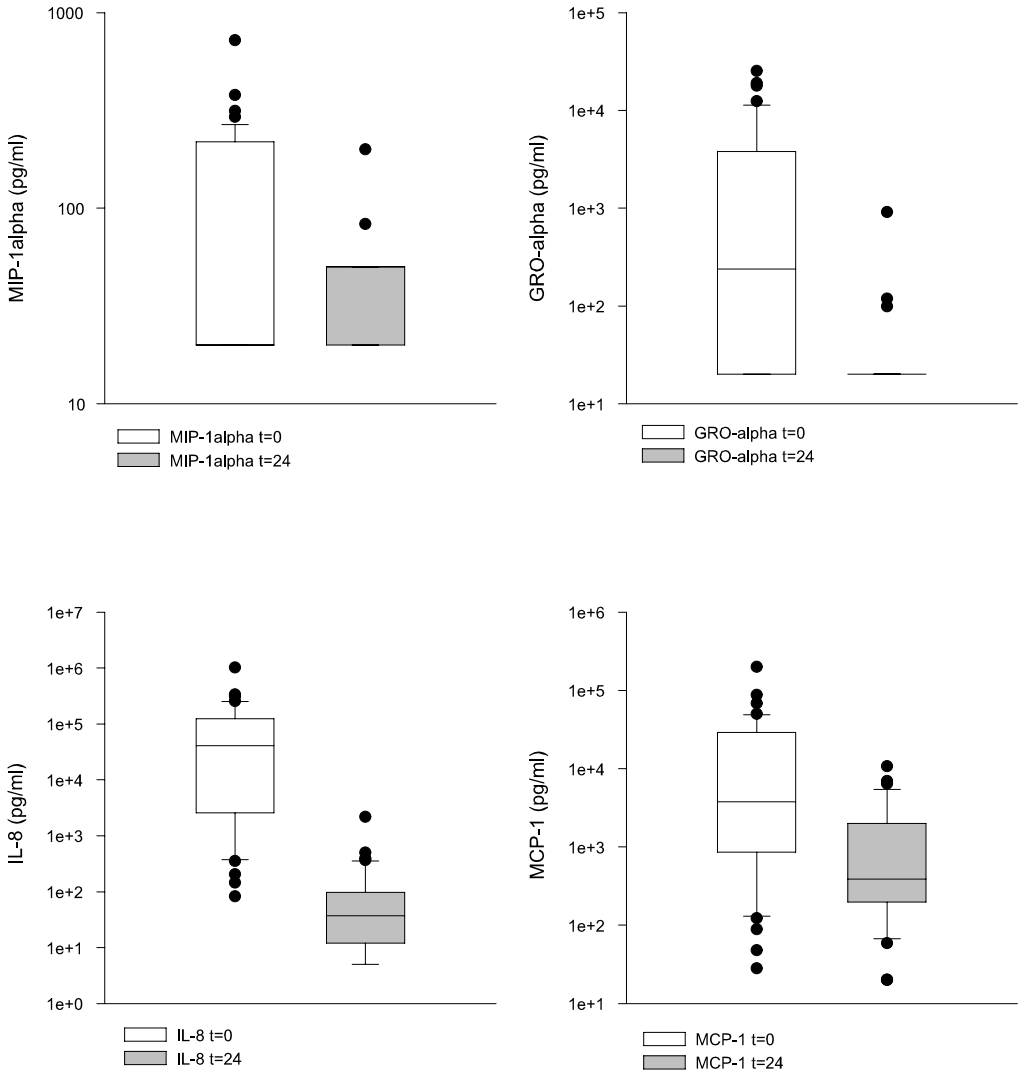

Figure 3. Chemokine levels in serum samples of children with meningococcal sepsis or septic shock on admission and obtained 24 hours after admission to the PICU.

Discussion

In meningococcal sepsis a complex network of cytokines, complement factors and coagulation and fibrinolysis factors are involved in the pathophysiology of disease as a response to the very high loads of LPS and meningococcal outer membrane proteins. Chemokines are involved in directing leucocytes to the site of inflammation and are probably necessary for the translation of the innate immune response against pathogens into a specific acquired response[18]. This study demonstrates the presence of extremely high levels of chemokines from the CC as well as the CXC family in sera obtained from children in the initial phase of meningococcal sepsis or septic shock. This implies a generalized up-regulation of both chemokine families in the early stage of meningococcal disease in children. Median levels of IL-8, MCP-1 and MIP-1α were higher than those described in patients with meningococcal meningitis[10, 11]. Furthermore, peak MIP-1α levels were 20 times and IL-8 levels 250 times higher than levels described in adult patients with sepsis[19].

MIP-1α and GRO-α were moderately elevated or even undetectable in moderately ill patients, but reached very high levels in the severely ill patients. MCP-1 and IL-8 serum levels were detectable in all patients and reached very high levels in severely ill patients, especially in non-survivors. Furthermore, there were strong significant differences between survivors and non-survivors in serum levels of MCP-1, MIP-1α and IL-8. Cut-off values with 100% sensitivity and 94-98% specificity in predicting outcome were calculated for these chemokines. This is in contrast with TNF-α, probably the most intensively studied cytokine in meningococcal disease, for which a cut-off value of 22.5 had a 78% sensitivity and 98% specificity. IL-6, another cytokine known to be involved in meningococcal sepsis was also higher in all non-survivors than in survivors[20, 21]. In our study, IL-6 had a cut-off value with a similar sensitivity and specificity as chemokines. The inverse correlation between MCP-1 and MIP-1α and the interval between the appearance of petechiae suggests that these chemokines play a major role in severely ill patients in whom the course of disease is more rapid than in other patients. Chemokine levels also correlated with disease severity, as indicated by the high correlations between disease severity scores and laboratory parameters. Correlations between chemokine levels and PRISM scores were higher than correlations between TNF-α and PRISM, indicating that serum levels of these chemokines are a better predictor for disease severity than TNF-α.

Common polymorphisms in the MIP-1α, MCP-1 and IL-8 genes have been recently discovered and are associated with an increased production of these chemokines[22-24]. Further research is needed to determine the role of these genetic polymorphisms in the severity of meningococcal disease in children.

In conclusion, levels of CXC and CC chemokines in the initial phase of meningococcal

sepsis can predict disease severity and outcome. They may be suitable candidates for implementation in prognostic scores based on laboratory parameters. The results of our study suggests that chemokines may play a key role in the pathophysiology of meningococcal disease and that chemokines as well as its receptors are potentially new targets for therapeutic approaches. Chemokine receptor antagonists, anti-chemokine antibodies and broad-spectrum chemokine inhibitors are currently under development[25, 26]. Future research will have to show their applicability in meningococcal disease.

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Chapter 2.4

Ex vivo chemokine production by peripherial blood monoclear cells from children with meningococcal sepsis

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Submitted

Abstract

In adult patients with sepsis or the systemic inflammatory response syndrome (SIRS) ex vivo stimulation assays show that monocytes as well as neutrophils suffer from endotoxin tolerance. This phenomenon has never been studied in children suffering from the most severe gram-negative invasive infection: meningococcal sepsis. The aim of the present study was to examine the release of tumor necrosis factor (TNF)-α, CC chemokines monocyte chemoattractant protein (MCP)-1 and macrophage inflammatory protein (MIP)-1α and CXC chemokines interleukin (IL)-8 and growth related gene product GRO-α of peripheral blood mononuclear cells (PBMC) retrieved from children with meningococcal sepsis upon stimulation with meningococcal outer membrane complexes (OMC).

PBMCs isolated from 20 children in the acute phase of meningococcal sepsis or septic shock on our pediatric intensive care unit (PICU), 24 hours, 3 and 7 days after PICU admission and from healthy adult volunteers were incubated with H44/76 OMCs for 4, 6 and 24 hours. All patients survived. Levels of TNF-α, MCP-1, MIP-1α, IL-8 and GRO-α were measured in cell culture supernatants using enzyme-linked immunosorbent assay (ELISA). Disease severity parameters including pediatric risk of mortality (PRISM) score and laboratory parameters were assessed.

IL-8 and MCP-1 production was significantly higher in patients than in controls and for IL-8 this persisted during the whole study period. TNF-α, GRO-α and MIP-1α production was always similar in patients and controls. MIP-1α, TNF-α and IL-8 production were inversely correlated with PRISM scores (-0.476, -0.489 and -0.529 respectively).

We conclude that PBMCs from children with severe meningococcal disease are still highly responsive to a relevant stimulus of meningococcal OMC and that the endotoxin tolerance phenomenon is not present in the acute phase of survivors of meningococcal sepsis.

However, production of TNF-α, MIP-1α and IL-8 are inversely related to PRISM scores, indicating that PBMC production capacity diminishes in the most severely ill patients.

Introduction

Meningococcal sepsis is one of the most life-threatening infectious diseases in childhood with peak incidences in infants and teenagers. During meningococcal sepsis, extremely elevated levels of meningococcal lipopolysaccharide (LPS), or endotoxin, can be detected in the blood. LPS is mainly present in the outer membrane vesicles formed by *Neisseria meningitidis* which are massively released in the bloodstream during septicemia. LPS plays a key role in the pathophysiology of meningococcal sepsis by induction of pro- and anti-inflammatory mediators by cells of the host immune system like neutrophils, monocytes and macrophages. However, other outer membrane components of *N. meningitidis* can also induce the production of cytokines[1,2.] As was shown in ex vivo whole blood stimulation assays monocytes as well as neutrophils from adult patients with sepsis or the systemic inflammatory response syndrome (SIRS) have a reduced capacity in producing cytokines like TNF-α, IL-8, IL-6, IL-1α and IL-1β[3-7]. This phenomenon is referred to as 'endotoxin tolerance' or immunoparalysis. It has been shown in patients with infections but also in patients undergoing cardiac surgery or suffering from multi-trauma[4, 8]. In children, endotoxin tolerance has not been studied intensively.

Furthermore, it is known that peripheral blood mononuclear cells (PBMCs) produce proinflammatory cytokines but also produce several chemokines. Chemokines are involved in chemoattraction and activation of leukocytes to the site of inflammation but also in the induction of cytokine production. They can therefore be considered as crucial determinants of inflammatory reactions and immunity[9-11]. We found that chemokine levels in acute phase sera from children with meningococcal sepsis or septic shock correlate with disease severity and outcome, indicating their potentially important role in meningococcal disease (C.L. Vermont, unpublished observations). The ex vivo production of chemokines in response to LPS or other outer membrane components of meningococci by PBMCs isolated in the acute phase of meningococcal disease has never been studied.

We were interested in endotoxin tolerance of PBMCs in children during the initial phase of meningococcal disease and the persistence of this phenomenon throughout the period of manifest sepsis. Therefore, we isolated PBMCs from children with meningococcal sepsis after admission on our pediatric intensive care unit (PICU)and 24 hours, 3 and 7 days thereafter for as long as children were admitted to the PICU. Isolated PBMCs were stimulated with outer membrane complexes of a *N. meningitidis* strain, containing LPS and several outer membrane proteins. The responsiveness of the PBMCs was studied by measuring cytokine and chemokine levels in the cell culture supernatant and correlating these levels with clinical and laboratory disease severity parameters.

Material and methods

Patients

Children with a clinical diagnosis of meningococcal sepsis or septic shock who were admitted to the PICU of Erasmus MC-Sophia were prospectively included in this study after written informed consent was obtained from their parents or legal guardians. Inclusion criteria for patients with meningococcal sepsis were: age between 1 month and 18 years, a petechial rash and/or purpura fulminans, tachycardia, tachypnea, a body temperature of < 36 or > 38.5˚C and the presence of an arterial catheter for blood collection. Meningococcal septic shock was defined as the clinical diagnosis of meningococcal sepsis plus either persistent hypotension despite adequate volume suppletion or two or more features of poor end-organ perfusion (pH <= 7.3, base deficit <-5 or plasma lactate > 2.0 mmol/l; arterial hypoxia defined as pO_2 <75 mmHg, a pO_2/FiO_2 ratio < 250 or TcO_2 saturation <96% in patients without over pulmonary disease, acute renal failure defined as urine output < 0.5 ml/kg/hr for at least 1 hour despite adequate fluid volume loading and without renal disease, or a sudden deterioration of baseline mental status not resulting from meningitis).
The Pediatric Risk of Mortality (PRISM) score, the Sepsis-related Organ Failure Assessment (SOFA) score and the Disseminated Intravascular Coagulation (DIC) score were assessed of all patients on admittance to determine disease severity. Laboratory parameters including white blood cell counts, platelet counts, bicarbonate and lactate concentrations and serum C-reactive protein (CRP) were measured at each sample time point.

Blood sampling and PBMC isolation

As soon as possible but at least within 6 hours after admission on the PICU blood was drawn from an arterial line (t=0). Subsequently blood samples were taken after 24 hours, 3 and 7 days for as long as the arterial catheter was in situ. Blood was collected aseptically in 10 ml Vacutainer Cell Preparation Tubes with sodium heparin (Becton Dickinson, Franklin Lanes, NJ) and processed immediately for peripheral blood mononuclear cell (PBMC) isolation as described by the manufacturer. In short, after centrifugation of the tubes at 800 *g*, plasma was removed and stored at -80˚C. The interphase containing PBMCs was removed and washed twice with RPMI 1640 buffer (Gibco, Grand Island, NY) supplemented with 1% penicillin/streptomycin, 20% fetal bovine serum and 1% glutamin (culture medium) by centrifuging for 15 minutes at 395*g*. After washing, the cell pellet was resuspended in 750 µl culture medium, after which an equal volume of culture medium containing 10% dimethylsulphoxide (DMSO) was added drop wise, while gently mixing by shaking the tube. Cells were then transferred to 1.5 ml sterile and pyrogen-free vials (Nalgene) and frozen at -80˚C in a

special container for slow-freezing. After 1-3 days, the samples were transferred to a -135˚C freezer and stored until assayed.
PBMCs of controls were isolated and stored similarly.

Preparation of outer membrane complexes
Outer membrane complexes (OMCs) of meningococcal strain H44/76 were isolated as described earlier[12]. The protein content was determined by using the bicinchoninic acid protein assay reagent (Pierce Chemical Co.), with bovine serum albumin as a standard.

Stimulation assay
The vials containing PBMCs were thawed quickly in a 37˚C water bath and transferred to sterile 15 ml tubes. Ten ml of culture medium was added and for 20 minutes cells were allowed to recover in the dark. Tubes were centrifuged for 10 minutes at 398g and the pellet was resuspended in culture medium. Cells were washed twice to remove DMSO by centrifuging for 10 minutes at 398g. After washing, PBMCs were counted and diluted with culture medium to obtain a suspension of $5.10^{E}5$ PBMCs/ml. PBMC viability was evaluated by tryptan blue coloring and was found to be >95% in each sample.
Aliquots of 200 µl/well were cultured in 96-well plates and stimulated with 0.1 µg/ml suspension of H44/76 OMCs or culture medium (negative control) in duplicate. In the same assay, control PBMCs were stimulated similarly. After 4, 6 and 24 hours, plates were centrifuged and cell culture supernatant was collected in polypropylene tubes (Micronic bv., Lelystad, the Netherlands) and stored at -80˚C until assayed.

Cytokine and chemokine assays
TNF-α, IL-8, MCP-1, MIP-1α and GRO-α were measured in cell culture supernatant and in plasma by enzyme-linked immunosorbent assay (ELISA). TNF-α and IL-8 were measured using ELISA kits from Sanquin (Amsterdam, the Netherlands) and MCP-1, MIP-1α and GRO-α were measured using Quantikine ELISA kits from R&D Systems Inc. (Minneapolis MN) according to the manufacturer's instructions. Culture supernatants were diluted in the appropriate buffer and when chemokine concentrations were above the upper limit of the standard curve of the assay, additional dilutions up to 1:1000 were made. The lower detection limit for GRO-α, MIP1α and MCP-1 was 20 pg/ml, for TNF-α 10 pg/ml and for IL-8 50 pg/ml.

Statistical analysis
Data are expressed as means ± sem. Student's t-tests were used to compare production of cyto- and chemokines between patients and controls, between two

subgroups of patients and within patients. Pearson correlation coefficients were calculated between chemokine production and disease severity parameters.

Results

Twenty patients were included in the study period between November 2001 to June 2002. Five of them met the criteria for meningococcal sepsis and 15 for meningococcal septic shock. After 24 hours samples of 17 patients were obtained, on day 3 of 11 patients and on day 7 four patients were still admitted to the PICU. All patients survived. The mean age of the patients was 7.0 years (SD 4.6). The mean PRISM score on admission was 19.7 (SD 10.4).
Release of IL-8 and MIP-1α was assessed after 4 hours of PBMC stimulation, GRO-α and MCP-1 after 24 hours and TNF-α after 6 hours of stimulation. Table 1 shows the mean release in patients and controls on time of admission, 24 hours, 3 and 7 days thereafter. In general, cytokine release induced by H44/76 OMCs in patients was similar or higher than in controls. On admission IL-8 and MCP-1 production was significantly higher in patients than in controls and for IL-8 this persisted during the whole study period. TNF-α, GRO-α and MIP-1α production was always similar in patients and controls. Unstimulated patient PBMCs produced consistently low levels of chemokines which were similar to those produced by control PBMCs (data not shown). We stratified patients into two groups of 10 according to their PRISM scores being higher or lower than 19.5 in order to compare patients with a moderate disease severity to those with very severe disease and prolonged illness. Table 2 compares the cyto- and chemokine release on admission between these two groups. MIP-1α production was lower in patients with high PRISM scores (independent sample T test,

Table 1. Cyto- and chemokine release of PBMCs from children with meningococcal sepsis on consecutive moments during PICU stay. TNF-α levels were assessed after 6 hours, IL-8 and MIP-1α after 4 hours and GRO-α and MCP-1 after 24 hours of stimulation with meningococcal H44/76 OMCs (0.1 μg/ml). Values are given as mean ± SEM.

Time		TNF-α (pg/ml)	IL-8 (pg/ml)	GRO-α (pg/ml)	MIP-1α (pg/ml)	MCP-1 (pg/ml)
Controls	(n=6)	1188±247	4495±523	10771±2568	5746 ±2191	2989±618
Patients	t=0 (n=20)	1105±300	12707±2088*	16530±3315	4420±1079	25902± 4962*
	t=24 (n=17)	1619±490	15484±3111*	16604±3278	5919±1379	5229±1042
	t=d3 (n=11)	1431±704	15011±3862*	16859±3954	7969±2149	10273± 2565*
	t=d7 (n=4)	668±74	12185±2650*	12257±7662	7585±4325	4271 ± 1674

* patients values significantly higher than controls

Table 2. Cytokine and chemokine production of PBMCs from children with meningococcal sepsis divided into two groups, either having a PRISM score< 19.5 or > 19.5. Values are given as mean ± SEM.

	Patients PRISM <19.5 n=10	Patients PRISM > 19.5 n=10	P value
TNF-α (pg/ml)	1679 ± 516	532 ± 203	0.053
IL-8 (pg/ml)	15769 ± 3434	9644 ± 2129	0.147
GRO-α (pg/ml)	20131 ±5045	12028 ± 3731	0.235
MIP-1α (pg/ml)	6530 ± 1855	2311 ± 670	0.047
MCP-1 (pg/ml)	21496 ±4422	30308 ± 8949	0.389

p=0.047) and a similar trend was seen for TNF-α production (p=0.053). Release of other chemokines was not significantly different.
Correlations were calculated between cyto- and chemokine production at timepoint t=0 and several disease severity parameters assessed on admission, such as PRISM, DIC and SOFA scores, WBC, serum CRP and lactate levels. MIP-1α production was strongly correlated with GRO-α, TNF-α and IL-8 production (Pearson correlation coefficients 0.634, 0.791 and 0.754 respectively). MIP-1α, TNF-α and IL-8 production were inversely correlated with PRISM scores (-0.476, -0.489 and -0.529 respectively) whereas no significant correlations were found with SOFA or DIC scores. Only TNF-α production was positively correlated with WBC (r=0.596).
MIP-1α production was inversely correlated with MIP-1α levels found in serum of the same patients (Spearman rank correlation r= -0.647) whereas none of the other studied cyto- and chemokine production correlated with their respective serum levels.

Discussion

In this study we found that PBMCs of children with severe meningococcal disease are still highly responsive to a relevant stimulus of meningococcal OMC. Patient PBMCs react with even higher chemokine production than control PBMCs. This is in contrast with publications on cytokine release by PBMCs in adult patients with sepsis due to a large variety of causative agents, in which PBMCs were found to be hyporesponsive to stimuli[3, 6]. One explanation for this finding could be that the rapid course of meningococcal disease is responsible for the absence of endotoxin tolerance. Meningococcal sepsis often develops in hours in otherwise perfectly healthy children, whereas sepsis in adult patients is frequently a process evolving within a few days. However, studies on in vivo exposure of healthy adults to LPS showed an effect of LPS on whole blood or PBMC cytokine production within a few hours[13, 14].

Furthermore, PBMCs collected 24 hours or 3 days after PICU admission of our patients did not show a reduced responsiveness compared to PBMCs collected on admission. However, intensive treatment of the patient has then already started and might blur the results. Another possibility is that PBMCs from children in general respond with a higher chemokine release compared to adults. Our control group consisted of healthy young adults. However, we did not find any correlation between cyto- and chemokine production and age in our patient group, in which age ranged from 6 weeks to 16 years. An alternative explanation may be that these children are genetically susceptible for high chemokine production upon contact with LPS or *N. meningitidis* in general due to genetic variation in their chemokine genes. However, the role of chemokine gene polymorphisms in susceptibility or severity of meningococcal disease has not yet been studied. Indeed, the lack of endotoxin tolerance seen in these children might be the reason why children suffering from meningococcal sepsis present with such a rapidly destructive disease process.
Since all of our patients survived the disease, we could not differentiate chemokine production and survival. However, PBMCs of the most severely ill patients as indicated by their PRISM scores on admission, had lower MIP-1α production and tended to have lower TNF-α production. Furthermore, production of MIP-1α, TNF-α and IL-8 inversely correlated with PRISM scores on admission. These results indicate that in the most severely ill children PBMCs are becoming increasingly less responsive to stimuli. Probably, these children have higher circulating levels of endotoxin, which can induce the phenomenon of 'endotoxin tolerance'. Since the chemokine production level in these patients is still higher than in controls, this phenomenon seems to be irrelevant in children surviving meningococcal sepsis. IL-10 is thought to be mediating monocyte hyporesponsiveness in patients with septic shock[15]. Although it is known that high levels of IL-10 are present in meningococcal sepsis patients, apparently this is not enough to prevent massive pro-inflammatory cytokine chemokine release.
High MIP-1α serum levels have been associated with severe meningococcal disease and non-survival (C.L. Vermont, unpublished observations). In the present study, we found an inverse correlation between MIP-1α serum levels and PBMC MIP-1α production after 4 hours of stimulation. This indicates that the MIP-1α-producing capacity of patient PBMCs is an indicator of disease severity as well. Apparently, in most severely ill patients with high measurable MIP-1α serum levels, PBMCs have become exhausted by the high production level and their residual MIP-1α-producing capacity is low. None of the other chemokine serum levels correlated with their production by stimulated PBMCs. Maybe, the role of other chemokine-producing cells in the production of these chemokines is more important or PBMC production capacity for these chemokines is larger.
We conclude that PBMCs from children with severe meningococcal disease are still

highly responsive to a relevant stimulus of meningococcal OMC and that the endotoxin tolerance phenomenon is not present in the acute phase of surviving meningococcal sepsis patients. However, production of TNF-α, MIP-1α and IL-8 are inversely related to PRISM scores, indicating that PBMC production capacity diminishes in the most severely ill patients.

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Chapter 2.5

Differences in LPS upregulation of ex vivo chemokine production of peripheral blood mononuclear cells from children with meningococcal sepsis

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Submitted

Abstract

The aim of this study was to determine the role of lipopolysaccharide (LPS) in the induction of chemokine production in peripheral blood mononuclear cells (PBMCs) from children with meningococcal sepsis.
PBMCs isolated from 20 children in the acute phase of meningococcal sepsis or septic shock on our pediatric intensive care unit (PICU) were incubated with outer membrane complexes (OMCs) of H44/76, a wild-type patient-derived meningococcal strain, and its LPS-deficient mutant strain pLAK33 for 4, 6 and 24 hours. Levels of tumor necrosis factor (TNF)-α, monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1α, interleukin (IL)-8 and growth related gene product(GRO)-α were measured in cell culture supernatants using enzyme-linked immunosorbent assays (ELISA).
Production of MIP-1α, TNF-α and GRO-α was always significantly higher in the H44/76 stimulated PBMC culture supernatant, whereas IL-8 production was significantly higher after 4 and 6 hours of H44/76 compared to pLAK33 stimulation and similar after 24 hours of stimulation. MCP-1 production by patient PBMCs after stimulation with H44/76 or pLAK33 OMCs was always similar. Using H44/76 OMCs instead of LPS-deficient OMCs, TNF-α and MIP-1α production was approximately 5 times higher, whereas IL-8 and GRO-α production only increased by a factor 2.
We conclude that the contribution of LPS to chemokine production differed per chemokine. LPS is most important in the induction of TNF-α and MIP-1α; IL-8 and GRO-α production is less dependent and MCP-1 production is independent of the presence of LPS.

Introduction

Sepsis caused by *N. meningitidis* is one of the most severe acute diseases in childhood, occurring especially in children below the age of 4 years and in adolescents. During meningococcal sepsis, massive amounts of meningococcal lipopolysaccharide (LPS), or endotoxin, can be detected in the blood. Endotoxin is mainly present in meningococcal outer membrane vesicles of *N. meningitidis* which are released in the bloodstream during septicemia and is responsible for the induction of pro- and anti-inflammatory mediators synthesis. However, other components of *N. meningitidis* such as outer membrane proteins (OMP), lipoproteins and capsular polysaccharide can also induce the production of these mediators[1, 2]. Furthermore, it is known that peripheral blood mononuclear cells (PBMCs) not only produce proinflammatory cytokines but also have the capacity of producing various chemokines. Chemokines belong to a family of more than 40 relatively small peptides and are mainly involved in chemoattraction, activation of leukocyte populations and in the induction of cytokine production. They can therefore be considered as crucial determinants of inflammatory reactions and immunity[3-5]. Chemokine subfamilies can be identified based on the highly conserved presence of the first two cysteine residues, which are either separated or not by other amino acids: the CC, CXC, CX_3C or the C chemokines[6]. We found that chemokine levels in acute phase sera from children with meningococcal sepsis or septic shock predicted disease outcome and correlated strongly with disease severity, indicating their potentially important role in meningococcal disease (unpublished observations). The role of LPS in the ex vivo production of chemokines by PBMCs in the acute phase of meningococcal disease and the correlation between serum chemokine levels and ex vivo PBMC production has not been studied previously.

We were interested in the role of LPS and meningococcal OMP in the induction of CC and CXC chemokines in PBMCs from children suffering from severe meningococcal disease. Therefore PBMCs from children with meningococcal sepsis were collected as soon as possible after admission on the pediatric intensive care unit (PICU) and 24 hours, 3 and 7 days thereafter for as long as children were still admitted to the PICU. Isolated PBMCs were stimulated with outer membrane complexes (OMCs) of a wild type *N. meningitidis* strain, containing LPS and several outer membrane proteins and with OMCs from its LPS-deficient mutant strain. Chemokine levels were measured in cell culture supernatant at several time points.

Materials and methods

Patients

Children with a clinical diagnosis of meningococcal sepsis or septic shock who were admitted to the PICU of Erasmus MC-Sophia were prospectively included in this study after written informed consent was obtained from their parents or legal guardians. Inclusion criteria for patients with meningococcal sepsis were: age between 1 month and 18 years, a petechial rash and/or purpura fulminans, tachycardia, tachypnea and a body temperature of <36 or >38.5˚C. Another inclusion criterion was the presence of an arterial catheter for blood collection to minimize discomfort for the patient. Meningococcal septic shock was defined as the clinical diagnosis of meningococcal sepsis plus either persistent hypotension despite adequate volume suppletion or two or more features of poor end-organ perfusion: pH <= 7.3, base deficit <-5 or plasma lactate >2.0 mmol/l; arterial hypoxia defined as pO_2 <75 mmHg, a pO_2/FiO_2 ratio < 250 or TcO_2 saturation <96% in patients without over pulmonary disease, acute renal failure defined as urine output <0.5 ml/kg/hr for at least 1 hour despite adequate fluid volume loading and without renal disease, or a sudden deterioration of baseline mental status not resulting from meningitis.

Blood sampling and PBMC isolation

As soon as possible but at least within 6 hours after admission on the Pediatric Intensive Care Unit blood was drawn from an arterial line. Blood was collected aseptically in 10 ml Vacutainer Cell Preparation Tubes with sodium heparin (Becton Dickinson, Franklin Lanes, NJ) and processed immediately for peripheral blood mononuclear cell (PBMC) isolation as described by the manufacturer. In short, after centrifugation of the tubes at 800*g*, plasma was removed and stored at -80˚C. The interphase containing PBMCs was removed and washed twice with RPMI 1640 buffer (Gibco, Grand Island, NY) supplemented with 1% penicillin/streptomycin, 20% fetal bovine serum and 1% glutamin (culture medium) by centrifuging for 15 minutes at 395*g*. After washing, the cell pellet was resuspended in 750 µl culture medium, after which an equal volume of culture medium containing 10% dimethylsulphoxide (DMSO) was added drop wise, while gently mixing by shaking the tube. Cells were then transferred to 1.5 ml sterile and pyrogen-free vials (Nalgene) and frozen at -80˚C in a special container for slow-freezing. After 1-3 days, the samples were transferred to a -135˚C freezer and stored until assayed.

Preparation of outer membrane complexes

Outer membrane complexes (OMCs) of meningococcal strain H44/76 and its LPS-negative mutant strain pLAK33[7] were isolated as described earlier[8]. The protein

content was determined by using the bicinchoninic acid protein assay reagent (Pierce Chemical Co.), with bovine serum albumin as a standard.

Stimulation assay
The vials containing PBMCs were thawed quickly in a 37˚C water bath and transferred to sterile 15 ml tubes. Ten ml of culture medium was added and for 20 minutes cells were allowed to recover in the dark. Tubes were centrifuged for 10 minutes at 398g and the pellet was resuspended in culture medium. Cells were washed twice to remove DMSO by centrifuging for 10 minutes at 398g. After washing, PBMCs were counted and diluted with culture medium to obtain a suspension of 5.10^E5 PBMCs/ml. PBMC viability was evaluated by tryptan blue coloring and was found to be >95% in each sample.
Aliquots of 200 μl/well were cultured in 96-well plates and stimulated with 0.1 μg/ml suspension of H44/76 OMCs or culture medium (negative control) in duplicate. In the same assay, control PBMCs were stimulated similarly. After 4, 6 and 24 hours, plates were centrifuged and cell culture supernatant was collected in polypropylene tubes (Micronic bv., Lelystad, the Netherlands) and stored at -80˚C until assayed.

Cytokine and chemokine assays
TNF-α, IL-8, MCP-1, MIP-1α and GRO-α were measured in cell culture supernatant by enzyme-linked immunosorbent assay (ELISA). TNF-α and IL-8 were measured using ELISA kits from Sanquin (Amsterdam, the Netherlands) and MCP-1, MIP-1α and GRO-α were measured using Quantikine ELISA kits from R&D Systems Inc. (Minneapolis MN) according to the manufacturer's instructions. Culture supernatants were diluted in the appropriate buffer and when chemokine concentrations were above the upper limit of the standard curve of the assay, additional dilutions up to 1:1000 were made. The lower detection limit for GRO-α, MIP-1α and MCP-1 was 20 pg/ml, for TNF-α 10 pg/ml and for IL-8 50 pg/ml.

Statistical analysis
Data are expressed as means ± standard error of mean (sem). Student's t-tests were used to compare production of chemokines induced by H44/76 OMCs and pLAK33 OMCs.

Results

Twenty patients were included in the study period between November 2001 to June 2002. Of these patients, 5 met the criteria for meningococcal sepsis and 15 for meningococcal septic shock. The mean age of the patients was 7.0 years (SD 4.6) and

all patients survived.
In general, LPS-containing H44/76 OMCs were more potent in the induction of chemokines than LPS-deficient OMCs (Figure 1). Production of MIP-1α, TNF-α and GRO-α was always significantly higher in the H44/76 stimulated PBMC culture supernatant, whereas IL-8 production was significantly higher after 4 and 6 hours of H44/76 compared to pLAK33 stimulation and similar after 24 hours of stimulation. MCP-1 production by patient PBMCs after stimulation with H44/76 or pLAK33 OMCs was always similar. TNF-α production reaches a platform after 6 hours of stimulation whereas the production of the other chemokines still significantly increased over the next 18 hours.
The difference between the 2 stimuli varied depending on the measured chemokine (Figure 1). Using H44/76 OMCs instead of LPS-deficient OMCs, TNF-α and MIP-1α production were approximately 5 times higher, whereas IL-8 and GRO-α production only increased by a factor 2.

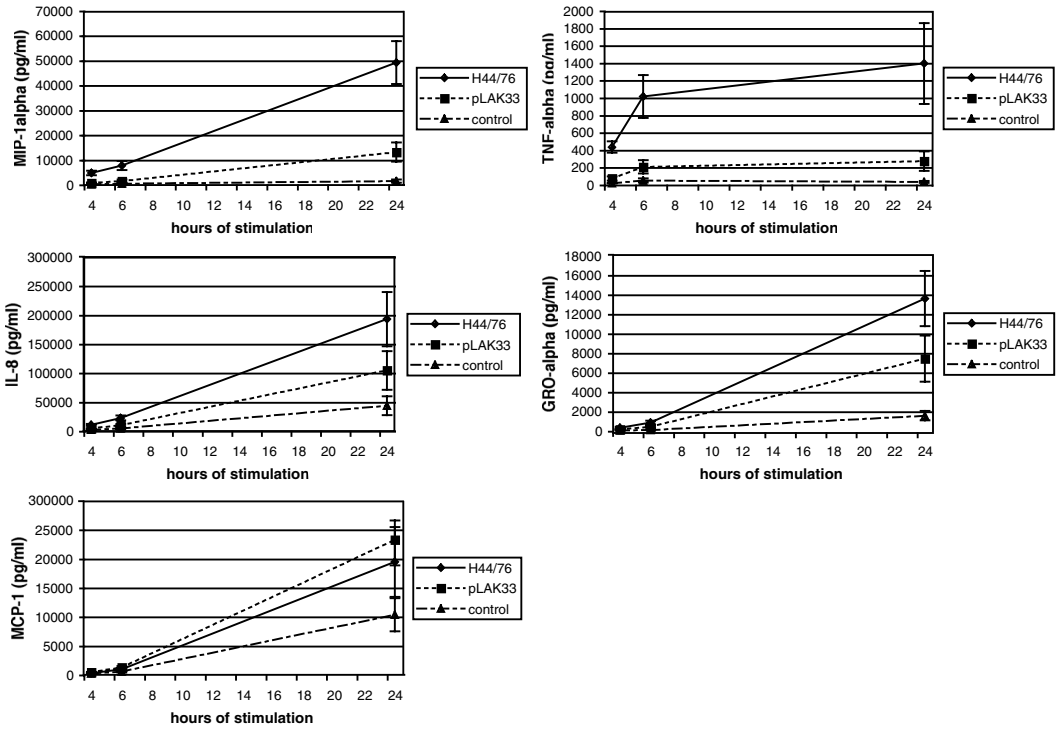

Figure 1. Mean production of TNF-α, MIP-1α, GRO-α, IL-8 and MCP-1 ± SEM of PBMCs from children in the acute phase of severe meningococcal disease. H44/76; stimulated with OMCs of wild type meningococcal strain H44/76, pLAK33; stimulated with OMCs from LPS-deficient mutant strain, control; unstimulated**.**

Discussion

We described in this study the contribution of LPS to the production of various chemokines by PBMCs. Due to limited amounts of patient PBMCs, we chose one concentration of 0.1 μg protein/ml of OMC suspension after pilot experiments using a range of concentrations. This concentration is within the range of concentrations used in earlier studies[1, 9].

Several authors have showed before that pro-inflammatory cytokine production does not require the presence of LPS. Meningococcal OMP were found to be the main factor in inducing the fulminant complement activation seen in meningococcal sepsis patients[1, 10, 11]. In an earlier study meningococcal OMCs of the LPS-deficient strain were inpotent inducers of IL-1β production by healthy human donor PBMCs, whereas in our study, these OMCs could induce substantial amounts of TNF-α and chemokines[1]. LPS-containing meningococci mainly upregulate toll-like receptor-4 (TLR-4), whereas the LPS-deficient strain activates the TLR-2 pathway[2, 9, 12].

The LPS-deficient strain was found to be a poor inducer of the nuclear factor kappa B (NF-κB) in a study of Dixon *et al.* [13]. NF-κB is thought to be the most likely molecule involved in the control of chemokine gene expression, since several binding sites for NF-κB have been found in the promoters of IL-8, GRO-α and MCP-1 genes[14-16]. Ciesielski *et al.* found that CC chemokines (e.g. MCP-1 and MIP-1α) expression induced by either TNF-α or LPS is mainly regulated through the NF-κB signaling pathway whereas CXC chemokines IL-8 and GRO-α induction by LPS does not require NF-κB[17]. This may explain the difference of only a factor 2 in IL-8 and GRO-α production between OMCs with or without LPS, and the major difference in MIP-1α-production. However, MCP-1 production was similar between the 2 stimuli and was also fairly high in the unstimulated PBMCs. MCP-1 appears to be readily produced by PBMCs without needing much stimulus. We found earlier very high levels of MCP-1 in sera from meningococcal sepsis patients (unpublished observations), corresponding with these findings. Maybe, serum factors, which are present in the culture medium, are able to stimulate MCP-1 production as well.

After 24 hours of stimulation, TNF-α, chemokines and other inflammatory mediators have reached very high levels that could co-stimulate the PBMCs and blur the role of LPS on itself. However, the difference between LPS-containing and LPS-deficient OMCs in stimulating capacity was consistently similar at all timepoints.

In conclusion, the contribution of LPS to chemokine production differed per chemokine, being most important in the induction of TNF-α and MIP-1α. The CXC chemokines IL-8 and GRO-α are less dependent on LPS and MCP-1 production is independent of the presence of LPS. This emphasizes the role of other immunomodulators present in *N. meningitidis* besides LPS in severe meningococcal disease.

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Chapter 2.6

PorA-specific antibody avidity in patients convalescing from meningococcal disease

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submitted

Abstract

Porin A (PorA), responsible for serosubtyping of *N. meningitidis,* is the main antigen of a candidate vaccine against serogroup B meningococci, which has been shown to induce high-avidity antibodies in children. In the present study we studied whether this avidity maturation is relevant for protection against disease by comparing antibody avidity after vaccination with that induced by natural meningococcal infection. Furthermore, we investigated whether antibody avidity after meningococcal disease is PorA-specific. Convalescent phase sera of 21 children with meningococcal septic shock caused by strains with PorA subtype P1.7-2,4 were used in this study. The Geometric Mean Avidity Index (GMAI) measured against a strain with the identical subtype as the infective isolate was significantly higher than against a strain with a heterologous PorA subtype or a PorA-negative mutant strain (57% versus 35% and 23% respectively, $p= 0.005$ and $p<0.001$). Geometric Mean Avidity Titers (GMATs) were highest for P1.7-2,4, corresponding with the highest GMAI. Serum bactericidal activity was lower than 4 in all convalescent sera. The GMAI after invasive meningococcal disease was lower than after vaccination with a monovalent P1.7-2,4 OMV vaccine. In conclusion, a PorA-specific antibody avidity maturation response was seen in meningococcal sepsis patients after convalescence. The GMAI after invasive meningococcal disease was lower than after vaccination with a monovalent P1.7-2,4 OMV vaccine, indicating that the immune response induced by this vaccine is relevant for protection against disease.

Introduction

Meningococcal meningitis and septicemia are important causes of morbidity and mortality in children, especially those younger than 2 years. Sequelae include loss of hearing, skin necrosis and occasionally amputation of limbs. Most cases of invasive meningococcal disease in the industrialized countries are caused by *Neisseria meningitidis* serogroup B. Candidate vaccines against this serogroup have not yet provided high efficacy rates in young children and infants. The search for a successful, widely protective meningococcal vaccine therefore still continues. The National Institute of Public Health and Environment (RIVM, currently Netherlands Vaccine Institute) developed two candidate outer membrane vesicle (OMV) vaccines, mainly containing porinA (PorA) as an antigen. PorA is responsible for the serosubtyping of *N. meningitidis*. A monovalent vaccine (MonoMen) expresses PorA subtype P1.7-2,4 whereas a hexavalent vaccine (HexaMen) contains six highly prevalent serosubtypes. Immunization of children with these vaccines induced a PorA-specific antibody response with a similar IgG isotype distribution as is seen after invasive meningococcal disease[1, 11]. We and others also showed that vaccination with MonoMen as well as HexaMen induces a significant antibody avidity maturation, which is a marker of the induction of immunological memory[2, 4, 5, 8, 11].
The immune response following invasive meningococcal disease is thought to be protective since recurrent meningococcal infections in otherwise healthy children are extremely rare. Antibodies found after systemic meningococcal infections are mainly directed against PorA, PorB, Opc and LPS[12, 13]. In children under 12 months, serum bactericidal activity (SBA) was poor when compared to children over 10 years of age, despite the presence of similar levels of IgG antibodies and a similar IgG isotype distribution[6]. Pollard *et al.* showed that differences in antibody avidity in infants are responsible for this striking observation[7]. These authors reported that the avidity of antibodies against meningococcal reference strain H44/76, which contains PorA serosubtype P1.7,16, was significantly lower in children younger than 1 year of age than in those older than 10 years. However, the PorA-specificity of the antibodies was not taken into consideration in this study. Therefore, differences in avidity maturation may depend on differences in PorA serosubtype of the strains causing disease in these children.
In the present study we analyzed immune memory after invasive meningococcal disease by measuring antibody avidity. We seek to investigate whether antibody avidity after natural meningococcal infection is serosubtype-specific and whether the quality of the immune response induced by vaccination as we previously measured by antibody avidity is relevant for protection against disease. To this purpose we selected convalescent phase sera from children who were all infected with *N. meningitidis*

serosubtype P1.7-2,4. Avidity maturation against the P1.7-2,4 antigen was measured and compared with the response against the heterologous antigen P1.7,16 and a PorA-deficient strain.

Patients and methods

Patients

Convalescent phase serum samples from children with meningococcal septic shock 1-3 months after admission to the Pediatric Intensive Care Unit of the Sophia Children's Hospital were obtained. The study was approved by the Medical Ethics Committee of the Erasmus MC. Written informed consent was obtained from the parents of the children. Sera of children with a positive blood culture for *N. meningitidis* serosubtype P1.4 were selected. The variable regions (VR) 1 and 2 of the PorA gene of these isolates were sequenced by the Netherlands Reference Laboratory for Bacterial Meningitis (Academic Medical Center Amsterdam/ RIVM) and showed that these isolates all had P1.7-2,4 as a VR1/VR2 combination.

Avidity ELISA

Antibody avidity was measured using thiocyanate[11]. In short, Immulon 2 (Dynex Technologies, Inc.) 96-well plates were coated overnight with 100 µl/well of a monovalent outer membrane vesicle (OMV) suspension at a concentration of 4 µg/ml. OMVs from strain H44/76 with serosubtype P1.7,16, as well as OMVs from its isogenic variants with serosubtype P1.7-2,4 and the PorA-negative isogenic mutant H1.5 were used as a coating. Sera were diluted 1:100 in PBS-0.1% Tween 80. A threefold serial dilution of serum samples was made in duplicate, so that the two halves of a plate contained identical serum dilutions. Plates were incubated for 90 minutes at 37°C. As a positive control, a serum sample from a volunteer who had been vaccinated with the hexavalent OMV vaccine in a phase I trial and had a high antibody titer against P1.7-2,4 and P1.7,16 was included on every plate. After three washes, 100 µl of a 1.5 M NaSCN solution dissolved in PBS was added to one half of each plate and plain PBS was added to the other half. After incubation for 15 minutes at room temperature all wells were washed three times and incubated with rabbit-anti-human IgG 1:5000 conjugated with horseradish peroxidase (HRP) for 90 minutes at 37°C. Subsequently after washing, TMB substrate was added, the reaction was allowed to proceed for 10 minutes and was stopped by adding 100 µl of 2 M H_2SO_4 per well. The absorbance at 450 nm was read using an EL312e Bio-Kinetics reader. IgG antibody titers were determined as the dilution yielding 50% of the maximal OD. Samples with antibody titers below the assay's detection limit were assigned a value of 50. Titers obtained after treatment with NaSCN were called "avidity titers". An Avidity Index (AI) was

expressed as the percentage of antibodies that remained bound at the antigen coat after treatment with sodium thiocyanate: AI = titer (NaSCN+)/titer (NaSCN-) x 100.

IgG isotype ELISA

IgG isotype distribution of the convalescent patient sera were measured by an OMV ELISA using isotype specific conjugates as described elsewhere[1]. Anti-human IgG_1, IgG_2, IgG_3 and IgG_4 antibodies conjugated with HRP were used from Sanquin (Amsterdam, the Netherlands). IgG isotype titers were measured as described for the avidity ELISA and samples with antibody titers below the detection limit were assigned a value of 50.

Serum bactericidal activity (SBA)

SBA titers were assessed as described previously[10]. Human plasma with no detectable SBA against any of the strains used in this study was used as an exogenous complement source. SBA titers were expressed as the reciprocal of the lowest serum dilution yielding ≥ 90 % killing after 60 minutes of incubation.

Statistical analysis

IgG titers, avidity titers and AIs were log-transformed and Geometric Mean Titers (GMT), Geometric Mean Avidity titers (GMAT) and Geometric Mean Avidity Indices (GMAI) with 95% confidence intervals were calculated. Differences in GMTs and GMATs against OMVs of meningococcal strains with different serosubtypes were analyzed by Wilcoxon signed rank tests. Differences in GMAIs were analyzed by paired sample t-tests.

Results

Serum samples from 21 children were used in this study. All children had a positive blood culture for *N. meningitidis*, serosubtype P1.7-2,4. The mean age of the patients was 6.3 years (SD 4.8 years). Figure 1 shows the Geometric Mean Titer (GMT) measured against OMVs of a meningococcal strain containing P1.7-2,4 or P1.7,16 as PorA antigen or a PorA-deficient strain in acute and convalescent serum samples. In the acute phase of disease GMTs were very low against all OMVs . In convalescent sera, there was no significant difference in GMTs against P1.7-2,4 and P1.7,16 ($p=0.071$). GMT against H1.5 was significantly higher compared to P1.7,16 but not when compared to P1.7-2,4 ($p<0.001$ and $p=0.063$ respectively).
The GMAT against P1.7-2,4 was significantly higher compared to the GMAT against P1.7,16 ($p=0.004$, Figure 2). GMTs were too low for assessment of GMAI in acute phase sera. However, in convalescent sera the GMAI against P1.7-2,4 was 57% which

is significantly higher than the GMAI against P1.7,16 (35%) and H1.5 (23%) ($p=0.005$ and $p<0.001$ respectively).
The IgG isotype distribution of the convalescent sera was dominated by IgG_1, followed by IgG_3, whereas no IgG_2 orIgG_4 was found (data not shown).
Serum bactericidal activity against strain H44/76 P1.7-2,4 did not reach titers higher than 2 in any of the convalescent sera, nor against strains H44/76 P1.7,16 or H1.5.

Discussion

In this study we show that the avidity of antibodies induced by a meningococcal infection in young children is specifically directed against the serosubtype of the disease-causing strain. Antibody avidity against OMVs of a strain with a different PorA subtype is significantly lower, and antibody avidity against OMVs of a PorA-negative mutant strain is even lower. These results indicate that a specific memory response against PorA is generated as a result of invasive infection and that this memory is not necessarily cross-reactive with other PorA subtypes. Since antibody GMTs in the acute sera were so low, GMAI could not be measured in these samples.
It has been well known that invasive meningococcal disease induces an antibody response mainly against PorA, but also against PorB, LPS and class 5 proteins. In this study, we focussed on studying strain-specific PorA-antibodies, since these have been found to be most relevant for bactericidal activity and protection from disease. Well-defined OMV suspensions were used as a coating in the avidity ELISA to measure antibody titers and the avidity index, in which PorA is the far most abundant antigen. These OMVs mainly contain either 1.7-2,4 or P1.7,16 PorA as a protein content, are depleted from PorB and contain only low amounts of LPS and class 5 proteins. Therefore, avidity maturation of antibodies against other antigens than PorA are not accurately measured in this set-up. However, the PorA-negative H1.5 OMVs contain large amounts of PorB and although earlier studies showed specific anti-PorB antibodies in convalescent-phase sera regardless of the serotype of the infecting strain, these antibodies were of low avidity[3].
We did not detect any significant serum bactericidal activity in the convalescent patient sera and therefore could not correlate SBA titers to GMAI. It has been shown previously that SBA is low after a natural meningococcal infection, especially in young children[6, 13]. Our group of patients had a mean age of 6.3 years, with only 3 children older than 10 years of age, and 7 under the age of 2 years, which may explain the absence of SBA in the convalescent sera. Furthermore, it should be noted that we use human complement to measure bactericidal titers. In general, human complement has been shown to measure lower SBA titers compared to baby rabbit complement[9].
The immune response after invasive disease in otherwise healthy humans is thought to be protective, despite the absence of SBA in convalescent patient sera. We compared the characteristics of the immune response in these patients to the immune response seen in children immunized with a monovalent P1.7-2,4 OMV vaccine. The IgG isotype distribution was similar between the two groups[11]. The GMAI in convalescent sera is lower in comparison with antibody avidity induced by the monovalent P1.7-2,4 OMV vaccine: 57% vs 73%. This indicates the fact that vaccination with this OMV vaccine induces even a better immunological memory than

invasive meningococcal disease and provides an encouraging view on the protection against meningococcal disease offered by this vaccine.

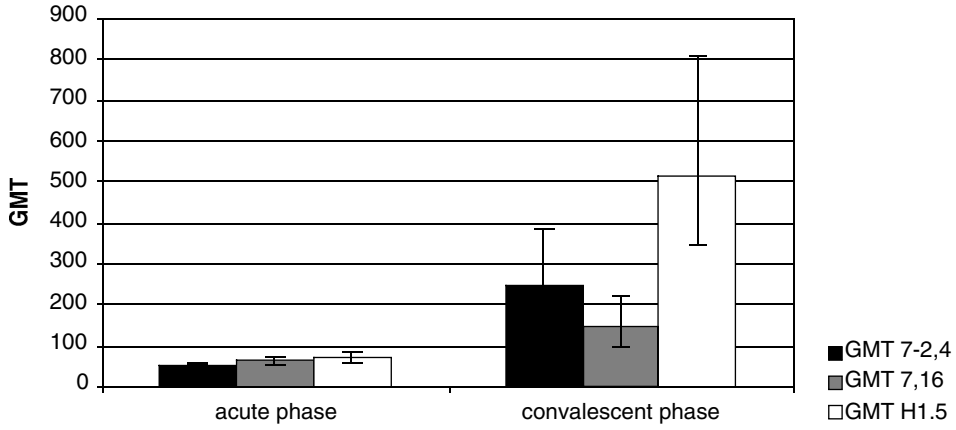

Figure 1. Geometric Mean Titer (GMT) of 21 sera in the acute phase and 1-3 months after sepsis with *N. meningitidis* P1.7-2,4 infection against outer membrane vesicles (OMVs) from strain H44/76 either expressing P1.7-2,4 or P1.7,16 PorA. Alternatively, the PorA-negative mutant H1.5 was used as source of antigen.

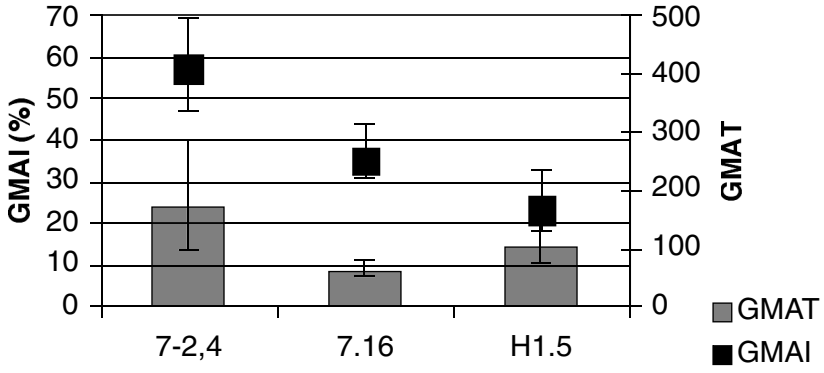

Figure 2. Geometric Mean Avidity Index (GMAI) and Geometric Mean Avidity Titer (GMAT) in convalescent patient sera 1-3 months after sepsis with *N. meningitidis* serosubtype P1.7-2,4.

Acknowledgements

We thank René Kornelisse and Ester de Kleijn for collecting patient serum samples and Wendy Keijzers for sequencing part of the PorA gene of the patient isolates.

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Chapter 3

Vaccination against *Neisseria meningitidis* serogroup B: correlates of protection

Chapter 3.1

Meningococcal serogroup B infections: a search for a broadly protective vaccine

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Expert Review Vaccines 2003 Oct; 2 (5): 673-681

Abstract

Meningococcal disease is in many West-European countries mainly caused by serogroup B. Recently a highly efficacious vaccine against infections caused by serogroup C has been introduced in Great Britain and the Netherlands. However, an effective vaccine against serogroup B has not yet become available. Outer membrane vesicle (OMV) vaccines against serogroup B were previously tested in large phase III trials, but showed a low efficacy in young children. In addition, the high variability of the vaccines' main component porin A potentially diminishes its efficacy. Therefore, several approaches in either optimizing these OMV vaccines or searching for novel, highly conserved antigens are currently under investigation. The sequencing of the meningococcal genome has provided new opportunities to detect additional immunogenic epitopes. In this review, the developments in the search for a broadly protective meningococcal serogroup B vaccine will be discussed.

Introduction

Infections caused by serogroup B meningococci remains an important cause of morbidity and mortality in children, despite the availability of effective antibiotic therapy and intensive care. The successful introduction of meningococcal serogroup C conjugate vaccines in the UK and the Netherlands has resulted in a dramatic decrease in serogroup C infections. However, the majority of meningococcal disease in Western-European countries and the USA, is caused by serogroup B. The possibility of a capsular polysaccharide switch after population-wide introduction of serogroup C conjugate vaccines requires high alertness. In order to eradicate meningococcal disease, development of a vaccine against meningococcal serogroup B, or a broadly protective vaccine against all disease-causing serogroups will remain necessary. The unraveling of the meningococcal genome in the year 2000 has greatly improved the possibilities to discover new vaccine candidates and has resulted in many new studies in this field. Candidate vaccines are studied in pre-clinical trials in animals. To assess their ability to induce functional protective antibodies, laboratory correlates of protection are needed. Usually, the presence of bactericidal antibodies in serum bactericidal activity (SBA) assays is considered as the best predictor for protection against serogroup A and C meningococci, but for serogroup B this relationship is not yet firmly established. Therefore, several other laboratory tests are used for the evaluation of potential vaccines, including animal protection models and opsonophagocytic assays. In this review, we provide an overview of present and potentially new meningococcal vaccines aimed at the prevention of serogroup B disease and discuss the developments in the field of the search for new vaccine candidates applicable as a broadly protective vaccine against *Neisseria meningitidis* serogroup B (see table 1).

Table 1. Overview of vaccine candidates for the development of a protective meningococcal serogroup B vaccine.

Antigen	Merits	Demerits	Ref.
OMV	Proven safety and immugenicity in humans, efficacy trials completed	Low efficacy in youngest children, main component (PorA) highly variable	1, 3
Recombinant porins	Passive protection in rats, incorporated in liposomes or micelles induce bactericidal antibodies	Functional antibodies only against strains with homologue porins	18-21
Mimics of capsular polysaccharide	Mab induced passive protection in animals	No immune response with peptide mimics in mice yet	28, 29
Inner core lipopolysaccharide	Relatively conserved, Mab is opsonic, bactericidal and showed passive protection in animals	Not all variants of LPS inner cores are covered	30, 32, 33
LPS molecular mimics	No toxicity problems	Peptide presentation is critical, bactericidal activity of induced antibodies not shown	34-37
NspA	Highly conserved, Mab is bactericidal, immunization with NspA shows active protection in mice	NspA is not essential for survival, thus fear of selective pressure. Accessibility of NspA hampered by high CPS expression	38-41
TbpA	Less variable than TbpB, active protection in animals	Antibodies induced in animals were not bactericidal	44, 45
TbpB	Capable of inducing bactericical antibodies and passive protection in animals	Rather variable among circulating meningococcal strains	42, 43, 45
Commensal *Neisseria* species	Evidence of cross-reactive protection by *N. lactamica* carriage, immunization with *N. lactamica* OMVs induced protective antibody in mice	Despite active protection, *N. lactamica* OMVs did not induce bactericidal antibodies in mice	46-48

Table 1, continued.

Antigen	Merits	Demerits	Ref.
GNA33	Surface-exposed, thought to be highly conserved	GNA33 turned out to be a mimic of PorA subtype P1.2	51-53
GNA2132	Passive protection conferred	High variability	54
NadA	Present in 50% of disease-causing meningococci; and in almost all isolates from hypervirulent lineages	Phase variable, surface-exposure hampered by CPS, variable bactericidal activity and passive protection in rats	55, 56
GNA1870	Seems highly conserved, induces bactericidal antibodies and passive protection	Three variants of GNA1870, little or absent cross-reactivity	57
P2086	Recombinant P2086 induces bactericidal antibodies	Two main subfamilies, little or no cross-protection	58
Hia homologue	Highly conserved, surface-exposed during invasion and carriage	No immunization studies done yet	60
App (Hap homologue)	Conserved, immunogenic in rabbits, bactericidal antibodies induced, antibodies present in sera from reconvalescing patients	No protection studies described yet	59
AspA	Well conserved, anti-AspA antibodies found in reconvalescent patient sera	Phase variable	61

Conventional vaccines against *N. meningitidis* serogroup B

Outer membrane vesicle (OMV) vaccines

Since the serogroup B capsular polysaccharide (CPS) strongly resembles polysialated glycoproteins which are expressed mainly in fetal brain tissue but also in adults, its immunogenicity is poor. Furthermore, it has been suggested that the use of CPS as a vaccine component may induce autoimmune antibodies. Therefore, the focus of vaccine development against serogroup B meningococci has been on the use of non-capsular antigens. One of the unique characteristics of meningococci is their ability to form outer membrane blebs, which contain outer membrane proteins as well as lipopolysaccharide (LPS). The principle of blebbing has been applied in vaccine development by preparing outer membrane vesicles (OMVs) by detergent extraction from meningococci. These OMVs can then be depleted from LPS to reduce toxicity. The first vaccines based on OMVs were prepared by the Finlay Institute in Cuba and the National Institute of Public Health in Norway. These two countries suffered from meningococcal serogroup B outbreaks mainly caused by serosubtypes P1.19,15 and P1.7,16 respectively. The Walter Reed Army Institute of Research produced an OMV vaccine based on a B:15 P1.3 strain as well. These OMV vaccines are the only vaccines against serogroup B that have been studied in large efficacy trials. The Cuban vaccine, which also contains serogroup C polysaccharide, is even registered in Latin American countries[1-3]. However, concerns were raised about their effectiveness in the youngest age-group. Another important drawback of these vaccines is the high variability of their main components, porins A (PorA) and B (PorB) and opacity proteins. This might mean that OMV vaccines only protect against isolates expressing the identical outer membrane proteins, making them extremely vulnerable for strong immunoselective pressure. To circumvent this problem, the Netherlands Vaccine Institute (former part of the RIVM) developed an OMV vaccine based on two strains each expressing three different PorA proteins. This hexavalent vaccine is safe and immunogenic in children and induced bactericidal titers in infants against most, but not all PorA subtypes included in the vaccine[4-7]. In order to provide a broad protection against as much serosubtypes as possible, a nonavalent vaccine is currently developed in cooperation with Wyeth, including the 9 most occurring subtypes in the industrialized countries. The Norwegian National Institute of Public Health is now co-operating with Chiron in producing a monovalent P1.4 OMV vaccine in order to control the ongoing P1.4 epidemic in New Zealand.

The large antigenic variability among meningococcal outer membrane proteins remains a complicating factor for the production of a broadly protective vaccine against all meningococcal B isolates. Currently, a new approach using OMVs as a vehicle for other polysaccharide or protein antigens is under investigation. In these OMVs

conserved outer membrane proteins or possibly other newly identified vaccine candidates can be up-regulated by replacing dispensable meningococcal genes with the gene of interest placed under a control of a strong promoter. Such a vaccine could then confer a cross-reactive immune response[8].
Alternative routes of OMV vaccine administration are also investigated. Intranasal administration would mimic the natural route of colonization and may induce mucosal antibodies besides a systemic immune response. Several studies in animals as well as in healthy adults have shown that intranasal OMV vaccination is safe and immunogenic and warrants further investigation in larger trials[9-12]. However, a comparative study of the functional antibody response after either intranasal or intramuscular vaccination in healthy adults demonstrated that intramuscular immunization induced significantly higher systemic IgG and SBA titers, whereas avidity maturation and an IgG_3 isotype response were totally absent after intranasal vaccination. Improvement of the immune response after intranasal immunisation might be achieved by altering both the vaccine formulation and the immunisation schedule[13]. Further improvement of OMV vaccines can be achieved by modifying the lipid A moiety of LPS, which reduces its toxic activity but does not affect the adjuvant activity of LPS[14]. Genetically detoxified LPS-mutant strains lacking either the *lpx*L1 or *lpx*L2 gene have been developed and OMVs from these strains have been tested for immunogenicity in animals. SBA titers were greatly reduced compared to the native strain, but the immune response was largely restored by adding aluminum adjuvant to the OMV preparations[15-17].

Recombinant porins and peptides
An alternative approach based on outer membrane proteins is the use of recombinant PorA produced in *E. coli* or *Bacillis subtilis* incorporated in liposomes, to promote native conformation of the protein, or reconstituted in the presence of detergent. These preparations induce functional antibodies in mice and protection in the infant rat infection model, but only against strains with the homologue serosubtype[18-21]. Therefore, multivalent liposomes containig 4 different recombinant PorA proteins were prepared by Humphries *et al*. and used in animal immunization studies, resulting in bactericidal sera against some of the subtypes but not against all[22]. Certain PorA subtypes seem to be immunodominant and further research is needed to enhance the immunogenicity of all subtypes included in such a multivalent vaccine. Similar results were found when recombinant PorB incorporated into liposomes or micelles preparations was used. An immunization study with these preparations in mice showed the induction of functional bactericidal antibodies. Addition of monophosphoryl lipid A (MPLA) as an adjuvant increased the immunogenicity and induced a broader range of IgG subclasses like IgG2a and IgG2b. However, the immune response was restricted

to the homologue PorB serotype and did not cross-react with heterologous serotypes[23].
The structure of one of the immunogenic loops of PorA was used for designing cyclic peptides by Oomen *et al.* The peptides were stable, conformationally correct and elicited bactericidal antibody responses in mice. This approach proves that loop epitopes of outer membrane proteins can be mimicked by designed peptides and might be useful in further vaccine development[24].

Capsular polysaccharide
Capsular polysaccharide (CPS) vaccines for the prevention of meningococcal serogroup B disease have been developed. However, the B polysaccharide is poorly immunogenic and the risk of inducing auto-immune antibodies that cross-react with glycosylated host antigens has hampered this approach. To improve its immunogenicity and in order to prevent the induction of autoimmune antibodies, the polysaccharide needs to be conjugated to a carrier protein and chemically modified from N-acetyl polysaccharide to N-propionyl polysaccharide. Even when conjugated to a protein carrier, bactericidal activity of the induced antibodies in several animal experiments depends on the choice of complement source[25]. Modified CPS bound to either recombinant porin B or covalently conjugated to OMVs induces bactericidal antibodies in monkeys. However, these vaccines have not been tested in humans[25, 26].
An interesting method to circumvent these problems is the use of molecular mimetics or mimotopes. These are peptides mimicking immunogenic epitopes of carbohydrate antigens without inclusion of regions of host cell mimicry[27]. Recently, Moe *et al.* described the use of molecular mimetics of group B polysaccharides. Several monoclonal antibodies that reacted with epitopes of the capsular polysaccharide, were distinct from host antigens and induced passive protection against experimental bacteremia were screened on large libraries of peptide phages and small molecules[28, 29]. Several peptides or peptoids were detected but to date, none of them induced a satisfactory immune response in mice immunization experiments.

Lipopolysaccharide
As opposed to CPS, *N. meningitidis* lipopolysaccharide (LPS) is immunogenic in humans since anti-LPS antibodies were found in sera from patients recovering from meningococcal disease. Up till now, 12 different LPS immunotypes have been described, and in addition to this variation, extensive phase variation of the outer core of LPS occurs. However, the inner core of LPS is relatively conserved, and might therefore be an interesting vaccine candidate. Plested *et al.*[30] discovered a Mab against an inner core LPS structure which is surface-accessible and present in 76% of a large panel of serogroup B meningococci. This inner core structure is represented by

a phosphoethanolamine at the 3-position of the β-chain heptose of the LPS inner core. The gene required for linking the phosphoethanolamine to the right position has been discovered as well[31]. The Mab has functional bactericidal and opsonic activity against the inner core structure and it was demonstrated that antibodies against the same structure were present in sera from patients re-convalescing from meningococcal disease[32]. Passive protection experiments in rats with this Mab showed protection against some but not all serogroup B meningococci which seemed to depend on the length of the LPS glycoforms present in the strain[33]. When immunogenic epitopes of the other variants of the LPS inner core are found, theoretically, a vaccine containing a few glycoforms covering all existing *N. meningitidis* immunotypes could be created. However, these other epitopes have not been detected so far and studies in humans with LPS inner core vaccines have not been performed yet.
Also, for LPS molecular mimics are being under investigation as vaccine candidates. Several phage display libraries have been screened for binding with a murine monoclonal antibody directed against immunotypes 3, 7 and 9 which account for the predominant immunotypes of disease-causing meningococcal isolates. Mice immunized with a heptapeptide conjugated to the carrier protein CRM_{197} elicited a moderate total IgG response against LPS measured by ELISA, mainly consisting of IgG_1[34]. A later study revealed that the presentation of the peptides is critical. Immunization with a cyclic peptide antigenic for the L3,7,9 Mab in mice elicited a better IgG response. So far, nothing is known about the bactericidal activity of these antibodies against circulating meningococcal strains[35-37].

NspA

In a search for new, highly conserved surface antigens, *Neisseria meningitidis* surface protein A (NspA) was discovered, being present on 99% of a large panel of meningococcal strains. A monoclonal antibody against this protein showed bactericidal activity in a dose-dependent manner and immunization of BALB/c mice with recombinant NspA protein actively protected the mice against a meningococcal challenge after 3 immunizations[38]. However, recent studies have shown that, although almost all meningococcal strains possess and express NspA, the accessibility of the protein seems to be limited by the amount of capsular polysaccharide expression. Strains with a strong expression of capsular polysaccharide B expression showed less binding of anti-NspA monoclonal antibodies and resistance to anti-NspA antibody-induced bactericidal activity[39]. Furthermore, a NspA-knockout mutant strain was still able to cause bacteremia and death in infant rats, indicating that NspA is not an essential virulence protein for meningococci[40]. Another recent study suggested that an NspA-based vaccine prepared from meningococcal outer membrane vesicles is more effective than the recombinant protein vaccine made in *E. coli* which was used in

earlier studies[41]. This makes NspA a suitable additive vaccine antigen in an OMV-based meningococcal B vaccine.

Transferrin binding proteins

Another group of widely conserved surface-exposed antigens is the *N. meningitidis* transferrin binding proteins A and B (TbpA and TbpB). They have an important role in the acquisition of host iron from human transferrin and are therefore essential for survival of the meningococcus in humans. TbpB is capable of inducing bactericidal antibodies and conferring passive protection in animals[42]. However, TbpB appeared to be more variable among circulating meningococcal isolates than TbpA[43]. Although recombinant TbpA did not induce a serum bactericidal immune response in mice, TbpA as a single antigen conferred protection in a mouse intraperitoneal infection model[44]. The combination of both TbpA and TbpB in their native complex has recently been found to be superior to each of the single recombinant proteins in conferring passive protection without a significant SBA response indicating that this might be a promising approach to develop a widely protective meningococcal B vaccine[45].

Commensal Neisseria species

A totally different but interesting approach for discovering a widely protective vaccine against all meningococcal serogroups is the use of commensal N*eisseria* species as a vaccine. It has been suggested for a long time that carriage of non-pathogenic Neisseria species, like *N. lactamica,* in early childhood induces natural immunity against meningococcal disease[46]. Therefore, a vaccine based on *N. lactamica* might be the ideal candidate for induction of a cross-reactive antibody response against all pathogenic meningococci. Oliver *et al.*[47] immunized mice with whole cells as well as with *N. lactamica* outer membrane vesicles and showed that OMVs induced similar IgG ELISA titers as *N. meningitidis* OMVs against a range of heterologous *N. meningitidis* isolates, but no bacteridal activity. Despite the lack of SBA titers, immunization with *N. lactamica* OMVs did protect mice against experimental infection with the same panel of meningococcal isolates, although the groups of mice were sometimes rather small (n=5). The specific antigen of *N. lactamica* responsible for this protection remains unexplained. Recently, O'Dwyer *et al.* used other commensal Neisseria species like *N. flavescens* and *N. cinerea* for transformation with a plasmid allowing various interesting antigens to be expressed in a neisserial background. Mice immunization studies with OMVs from these transformed strains containing NspA are under investigation and several other candidate vaccine antigens can be envisioned to be investigated in this interesting novel method[48]. Another advantage of this method would be the attractiveness of large-scale production with a non-pathogenic *Neisseria* species.

Genome-derived new vaccine candidates

Genome-derived neisserial antigens

The genomes of two pathogenic meningococcal strains have recently been sequenced: serogroup B strain MC58[49] and serogroup A strain Z2491[50]. The availability of the meningococcal genome has permitted the development of a new strategy called reverse vaccinology: a systematic search of the meningococcal genome for genes encoding possible vaccine antigens, such as proteins located on the bacterial surface. Pizza *et al*. used this process of 'genome mining' and identified several suitable genes[51]. These genes have been cloned and expressed in *E. coli* and the recombinant proteins were purified and injected into mice. Murine serum antibodies against the purified proteins were subsequently analyzed for surface-exposure and bactericidal against 3 meningococcal strains. This approach has led to the identification of 7 candidates that were all present and surface-exposed in a large panel of 31 meningococcal isolates.

One of the novel surface-exposed proteins discovered by the genome sequencing project is the membrane-bound lipoprotein GNA33 (genome-derived *Neisseria* antigen 33). This protein was the first to be further investigated since it induced bactericidal titers similar to those induced by meningococcal OMVs and was highly conserved. GNA33 turned out to be a lytic transglycosylase molecular mimic of an epitope on loop 4 of PorA subtype P1.2[52]. A monoclonal antibody against GNA33 was prepared by immunizing CD1 mice with recombinant GNA33 protein. This Mab is capable of conferring passive protection in infant rats against several P1.2 serogroup B strains although the bactericidal activity of this Mab is lower than the commonly used anti-P1.2 Mab[53]. The major advances of the use of a recombinant molecular mimetic antigen like GNA33 is that it is easily prepared in large amounts by expressing the protein in non-infectious bacteria and that it does not need refolding or reconstituting in liposomes to attain immunogenicity. Possibly, mimetics of other PorA epitopes can be created as well, and a multivalent meningococcal vaccine can be prepared. Adaptation of such a multivalent vaccine to currently new circulating disease-causing subtypes might be easier than adjusting a multivalent OMV vaccine. However, such a vaccine would still have the same drawbacks as PorA-based OMV vaccines have, for it will never cover all circulating disease-causing subtypes.

Another genome-derived new antigen discovered by Pizza *et al*.[51] is GNA2132. This protein was found to be much more variable among *N. meningitidis* strains than GNA33. When mice were immunized with recombinant GNA2132, bactericidal titers against a set of serogroup B and C meningococcal strains were low. Despite the absence of significant bactericidal activity, passive protection was conferred by pooled anti-GNA2132 serum against these strains[54].

NadA

NadA was discovered by screening of the meningococcal genome and it shows strong resemblance to a class of adhesins present in other pathogens like *M. catarrhalis* and *Yersinia* species that are known to be important virulence factors. This suggests a role for NadA in bacterial adhesion and host-cell interaction. NadA was present in approximately 50% of a large group of disease-associated meningococcal isolates and in almost all isolates of a subset of strains of hypervirulent lineages. Sequence of the NadA gene revealed the presence of 3 highly conserved alleles. The protein seems to be exposed to the cell surface and available for antibody binding. However, in the same experiments it was also shown that the expression of NadA varied during the growth of the bacterium and that the presence of a high amount of capsular polysaccharide seemed to hamper the surface-exposure of the protein and therefore the possibilities for antibody binding. The variation in antigen expression level was also reflected in a variable bactericidal activity of anti-NadA serum against different *N. meningitidis* strains and passive protection experiments in infant rats[55,56].

Lipoproteins

Most recently, GNA1870 was investigated and described by Masignani *et al.*[57]. GNA 1870 is a surface-exposed lipoprotein present on 100% of 71 tested strains of *N. meningitidis*. Strains can be divided in high, intermediate and low expressors of the protein, but anti-GNA1870 sera induced by the recombinant protein kill even the low expressors. The same serum induces passive protection in an infant rat model. Three variants of the protein were discovered and antiserum induced against each variant showed low or absent cross-reactivity with the other variants. However, inclusion of the 3 known variants of GNA1870 so far in a vaccine, could theoretically protect against all *N. meningitidis*, regardless of their serogroup. Therefore, this protein certainly will be intensively studied in the near future and studies in humans are expected.
Screening of the genomic sequence of a serogroup A meningococcal strain by Bernfield *et al.*[58] revealed the P2086 gene, an outer membrane lipoprotein. Sequencing of many meningococcal strains showed that there are two main subfamilies of this protein. The recombinant P2086 proteins from several meningococcal strains were expressed in *E. coli*, isolated and used to immunize mice. Antisera induced against one subfamily showed bactericidal activity against strains from the same subfamily and occasionally against a strain from the other subfamily. This response was independent from the serosubtype of the strains and therefore the authors suggest that a mixture of PorA and P2086 recombinant proteins in a vaccine could elicit a broadly protective immune response[58].

Autotransporters

The autotransporter protein family has been investigated for their vaccine potential since these proteins are either surface-exposed or secreted and often associated with virulence factors[59]. However, until the complete meningococcal genome was sequenced in 2000, information on neisserial autotransporter genes was limited. The meningococcal genome sequence data have been searched for novel autotransporters by several investigators. Van Ulsen *et al.*[60] found two neisserial autotransporters, which were homologous to *Haemophilus influenzae* adhesion and penetration protein (Hap), and *Haemophilus influenzae* adherence protein (Hia). The genes for these proteins were highly conserved among *N. meningitidis* isolates and shown to be surface-exposed and expressed during meningococcal invasive disease and carriage[60]. The recombinant Hap homologue protein, called App for adhesion and penetration protein by Hadi *et al.*[59], was used for immunization experiments in rabbits, in which it was immunogenic and cross-reacting with a panel of meningococcal isolates tested. An additional novel autotransporter protein, named autotransported serine protease A (AspA) is well conserved in serogroup A, B and C meningococci and antibodies against this protein were found in patient reconvalescing sera. However, AspA was also shown to be phase variable and therefore less suitable as a sole, vaccine candidate[61].

Functional genomics

A new and not yet intensively studied approach is the use of DNA microarrays carrying the entire meningococcal genome and analyze gene up-regulation upon interaction with host cells, like human epithelial and endothelial cells and upon exposure to human serum[62-64]. Several interesting new adhesion-induced surface antigens which were able to induce bactericidal antibodies in mice were found as possible vaccine candidates[64]. These antigens, of which two are hypothetical proteins were highly conserved among a set of meningococcal strains. Other applications of this microarray technique, like the search for invasion-associated genes, can be easily envisioned and will undoubtedly be explored in the near future.

Another elegant genomic approach is the use of signature-tagged mutagenesis by Sun *et al.*[65]. This method uses a large number of insertional mutants, each tagged with a unique sequence identifier, which are screened for their ability to cause systemic disease in a rat model. The inserted genes of the mutants that failed to establish systemic meningococcal infection were identified. Besides some genes previously known for their relation with virulence, 65 genes with previously unknown involvement with meningococcal pathogenesis were identified. Most of these genes encode for proteins predicted to be surface-located and form the basis for further research into their suitability as vaccine targets. A similar approach was followed by Pelici *et al.,* who

constructed a library of meningococcal mutants of a serogroup C strain with defined knockouts in almost every non-essential gene. This library can be used to identify the genes important for several processes in causing meningococcal disease like adhesion or invasion[66].

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Chapter 3.2

Neisseria meningitidis serogroup B: laboratory correlates of protection

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FEMS Medical Microbiology and Immunology 2002; 34/2 pp 89-96

Abstract

Meningococcal disease in the Western countries is frequently caused by *Neisseria meningitidis* serogroup B. Major efforts have been made to develop a safe and efficacious vaccine against this serogroup which is suitable for use in infants and young children. To assess the quality of the immune response after vaccination with candidate vaccines, laboratory correlates of protection are needed. For serogroups A and C, serum bactericidal activity (SBA) is a well established predictor for protection but for serogroup B other mechanisms besides SBA may be also probably involved in conferring protection from disease. Several laboratory methods for identification and evaluation of the immunogenicity of possible vaccine antigens are described in this review.

Meningococcal B infections and natural immunity

Neisseria meningitidis is an obligate human pathogen and is the most prevalent serogroup causing meningococcal disease in the Western countries. In the majority of cases it only colonizes the host for a period of time but it can also invade and cause systemic diseases like sepsis and meningitis. Mostly young children under 4 years of age and adolescents are at risk for this disease and mortality rates are up to 40 % for sepsis. Meningococcal carriage is a natural immunizing process resulting in the induction of systemic protective antibody responses[1]. Acquired immunity to meningococcal antigens involves specific B and T cells. Antigen specific B cells can produce serum IgM and IgG and secretory IgA (sIgA) antibodies. The induced serum antibodies play an important role in the clearing of bacteria by direct complement lysis or by opsonophagocytosis. The role of mucosal IgA against colonization and infections is still unclear. However, serum IgG may diffuse into mucosal surfaces and act like sIgA to prevent meningococcal carriage and/or infection. Since repeated invasive infections with meningococci are extremely rare in otherwise healthy individuals, but it is unsure if this is because of a protective immune response induced by systemic disease or because of the very small chance of invasion of another virulent meningococcal strain. Goldschneider *et al*. showed that the presence of bactericidal antimeningococcal antibodies and the resistance to meningococcal disease is correlated at all ages[2]. The relationship between serum bactericidal activity (SBA) and protection from meningococcal disease is particularly established for antibodies against serogroup A and C capsular polysaccharides[3]. This knowledge has been used to evaluate the immune response of recently developed meningococcal A and C conjugate vaccines, and enables prediction of protection by these vaccines. The capsular polysaccharide (PS) of serogroup B is poorly immunogenic, probably because it has strong similarities with structures on human fetal neural cells. It is therefore likely that the immune responses against surface exposed proteins and polysaccharides other than capsular PS are important in immunity against serogroup B. Following systemic infection with meningococcus serogroup B, antibodies against PorA, PorB, LPS, class 5 OMP's and H.8 antigens can be found, but it's not known which of these contributes to the natural acquisition of protective immunity[4-6]. The identification of antigens eliciting long-lasting protective immunity against *Neisseria meningitidis* serogroup B (Men B) is still in progress and the applicability of SBA as a serologic correlate has not yet been established. Therefore, to evaluate the immune response induced by candidate Men B vaccines, other laboratory correlates of protection are urgently needed.

Vaccines and clinical studies

Polysaccharide vaccines against serogroup A, C, W135 and Y, including a tetravalent vaccine containing all 4 serogroups, have been widely used in high risk populations, such as inhabitants of sub-Saharan countries and individuals with deficiencies in (late) complement pathway components. However, children below the age of 2 years, who are at highest risk for contracting meningococcal disease in developed countries, are unable to develop a T-cell dependent antibody response against polysaccharide antigens, resulting in an absence of immunological memory. Furthermore, development of serogroup B polysaccharide vaccines in humans has been limited because of the theoretical risk that these vaccines will overcome immune tolerance and induce autoimmunity. Therefore, further development of these kind of vaccines must be undertaken carefully. Jennings *et al.* developed a chemically modified polysaccharide that contains N-propionyl groups in stead of the N-acetyl groups found at position C5 of the sialic acid residues[7,8]. This modified polysaccharide has been coupled to various carrier proteins and was immunogenic in mice and non-human primates. Concern over the safety and poor immunogenicity of B capsular polysaccharide vaccines has focused attention on other cell-surface antigens as vaccine candidates (see table 1). Outer membrane vesicle (OMV) vaccines have been developed and tested in clinical field studies. Three monovalent OMV vaccines have been developed from wild type strains of serogroup B meningococci that have caused outbreaks of disease: the Finlay Institute in Cuba B4:P1.15 OMV; VA-mengoc-BC; The Walter Reed Army Institute (WRAIR) in USA:B15:P1.3 and the National Institute of Public Health (NIPH) in Norway B15:P1.7,16 OMV. Several efficacy trials have been conducted with these OMV vaccines (see table 2). The protective efficacy was age dependent: for the Cuban OMV vaccine in Brazil 70% for children older than 4 years, while in younger children no efficacy was demonstrated[9]. The protective efficacy of the Norwegian vaccine was 57% in secondary school pupils[10]. In Chile the WRAIR vaccine efficacy was 70 % in the volunteers aged from 5-21 year, but no protection was shown in children aged between 1 and 4 years[11]. In most of these studies IgG titers and SBA titers have been measured. All three vaccines contain outer membrane proteins (OMP) derived from the outer membrane vesicles (OMV) of the organism. However, each vaccine is manufactured from a single bacterial isolate and thus a single PorA OMP serosubtype. There are numerous B serosubtypes causing invasive disease in Europe and the protection afforded by an OMP vaccine is thought to be largely serosubtype specific. However, this has been disputed and direct evidence of restriction of efficacy to homologous serosubtypes is lacking[12]. One potentially relevant factor determining induction of cross-reacting antibodies to non-vaccine serosubtypes may be the presence of high molecular weight OMPs other than PorA OMP[9,13-15].

Table 1. Antigens of Men B as possible vaccine candidate.

Antigens Major outer membrane proteins:	**Properties**
Porin PorA (class 1: (Sero)subtype	Highly diverse, Mab bactericidal and protective
Porin porB (class 2/3: (sero)type	limited diversity, Mab bactericidal, not protective
RmpM (class 4)	conserved
Opc/Opa (class 5)	highly variable
Transferrin binding protein Tbp2 (iron-regulated)	variation
Iron-regulated OMP FrpB	variation?
Lipopolysaccharide Pili	limited diversity, variation Mab bactericidal highly variable
Minor outer membrane protein NspA	Conserved, antibodies bactericidal

A multivalent PorA vaccine has been genetically engineered by the RIVM in the Netherlands. This hexavalent PorA OMV vaccine contains two trivalent vesicles each expressing 3 different PorAs. Clinical studies have shown this vaccine to be safe and immunogenic in infants, toddlers and school children[16,17]. Other protein vaccine candidates are the transferrin binding protein TbpB, which is important for the acquistion of iron from human transferrin, the iron-regulated OMP FrpB and the neisserial surface protein A (NspA). However, large clinical trials using these vaccines in humans, especially in children, have not been conducted so far. Another candidate as a vaccine antigen is the meningococcal lipopolysaccharide (LPS). *N. meningitidis* expresses a number of different glycoforms of LPS and the expression differs in invasive versus carriage isolates. No clinical data are yet available, but preclinical data show the induction of opsonic rather than bactericidal antibodies by detoxified LPS or LPS conjugate vaccines[18]. Recent publication of the genomes of two meningococcal isolates offers new opportunities for the identification of candidate antigens like

Table 2. Vaccine efficacy studies.

Vaccine	Study	% responders (homologous, 4-fold rise in SBA (95% CI)	Efficacy (95 % CI)	Ref.
Finlay; Purified total OMP in proteoliposome with added high mol. wt OMP50μg (B:P1.15)	Cuba, teenagers, double-blinded placebo control, 2 immunizations, 16 months study	30 % (12-54%)	83 % (42-95 %)	[58]
Finlay	Brazil, case control, 2 immunizations, 12 months follow-up	22% 45 % 52 %	-37 % (100-73 %) (3-23 mths) 47 % (-72-84 %) (24-47 mths) 74 % (16-92%) (48-83 mths)	[9]
Norwegian, total OMP in vesicle (OMV) formulation (SIFF) 25 μg (B:15:P1.7,16)	Norway, teenagers, randomized at school level, double-blinded, placebo control, 2 immunizations, almost 3 year study period	46 % (34-59%)	57,4 % (lower limit 27%)	[10]
Purified class 1,3,4 containing OMP (WRAIR) 100 μg (B:15:P1.3)	Chile, 1-21 years, randomized, double-blinded, placebo control, 2 immunizations, 20 months follow-up	41% 83%	-39% (1/4 yrs) 70% (5/21 yrs)	[11]

autotransporters, adhesins and lipoproteins which can possibly act as new vaccine components.

Correlations between vaccine efficacy and laboratory assays

Acquisition of bactericidal antibodies has been shown to correlate inversely with protection from meningococcal disease incidence for serogroup B as well as for A and C [2]. For Men A and C a fourfold increase in SBA or the maintenance of serum anti-polysaccharide IgG level of 2 μg/ml[19] are accepted as surrogates for protection for

these serogroups with SBA being the gold standard[20]. However, whether titers of anti-serogroup B meningococcal bactericidal antibody correlate with protection from meningococcal disease is unknown. It has been suggested that SBA underestimates the clinical efficacy of OMV vaccines and that protection against group B infection may also be due to opsonic antibodies and to innate immune responses, which are not demonstrated in the SBA[13,21-23]. Therefore, assays other than SBA that demonstrate opsonic activity or whole blood bactericidal activity have been suggested as more appropriate markers of protection than SBA.
The lack of an accepted surrogate of protection hampers vaccine development against Men B disease. Correlates of protective immunity to Men B in humans are helpful for identifying protective antigens, demonstrating the immunogenicity of a vaccine candidate and its potential efficacy. With these correlates, studies concerning optimization of the dose, vehicle, adjuvant and schedule of immunization can be performed. Potential correlates can be proposed on the basis of animal models and *ex vivo/ in vivo* studies in humans. Most critical is their validation; ultimate validation will require correlation with protection in a phase III efficacy trial of an effective vaccine. A selection of the most promising correlates should be made for evaluation in phase I and II and ultimate phase III vaccine trials.

Assays to measure immunity to Men B antigens

In all assays measuring immunity against Men B, a variety of target bacterial strains or antigens are used. Wild type strains, isogenic vaccine strains or strains containing modified LPS or outer membrane proteins as well as specific antigens like OMVs or outer membrane proteins are all used in order to measure the broad or specific immune response, depending on the antigen(s) being studied. This complicates the comparison of results between laboratories studying different vaccine candidates, but is necessary to study the specificity of the immune response against a vaccine antigen.

Functional assays

SBA

In the serum bactericidal assay (SBA), meningococci are lysed by serum antibody together with a human or animal complement source through the classical complement activation pathway. SBA titers for each unknown sample are expressed as the reciprocal serum dilution yielding ≥50% or 90% killing as compared to the number of target cells present before incubation with serum and complement. In assessing the immune response against meningococci, SBA has been the most widely used method. Usually, in measuring immunity after vaccination against serogroup B, not only the absolute value of SBA titers is assessed, but also the percentage of vaccinees who

respond with at least a 4-fold increase in SBA after vaccination. After the work of Goldschneider *et al*., the SBA has been adopted as the "gold" standard for immunity against serogroup A and C. There is some evidence that SBA correlates with protection against serogroup B meningococcal disease[11,24]. However, in a large vaccine efficacy trial studying two efficacious OMP vaccines it was shown that measurement of SBA against serogroup B as a correlate of protection might underestimate clinical efficacy[13]. Probably, other mechanisms besides the bactericidal response can also provide protective immunity against serogroup B meningococci. For serogroup A and C a standardized assay was designed using rabbit serum as a complement source, since this is readily available[25]. However, the use of infant rabbit complement results in much higher SBA titers than the use of human complement and a recent paper showed that this assay lacks sensitivity in predicting a protective titer as assessed by Goldschneider *et al* using human complement[26]. For MenB the bactericidal antibodies with rabbit complement appear to be mainly directed against group B polysaccharide whereas antibodies bactericidal with human complement are primarily directed against subcapsular antigens[27]. For evaluation of MenB vaccines, the use of human complement in SBA is therefore obligatory. Another significant disadvantage of the conventional SBA test is the labor-intensiveness of plating and visually counting of target bacteria. Computerized colony counters are available, but the process can not be totally automated and is therefore difficult to standardize and time-consuming. Various researchers have reported new methods to by-pass these difficulties by adding a colorimetric or fluorescence-based indicator to the reaction mixture in the wells after the incubation of serum and complement with bacteria. The colorimetric SBA is based on the ability of viable bacteria to consume glucose, leading to acid production and therefore lowering the pH, which can be detected by a color change of a pH indicator. The fluorescence SBA uses an reduction-oxidation indicator to detect surviving bacteria and both assays can be read visually or by a fluorescence or spectrophotometric plate reader. Titers obtained by both assays correlated strongly with titers obtained by a conventional SBA method[28,29]. This makes the assays a fast, easy and more reliable alternative to the conventional SBA, and thus more suitable for large-scale testing.

Whole blood killing assay

A whole blood assay (WBA) has been developed to assess the total killing capacity of human blood after vaccination or infection. A WBA does not only measure the complement-depending antibody mediated killing, but also the opsonophagocytic activity of blood, using the endogenous complement and cellular components of the immune system. The assesment of protective immunity in children convalescing from meningococcal disease showed that WBA detects bactericidal activity in more patients

than the SBA, as well as a significant cross-reactivity between serosubtypes[23]. The same result was shown recently in 105 infants immunized with a monovalent OMV vaccine[12]. Thus, WBA seems to be a more sensitive measure than SBA, although the two methods are not directly comparable. The correlation between WBA killing and protection against meningococcal disease is currently unknown. The test provides some logistic difficulties since it has to be performed within 2 hours after blood collection. Therefore, it will be difficult to address the role of the WBA as a better predictor of vaccine efficacy in large phase III trials. Another complication is the need of fresh blood of healthy, non-vaccinated individuals as a negative control.

Opsonophagocytosis

The importance of opsonophagocytic activity as a host defense mechanism, especially against serogroup B meningococci was shown by Ross *et al.*[30]. Opsonophagocytic assays have been developed using freshly isolated human polymorphonuclear cells (PMNs) or PMN cell lines as a source of phagocytic cells. Together with serum antibody and complement opsonization and ingestion of antigen-coated fluorescent beads, ethanol-fixed meningococci labeled with fluorescein isothiocyanate (FITC) or heat-killed meningococci occurs, after which phagocytosis can be quantitated by flow cytometry, chemiluminescence or measurement of oxidative burst activity. FITC-labeling or heat-killing of bacteria might destroy epitope conformation and therefore not reflect the epitope exposition on live bacteria[31]. Antigen-coated beads probably present antigens similar to the native conformation and has the advantage that antigen specificity of serum opsonic activity can be studied as well[22,32]. Serum opsonic activity has been shown to correlate with IgG titers against OMVs, PorA and PorB measured by ELISA[22,33] and SBA[34]. The measurement of serum opsonic activity is expected to be of additional value to survey a vaccine trial, although so far it has not been tested in any efficacy trial yet.

T cell responses

Although protective immunity against meningococci mainly relies on antibody-mediated effector functions as serum bactericidal activity and opsonophagocytosis, T-cells play an important role in the regulation of the immune response, including stimulation of B-cells for antibody production. T-cells are necessary for the establishment of immunological memory and to promote activation of phagocytic cells, thereby facilitating the uptake and destruction of meningococci. T helper cells can be divided into Th1 and Th2 subpopulations on the basis of different cytokine production and effector functions. Human T-cell response induced by vaccination can be measured by a T-cell proliferation assay. In this assay, human peripheral blood mononuclear cells are incubated with meningococcal antigens like OMVs, porins or heat-killed

meningococci and pulsed with [^{3}H] thymidine. Recognition of antigens will stimulate T-cells to respond and proliferate, which leads to incorporation of [^{3}H] thymidine into DNA of the dividing T-cells. The amount of incorporated radioactivity measured by a scintillation counter is a measure of T-cell proliferation. Futhermore, cytokines produced by T-cells can be measured in cell supernatants upon stimulation with meningococcal antigens and the proportion of memory T-helper cells after vaccination can be measured. In vitro T cell proliferation to PorA, Opa, Opc and OMV have been reported in normal adults[35] and after vaccination[36,37] or infection[38]. Naess *et al*. found that vaccination with the Norwegian OMV vaccine induced strong T-cell responses specific for OMV, PorA and to a lesser extent PorB, which correlated strongly with anti-OMV IgG titers measured in ELISA[37]. Also, vaccination increased the proportion of memory T-helper cells. The cytokine pattern after meningococcal infection appears to be age-dependent, skewing from a Th1 to a Th2 pattern upon age[38]. The only report about T-cell cytokine patterns after vaccination points towards stimulation of a T-helper 1 response in immunized adults[39].

4.2 Non-functional assays

ELISA

Specific amounts of antibodies can be measured by ELISA methods, either using whole cells outer membrane vesicles (OMVs) or purified proteins like PorA as an antigen coat. This is a quantitative indication of the immune response after vaccination. However, several efficacy trials have provided evidence that ELISA titers are an inaccurate correlate of protection[11,13]. Correlations between total IgG titers and SBA after vaccination vary with differences in the administered vaccine and the antigen coat in ELISA[24,40-42]. The explanation for this poor correlation might be the difference in bactericidal capacity of the induced IgG subclasses; IgG1 and IgG3 are the most effective antibodies for complement-mediated killing of meningococci. Several studies showed that these subclasses dominate the response following infection as well as after immunization with OMV vaccines[40,41,43-45]. Only IgG1 correlated moderately with SBA titers and strongly with opsonophagocytic activity measured as respiratory burst[40,42]. Another possible explanation for the weak correlation between SBA and ELISA titers may be that antibodies are of low affinity, making them less capable of effectively binding and killing bacteria. A qualitative ELISA method has been developed to measure the relative avidity of antibodies. Antibody avidity, a measure of the functional affinity of serum antibody to bind to antigen, can be measured using thiocyanate as an agent to dissociate weak bindings between antigen and antibody in ELISA. In vaccination studies using the meningococcal C conjugate vaccine, the functional importance of avidity maturation has been shown[46]. Furthermore, a high avidity index indicates successful priming with the Hib conjugate vaccine and high

avidity antibodies after vaccination with the Hib or a pneumococcal conjugate vaccine appear to protect animals from disease[47-49]. For meningococcal serogroup B, it was shown that the monovalent RIVM OMV vaccine induced a clear avidity maturation in toddlers and that there was a moderate correlation between avidity index and SBA[41]. At least one of the six serosubtypes included in the hexavalent RIVM vaccine also induced a significant avidity maturation in infants (R.Borrow, personal communication). Although the value of antibody avidity has not yet been proven in an efficacy trial, it appears to be a promising candidate for predicting long-lasting protective immunity after vaccination.

Immunoblot

Analysis of serum antibody by immunoblotting reveals the specificity of antibodies to several meningococcal antigens on the same blot. It has been mainly used to measure the antibody response after immunization with the Norwegian OMV vaccine or after systemic meningococcal disease[14,50]. An immunoblot of sera from patients reconvalescing from meningococcal disease provides the possibility to investigate which antigens induce antibody. In reconvalescent patient sera the majority of antibodies were directed against class I, III and V proteins[14]. High IgG levels against PorA and PorB in vaccinee or patient sera measured by immunoblotting correlates well with IgG measured in ELISA, but when antibody concentrations were low, immunoblot measurements did not correlate with ELISA titers[50]. Summarizing, immunoblotting is a useful method for comparing the specificity of immune response induced by various vaccines. A disadvantage of the method is that it requires boiling of the antigen coinciding with loss of its native structure and therefore (part of) its antigenicity. Furthermore, immunoblotting is a labor-intensive method, making it less suitable as a laboratory assay in large clinical vaccine trials.

Animal models

Only in animal models of infection the complexity of the interactions of the bacteria with whole tissues and the humoral and cellular immune systems can be assessed. Animal infection models are also of great importance for the assessment of the protective efficacy of existing and candidate vaccines. As humans are the only natural host for Men B, it has proved to be difficult to establish animal infection models. Infant mouse models using an intravenous (i.v.) and intraperitoneal (i.p.) route of infection have been developed. Both models require an additional iron source (e.g. iron dextran, human transferrin)[51,52]. These models have been applied in vaccine development for studying various aspects of meningococcal virulence and the protective activity of antibodies. However, the natural route of infection in humans is the intranasal route. After intranasal infection of infant mice, nasal colonization was established, but lung

colonization was necessary for the development of bacteremia[53]. This is not comparable with the course of disease in humans. Also there was still a need for intraperitoneal iron dextran injection to enhance meningococcal strain virulence resulting in infection[53,54]. The advantages of using infant rat models are that no additional iron source is necessary, lower inocula are required and the development of both bacteremia and meningitis can be determined by sampling of blood and cerebrospinal fluid. However, also this model can only be used for passive protection studies and studying pathogenesis of meningococcal bacteremia and meningitis[55-57].
In conclusion, although much valuable information can be obtained by animal models on the virulence of bacterial strains and mutants protection after passive immunization with specific antibodies, the available models are not yet suitable as a model for human meningococcal disease and active immunization.

Concluding remarks

The search for a protective vaccine against meningococcal serogroup B is hampered by the lack of knowledge about the acquisition of natural immunity against this serogroup. The relative contribution of various isotypes of antibodies and antigenic-specific antibodies found after systemic infection to protection is not fully understood. Also, the relative importance of various antigens as protective antigens and thus suitable for application in a vaccine needs further research. A candidate vaccine has to be tested extensively for its immunogenicity and efficacy. Several laboratory methods are now available to measure immunity against Men B. So far, SBA has always been considered to be the best, but not ideal, method, and several other assays have been evaluated. These seem to be suitable as additional measurements of protection, but it remains uncertain if these correlate with vaccine efficacy better than SBA. The whole blood assay is probably most representative of the in vivo interaction between immune system and bacteria, but so far not suitable in large-scale trials.
In conclusion, at the moment a combination of several assays is recommended for evaluation of the immune response after vaccination, in order to assess the quality of a Men B vaccine candidate. Animal models mimicking Men B disease can provide much valuable additional information in that respect; however all the available models have some shortcomings in comparison with the natural course of disease in humans.

Acknowledgements

We thank Loek van Alphen and Hans Rümke for critical reading of the manuscript.

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Chapter 3.3

Antibody avidity and IgG isotype distribution following immunization with a monovalent meningococcal outer membrane vesicle (OMV) vaccine

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Infection and Immunity 2002; 70: 584-590

Abstract

The avidity maturation and IgG isotype distribution of antibodies after vaccination with a meningococcal B outer membrane vesicle (OMV) vaccine were evaluated as indicators for protective immunity. Pre and post vaccination sera from134 healthy toddlers (2-3 years of age) immunized with a monovalent meningococcal B OMV (serosubtype P1.7-2,4) vaccine adjuvated with $AlPO_4$ or $Al(OH)_3$ were analyzed using ELISA methods. Vaccinations were given 3 times with an interval of 3-6 weeks or twice with an interval of 6-10 weeks. A booster was given after 20-40 weeks. The avidity index (AI) of antibodies increased significantly during the primary series of vaccinations and after the booster. No differences in AIs were seen between the two vaccination schedules or adjuvants. After vaccination, IgG1was the predominant IgG isotype followed by IgG3. No IgG2 or IgG4 was detected. There was a strong correlation between SBA and ELISA titers: $r=0.85$ ($p< 0.0001$) for total IgG, $r=0.83$ for IgG1 ($p< 0.0001$), $r=0.82$ for IgG3 ($p<0.0001$) and $r=0.84$ ($p< 0.0001$) for the avidity titer. When two subgroups were compared with similar anti-OMV IgG levels before and after the booster vaccination, the higher AI after the booster vaccination was associated with a significantly increased SBA. We conclude that avidity maturation occurs after vaccination with a monovalent meningococcal B OMV vaccine, especially after boosting, indicated by a significant increase of the AI. Vaccination with the monovalent OMV vaccine induced mainly IgG1 and IgG3 isotypes, which are considered to be most important for protection against meningococcal disease. An increase in AI of antibodies is associated with increased SBA, independent of the level of specific IgG and the IgG isotype distribution. Measurement of the AI and IgG isotype distribution of antibodies after vaccination can be a supplementary method for predicting protective immunity to be evaluated in future phase III trials with meningococcal serogroup B vaccines.

Introduction

Neisseria meningitidis is an important cause of meningitis and septicemia worldwide. In many countries in Western Europe, *N. meningitidis* serogroup B is most frequently isolated from these seriously ill patients. In the struggle against meningococcal disease caused by this serogroup, great efforts have been made towards the development of a protective vaccine. The group B capsular polysaccharide is poorly immunogenic since it shows strong antigenic resemblance to structures expressed on human fetal neural cells[12]. As a consequence, a serogroup B polysaccharide vaccine may induce antibodies cross-reactive with human tissues. Therefore, vaccines containing outer membrane proteins have been developed and have been shown to induce protective immune responses[3,11]. At the National Institute for Public Health and the Environment (RIVM), a vaccine was developed consisting of two outer membrane vesicle (OMV) preparations, each expressing three different PorA proteins representing the majority of circulating serosubtypes in the Netherlands and other countries in Europe[7]. This vaccine has been tested in several phase I and II trials and has proven to be safe and immunogenic[6,8]. Since serosubtype P1.7-2,4 is the cause of a current epidemic in New Zealand and is the most prevalent serosubtype in the Netherlands as well, a monovalent vaccine with a double expression of this PorA has also been constructed at the RIVM. This vaccine appeared to be safe and immunogenic in toddlers; over 90 % of vaccinated children showed a fourfold increase in serum bactericidal activity (SBA)[9].

There is a great need for well-defined markers for immunity induced by vaccination. These markers could serve as surrogates of vaccine protective efficacy and would be helpful for the quick introduction of new or improved vaccines in the future. Measurement of total IgG titers using specific ELISA does not provide any information on the functionality of the antibodies. A fourfold rise in SBA after vaccination has been widely used to evaluate immunogenicity and vaccine efficacy of various meningococcal B vaccines. However, Perkins *et al.*[23] showed that a fourfold rise in SBA appears to underestimate clinical efficacy. In addition, SBA titers and IgG ELISA titers in sera obtained after vaccination with the RIVM hexavalent OMV vaccine correlated poorly[10]. An explanation for a poor correlation between SBA and ELISA may be that only high avidity antibodies are bactericidal. For_vaccination with meningococcal C conjugate vaccines, the functional importance of antibody avidity maturation after vaccination has recently been demonstrated by Richmond *et al.*[27]. Several studies with conjugate vaccines against *Streptococcus pneumoniae* and *Haemophilus influenzae* type b (Hib) have also shown that vaccination induces an increase in antibody avidity[2,14,28] and that low concentrations of passively administered high avidity antibody can protect experimentally infected animals from disease[20,31]. Most investigators use an ELISA

method using sodium thiocyanate (NaSCN) as an agent to discriminate weak binding between antibody and antigen from high affinity binding[25]. By calculating an avidity index (AI), the relative avidity of specific antibodies in serum can be compared in the course of a vaccination trial.
Functional activity of antibodies also depends on IgG isotypes[19]. IgG1 and IgG3 are most effective in complement binding and activation[5], and IgG2 may also contribute to protection against meningococcal disease[1]. Furthermore, affinity differences have been found in antibodies with similar antigen-binding specificities but different IgG isotypes[24]. IgG1 and IgG3 are mainly directed at protein antigens, whereas IgG2 is predominantly found after vaccination with polysaccharide antigens in adults[15,16,29].
The aim of this study was to compare SBA with the AI and IgG isotype distribution and to evaluate antibody avidity maturation as an indicator for protective immunity after vaccination with the monovalent P1.7-2,4 OMV vaccine. The AI was determined by an ELISA technique using NaSCN and the IgG isotype distribution by an isotype specific ELISA[21,26]. The influence of different adjuvants and immunization schedules on avidity maturation and IgG isotype distribution was also examined.

Materials and methods

Vaccine and subjects

In this study serum samples were used from a previously performed randomized, blind and comparative clinical phase II trial in toddlers using a PorA-based meningococcal OMV vaccine against the serosubtype P1.7-2,4[9]. This vaccine is made from strain F91 that contains duplicate copies of the PorA gene and has no expression of class 3 and 4 proteins. The vaccine was either adsorbed with $AlPO_4$ or with $Al(OH)_3$ and two different vaccination schedules consisting of two or three vaccinations in the primary series and one booster vaccination were evaluated. In the 2+1 schedule 2 vaccinations were given with a time interval of 6 to 10 weeks, whereas in the 3+1 schedule 3 vaccinations were given with a time interval of 3 to 6 weeks. In both schedules a booster vaccination was given 20 to 40 weeks after the primary series. Blood samples were drawn by venipuncture before each vaccination and 4 to 6 weeks after the last vaccination of the primary series and after the booster. Accordingly, 5 and 6 blood samples were obtained from children immunized with a 2+1 or a 3+1 schedule respectively. In total, 134 toddlers participated in this study. The trial was designed to be able to detect a 40 % difference between immune responses in the different groups with alpha=0.05 (two-sided) and 80% power with 30 children per group.

SBA

SBA was measured as reported previously[9]. In short, the serosubtype P1.7-2,4 isogenic variant of *N.meningitidis* serogroup B strain H44/76 was grown on a GC-agar plate containing 1% isovitalex (SVM, Bilthoven, the Netherlands) at 37° C for 18-20 hours in 5% CO_2. Single colonies were picked and suspended in 2 ml of Müller Hinton Broth (MHB) (SVM, Bilthoven, the Netherlands). From this suspension a 20 ml flask with MHB was prepared with an OD_{620} of 0.07-0.08. The bacteria were grown until an OD_{620} of 0.22-0.24 ($\approx 10^9$ cfu/ml) was reached. This culture was diluted in Gey's Balanced Salt Solution (GBSS) (Sigma, St. Louis, USA) with 0.5% Bovine Serum Albumin (BSA) (ICN, Irvine, USA) to $\approx 10^5$ cfu/ml. To each well of a 96-well microtiter plate 6 µl of this dilution was added to 12 µl of 2-fold dilutions of heat inactivated sera in GBSS/BSA. After 10-15 minutes 6 µl complement (40% (v/v) in GBSS/BSA, final concentration 10 % (v/v)) of a negative human donor was added. Time zero plates were incubated overnight in triplicate as follows: 7 µl of a well with only bacteria, complement and GBSS/BSA was spread on a GC agar plate with 1% isovitalex. The microtiter plates were then incubated at 37°C, 5% CO_2 for 60 minutes. Subsequently, 7 µl of the suspension in each well was spotted onto GC agar plates with 1% isovitalex. After 18-20 hours incubation at 37°C and 5% CO_2 the number of cfu on time zero plates was counted. The average number of cfu was set at 100%. Then the plates from the serum dilutions were counted and the serum bactericidal titer was reported as the reciprocal of the lowest serum dilution yielding ≥ 90% killing.

Avidity ELISA

The AI was determined using an ELISA method as described by Anttila *et al.*[2] with some minor modifications. Briefly, Immulon 2 (Dynex Technologies, Inc.) 96-well plates were coated overnight with 100 µl/well of a P 1.7-2,4 monovalent OMV suspension at a concentration of 4 µg/ml. Sera were diluted 1:100 in PBS-0.1% Tween 80 and a threefold serial dilution of serum samples was incubated for 90 minutes at 37° C. One plate contained all samples from a single child in duplicate. As a positive control, a serum sample from a volunteer who had been vaccinated with the hexavalent OMV vaccine in a phase I trial and had a high antibody titer against P1.7-2,4 was included on every plate. Sodium thiocyanate (NaSCN) was used to dissociate low avidity antigen-antibody binding. To determine the optimal assay conditions for measuring the AI, different concentrations of NaSCN (ranging from 0 - 3 M) were tested first on a subset of serum samples drawn at different time points. A 1.5 M solution gave a strong reduction in ELISA titer of some samples whereas the ELISA titers of other samples were not affected. Therefore we considered 1.5 M as a good discriminating concentration. After three washes, 100 µl of a 1.5 M NaSCN solution (dissolved in

PBS) were added to one half of each plate and plain PBS was added to the other half. After incubation for 15 minutes at room temperature all wells were washed three times and for detection of antibody binding the plates were incubated with rabbit-anti-human IgG 1:5000 conjugated with horseradish peroxidase conjugate for 90 minutes at 37°C. Subsequently after washing, TMB substrate was added, the reaction allowed to proceed for 10 minutes and was stopped by adding 100 µl of 2 M H_2SO_4 per well. The absorbance at 450 nm was read using an EL312e Bio-Kinetics reader. IgG antibody titers were determined as the dilution yielding 50% of the maximal OD. Samples with antibody titers below the assay's detection limit were assigned a value of 50. Titers obtained after treatment with NaSCN were called "avidity titers". An Avidity Index (AI) was expressed as the percentage of antibodies that remained bound at the antigen coat after treatment with sodium thiocyanate: AI = titer (NaSCN+)/titer (NaSCN-) x 100[2]. As a control for antibody specificity, pre-vaccination, post-primary series and post-booster serum samples from 24 toddlers were also used in an OMV-ELISA measuring total IgG against OMV's of the PorA negative mutant strain H1.5.

IgG isotype ELISA

IgG isotype specific antibody titers were determined by OMV-ELISA using isotype specific conjugates as described previously[10]. In short, plates were coated and sera were incubated in the same way as described for the avidity ELISA. After serum incubation, plates were washed three times and incubated with mouse-anti-human conjugate specific for each of the various IgG isotypes (clone numbers HP 6188 for IgG1, HP 6014 for IgG2, HP6095 for IgG3 and HP6196 for IgG4, CLB, the Netherlands)[17,18]. After incubation for 90 minutes at 37°C, plates were washed, TMB substrate was added, and after 15 minutes the reaction was stopped by adding 100 µl of 2 M H_2SO_4 per well. Plates were read in an EL312e Bio-Kinetics reader at 450 nm and IgG antibody titers were determined as the dilution yielding 50% of the maximal OD.

Statistical analysis

Antibody titers were log-transformed before statistical analysis. To determine which immunization schedule and vaccine adjuvant induced the highest overall SBA and antibody titers in the course of vaccination, values of total IgG, IgG1, IgG3, the avidity titer and SBA titer from sera obtained at four time points each were added up for every child. Means of these sums were calculated for each group and a variance analysis was performed using SAS software. Titers before the primary series were generally undetectable and therefore excluded from the analysis, as well as the extra titers obtained during the primary series of the 3+1 schedule since this time point was not shared by all trial participants. Spearman's rho correlation coefficient was used for

calculation of correlations between the different IgG antibody titers, AI and SBA results, using SPSS. Parametric tests were used to calculate differences in AI between time points and different groups, and non-parametric tests were used to calculate differences in IgG and SBA between groups.

Results

Total anti-OMV IgG titer

Total anti-OMV IgG titers increased during the primary series from 308 after one vaccination to 5318 after the primary series (Fig. 1). In the pre booster samples, total anti-OMV IgG dropped significantly to 741. After a booster vaccination, total anti-OMV IgG reached a mean titer of 15230. Variance analysis showed that total anti-OMV IgG levels were highest following a 3+1 schedule ($p< 0.0001$) with $AlPO_4$ as an adjuvant ($p= 0.0002$) (data not shown). Total IgG titer of serum samples drawn before the vaccination trial, after the primary series and post-booster of 24 toddlers were measured against PorA-negative OMVs as a control for antibody specificity. Mean total IgG titer against these OMVs were 677 before vaccination, 881 after the primary series and 911 post booster (Fig. 1), indicating that the IgG titer against P1.7-2,4 OMVs was PorA specific.

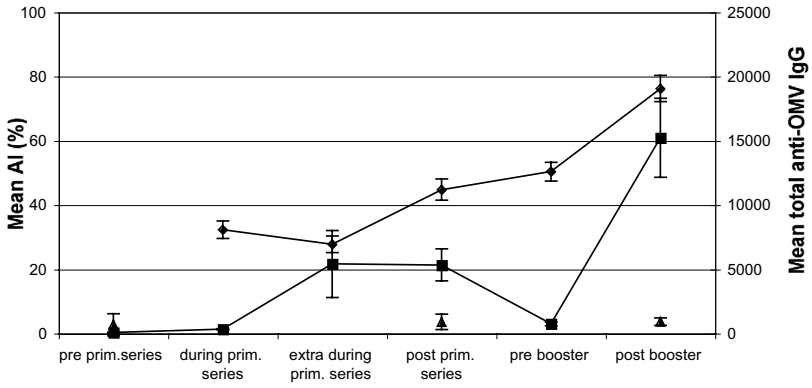

Figure 1. Mean Avidity Index (AI) expressed as a percentage (diamonds), mean total IgG level against P1.7-2,4 OMVs (squares) and mean anti-OMV IgG level against PorA- negative OMVs (triangles) in sera of 134 toddlers after immunization with a monovalent Men B OMV vaccine. Error bars indicate 95 % confidence intervals.

Avidity

As found for the total anti-OMV IgG titer, variance analysis showed that the avidity titer was highest in the group using the 3+1 vaccination schedule and $AlPO_4$ as an adjuvant, indicating that using this schedule and adjuvant resulted in significantly more avid antibodies (p=0.0005 and p=0.0243).

Mean AIs for all children are shown in figure 1. Included are only values for serum samples containing measurable specific IgG antibodies, since it is not possible to calculate an AI when anti-OMV IgG titers are undetectable. Only 4 out of 134 toddlers had a detectable anti-OMV IgG titer before the first vaccination, with an AI of 34, 40, 50 and 53 %, suggesting previous natural exposure to meningococcal antigens. The AI values of these 4 sera are not plotted in the figure. All other serum samples obtained during the trial had detectable antibodies and were included in the analysis. The mean AI after the primary vaccination series was 44.8 % (confidence interval (CI) 41.5-48.0). Before the booster, the AI rose significantly to 50.0 % (CI 47.5-53.2) (paired sample t-test, p= 0.015). After the booster vaccination the mean AI increased further to 76.2 % (CI 72.2-80.3) (p< 0.0001). Although IgG antibody titers dropped in the period after the last vaccination of the primary series, the AI of these antibodies increased (Fig. 1), whereas AI increased over time for the 4 groups receiving different adjuvants and following different immunization schedules (Fig. 2). The differences between these

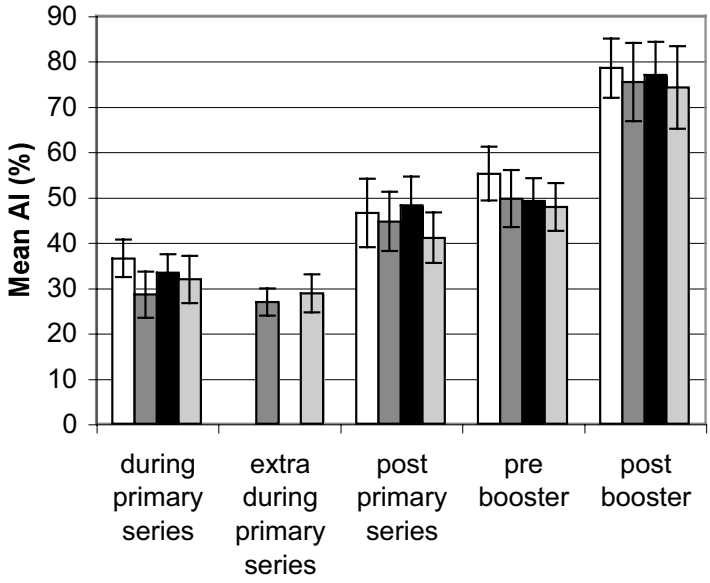

Figure 2. Mean AI expressed as a percentage for each group. The four bars represent the two different vaccine adjuvants and the two different vaccination schedules.

$Al(OH)_3$: aluminium hydroxide; $AlPO_4$: aluminium phosphate; 2+1: two primary vaccinations followed by a booster vaccination (□ = $Al(OH)_3$, ▒ = $AlPO_4$); 3+1: three primary vaccinations followed by a booster vaccination (■ = $Al(OH)_3$, ▒ = $AlPO_4$). Error bars indicate 95 % confidence intervals.

groups in AI were not significant after the booster vaccination (ANOVA, p=0.746).
To investigate the importance of the number of vaccinations in stimulating a high AI, two subgroups were selected with a similar mean total anti-OMV IgG titer. One group of children had a mean total anti-OMV IgG titer of 3.4 (95% CI: 3.3-3.5) after the primary series (n=86) whereas the other group had a mean total anti-OMV IgG of 3.4 (95% CI: 3.3-3.6) post booster (n=21) (Mann Whitney U test, p= 0.215). AI and SBA titers were compared between these two groups. Both AI and SBA were significantly higher in the post-booster group than in the post primary group: 73.6 vs. 45.3 % (p< 0.0001) and 1.0 vs. 0.64 (p= 0.009) respectively, indicating that avidity maturation upon boosting was independent of the IgG titer.

IgG isotype distribution
Figure 3 shows the distribution of IgG isotypes in the 4 different groups. In every sample, IgG1 was the predominant isotype. IgG3 was also found at high levels, whereas no IgG2 or IgG4 could be detected.
Variance analysis showed that levels of IgG1 as well as IgG3 were both higher using a 3+1 schedule than using the 2 + 1 schedule (p=0.0007 and p=0.0008 respectively). $AlPO_4$ as an adjuvant induced significantly higher IgG1 titers than $Al(OH)_3$ (p= 0.0002 and p= 0.0033). Specific IgG3 titers did not differ between the two adjuvants (p= 0.07).

Correlations between SBA, AI and ELISA titers
SBA levels for the sera were reported previously[9]. Briefly, 66 % of all toddlers showed a fourfold or higher increase in SBA after the primary series and this percentage increased up to 97 % after the booster. We used these results to correlate ELISA and AI results to SBA for each individual serum sample (figures 4 A, B and C). The SBA titers and ELISA titers correlated strongly: r=0.85 for total IgG, r=0.83 for IgG1, r=0.82 for IgG3 and r=0.84 for the avidity titer (p< 0.0001). Correlation between SBA and AI was markedly lower but still significant (r=0.36, p< 0.0001).

Discussion

This study showed clearly that the AI of PorA specific antibodies increased during the course of vaccination with a monovalent meningococcal OMV vaccine, even when IgG titers fell after the primary series. To our knowledge, this is the first study showing avidity maturation of antibodies elicited by a meningococcal group B vaccine in children.
To measure the AI we used NaSCN in ELISA at a concentration of 1.5 M. For antibodies against polysaccharides, Anttila *et al.* (2) used 0.5 M NaSCN in their assay

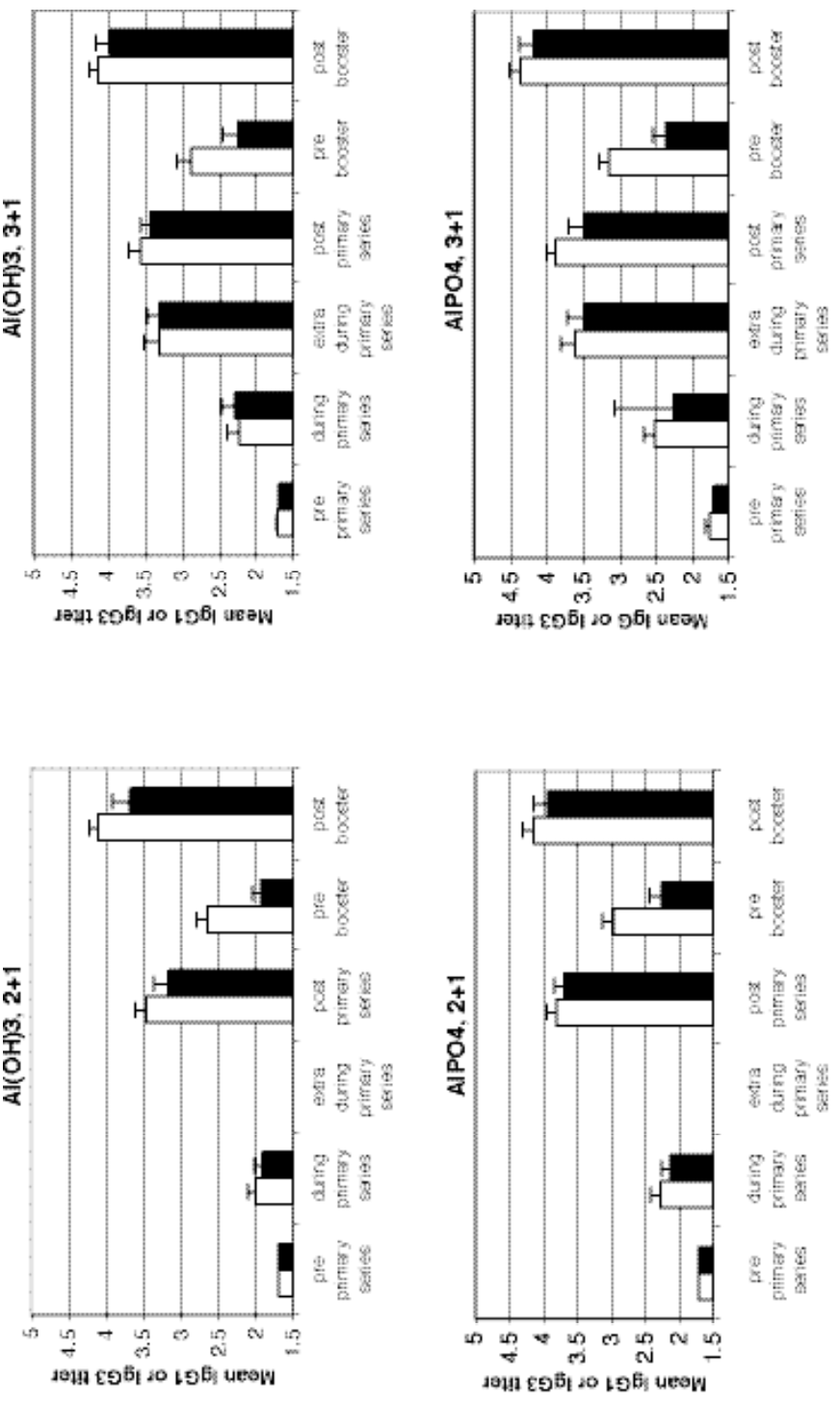

Figure 3. Serum [10] log IgG isotype concentrations against P1.7-2,4 OMVs for each group. No IgG2 or IgG4 was found.
A1 $(OH)_3$: aluminium hydroxide, A1PO_4: aluminium phosphate, 2+1: two primary vaccinations followed by a booster vaccinatiation;
□ = IgG1, ■ = IgG3. Error bars indicate 95% confidence intervals.

Figure 4 A

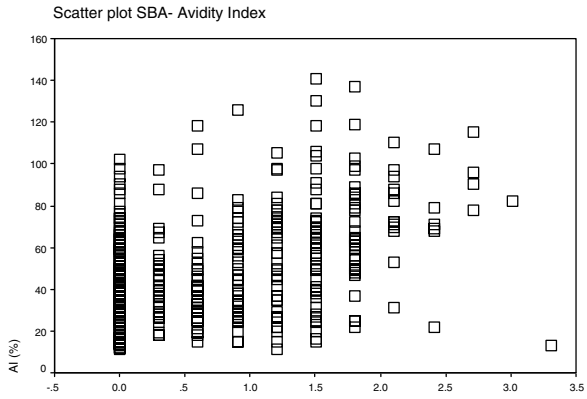

Figure 4 B

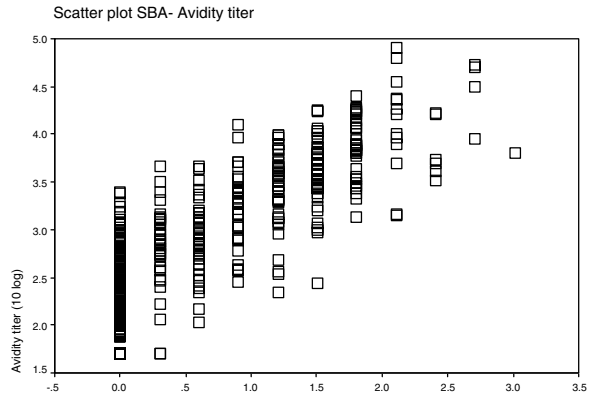

Figure 4 C

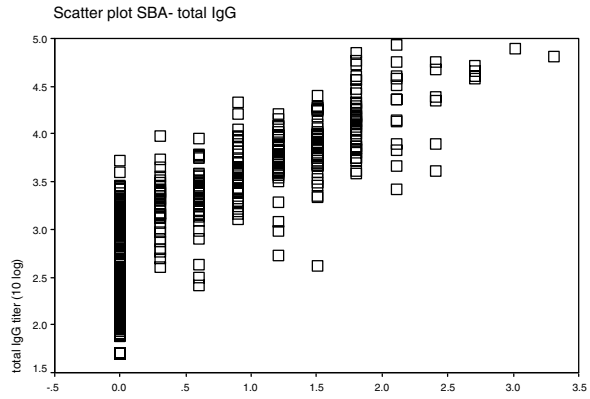

Figure 4. Scatter plots of ^{10}log serum bactericidal titers plotted against AI (figure 4 A, r=0.36), ^{10}log avidity titer (figure 4 B, r=0.84) and ^{10}log total IgG titer (figure 4 C, r=0.85).

to measure the AI of antibodies elicited by various pneumococcal vaccines, while Goldblatt *et al.*[14] used a mean concentration of up to 0.6 M of NaSCN in their elution ELISA. We found the best resolution between high and low avidity antibodies using a 1.5 M concentration, comparable with Pollard *et al.* (25) who used 1.6 M NaSCN to measure the AI after infection with serogroup B meningococci. This difference in molarity shows that the avidity of antibodies against proteins such as PorA is in general higher than that of antibodies against (conjugated) polysaccharides. The pH of the high molarity solution was reduced to 6.75, compared to 7.4 of PBS alone. However, Goldblatt *et al.*[13] showed that pH has no effect on the thiocyanate ion. Although antibody avidity is an important surrogate of protective efficacy for several vaccine types, vaccines differ in their ability to evoke avidity maturation. Anttila *et al.*[2] showed that boosting with a pneumococcal polysaccharide vaccine did not induce an increase in the AI. This was in contrast with the response seen after a booster with pneumococcal conjugate vaccine, which induced a strong increase in AI, indicating that maturation of antibodies was induced by this vaccine. The AI as a determinant of protective efficacy was also reported by Usinger *et al.*[31] who showed an inverse correlation between the magnitude of avidity and the amount of antibody required to protect mice against lethal pneumococcal bacteremia. Furthermore, Goldblatt *et al.*[14] showed that the AI could be used as a surrogate marker of successful priming by Hib conjugate vaccines. Children with anti-Hib IgG titers below the protective level of 1.0 μg/ml after primary immunization had antibodies with significantly lower AIs after the booster than children who showed a high antibody titer after priming. After systemic infection with serogroup B meningococci the mean avidity of antibodies in infants was markedly lower than in children older than 10 years[25]. This finding correlated with the absence of SBA in the serum samples of the infants and the presence of SBA in the sera of children older than 10 years[25]. In our study, the booster vaccination induced an increase of the mean AI of anti-OMV IgG up to 76%. By using OMVs of a PorA negative strain HI-5 as an antigen in ELISA the antibodies induced by the RIVM vaccine were shown to be mainly PorA specific (Fig. 1). Less than 10% of antibodies appeared to be directed against other antigens such as LPS or other outer membrane proteins. To differentiate an increase in antibody titers from an increase in AI upon boosting, the AI of pre and post booster sera with similar anti-OMV IgG titers were compared. Although IgG titers were equal, the post-booster group had a much higher AI. This was associated with a significant increase in SBA titer. This finding indicates that the number of vaccinations a child received was more important for avidity maturation than the level of IgG and that an increase in avidity after boosting had a strong effect on SBA. A booster vaccination seems to be necessary for optimal antibody maturation. Although correlation between SBA and AI was rather low (r=0.36), an increase in AI clearly coincided with an increase in SBA. However, in the pre-

booster samples AI significantly increased, while anti-PorA antibody titers dropped (Fig. 1), as well as SBA[9]. This phenomenon was also seen in studies using meningococcal C and Hib conjugate vaccines[4, 14]. The authors of these studies suggested that avidity maturation after priming is associated with the establishment of immunological memory and that because of this memory, protection against disease at that time point is possible. Analogously, the avidity maturation after a primary series of meningococcal B OMV vaccinations may predict immunological memory and long-term protection, but this can only be proven in a phase III efficacy trial. To confirm that the avidity maturation in our study was induced by vaccination and was not an effect of aging, we tested a selection of serum samples from a control group of an earlier study. These toddlers were vaccinated with hepatitis B vaccine instead of a hexavalent OMV vaccine and received one dose of monovalent OMV vaccine 2 and a half years later (age 5-6 years old). Mean AI in this group was much lower than mean AI after the booster vaccination in this study (29.9%, CI 23.5-36.3%, n=10).

Variation in immunization schedules and different aluminium adjuvants had no effect on the post booster AI. Variance analysis revealed that the difference between adjuvants was not significant (p= 0.13) during the course of vaccination. However, the 3+1 schedule stimulated higher anti-OMV IgG titers resulting in significantly higher SBA titers (p= 0.03), in accordance with previous results[9].

The isotype distribution of the immune response after immunization with an OMV vaccine was dominated by IgG1 and IgG3, in agreement with data described in earlier studies (22). As shown in figure 3, the distribution of IgG1 and IgG3 did not change during the vaccination trial, except for a significant decline of IgG3 in the pre-booster samples. This phenomenon was probably due to the shorter half-life of IgG3[22]. Thus, avidity maturation is not related to a shift in IgG isotype distribution during the course of vaccination. We did not detect specific IgG2 in any sample. An earlier study performed in our laboratory showed low IgG2 levels detected by whole cell ELISA after immunization with a hexavalent OMV vaccine[10]. This difference may be explained by differences in coating conditions, as we used OMVs for ELISA plate coating instead of whole cells. OMVs contain low amounts of *galE* lipopolysaccharide (LPS) whereas whole cells contain the wild type LPS (immunotype L3). Perhaps the IgG2 antibodies were directed against LPS or other proteins or polysaccharides present in whole cell preparations.

Previously, we could not find a good correlation between SBA and specific IgG or IgG subclasses using whole cell ELISA after vaccination with a hexavalent vaccine. Correlations ranged from non-significant up to 0.64 for total IgG or IgG subclasses[10]. However, in a study using the Norwegian monovalent P1.7,16 OMV vaccine, significant correlations were found between total IgG, IgG1, IgG3 and SBA[22]. In this study, also using a monovalent vaccine, the correlations with SBA were high, varying from 0.82 for

IgG3 and 0.83 for IgG1 to 0.85 for total IgG ($p<0.0001$). Correlation between SBA and the avidity titer was similar: $r= 0.84$ ($p<0.0001$). Probably, most antibodies induced by a monovalent vaccine are indeed functionally active whereas not all antibodies induced by the hexavalent vaccine are. Furthermore, the whole cell ELISA, which was used in the hexavalent study, may not be a very distinctive method to measure the specific response against PorA compared with an OMV ELISA. We found high background signals using whole cell ELISA, even in pre-vaccination samples. It seems that whole cells bind more non-bactericidal antibodies than OMVs.
We conclude that a meningococcal B monovalent OMV vaccine against serosubtype P1.7-2,4 induced high levels of functional, PorA specific IgG antibodies indicated by a strong correlation between ELISA titers and SBA. Avidity maturation of PorA specific antibodies occurred during the course of vaccination, especially after boosting. The AI of antibodies after the booster vaccination was comparable for 2+1 and 3+1 vaccination schedules and for various forms of aluminum adjuvants. Avidity maturation was independent of the level of IgG and age at vaccination. Serum antibodies with high AI were more effective in SBA than low AI antibodies. This cannot be explained by differences in IgG isotypes, since IgG1 predominated during the course of vaccination followed by IgG3 and a booster vaccination did not change this pattern. This study indicates that SBA of serum after vaccination depends on PorA specific antibody concentrations in particular and that there is an additional effect of AI. Measurement of AI should be implemented in future efficacy trials with serogroup B meningococcal vaccines to investigate its potency as a predictor for immunological memory and long term protection.

Acknowledgements

We thank dr. N.J.D. Nagelkerke for help with statistics, and prof. dr. B.A. Adler for critical reading of the manuscript.

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Chapter 3.4

PorA-specific differences in antibody avidity after vaccination with a hexavalent meningococcal B outer membrane vesicle vaccine in toddlers and school children

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Accepted for publication in Vaccine, 2004

Abstract

A clinical phase II trial with an experimental hexavalent OMV vaccine (HexaMen) containing 6 different PorAs was carried out in toddlers (2-3 years) and schoolchildren (7-8 years) in the Netherlands. HexaMen exists of two OMVs each containing 3 different PorA types. The serum bactericidal activity (SBA) after vaccination against the 6 PorAs was significantly different and was higher in toddlers than in schoolchildren. After vaccination the SBA against P1.5-2,10 was 4-6 times higher than against P1.7-2,4. The aim of this study was to test whether the differences in SBA could be explained by a difference in subtype-specific antibody avidity maturation. The avidity index of antibodies against 3 subtypes (PorA types P1.5-2,10; P1.12-1,13 and P1.7-2,4) was measured by ELISA and evaluated in relation to SBA. A significant avidity maturation for the 3 PorA subtypes was found. This maturation is most pronounced for P1.5-2,10 (mean AI = 72%), correlating with the highest SBA titres. Generally, the avidity titre correlated best with SBA. No differences in avidity indices against the 3 tested PorAs were found between toddlers and school children indicating that avidity maturation induced by this vaccine is not age-dependent.

Introduction

The Netherlands Vaccine Institute (NVI, formerly part of the National Institute of Public Health and the Environment) developed two candidate vaccines against *N. meningitidis* serogroup B based on outer membrane vesicles (OMVs) mainly containing porin A (PorA). A monovalent OMV vaccine (MonoMen) is based on a genetically engineered meningococcal strain containing two PorA subtype P1.7-2,4 genes, the most prevalent subtype among disease-causing meningococcal isolates in the Netherlands. This vaccine was safe and immunogenic in Dutch toddlers[1]. Immunisation with MonoMen mainly induced immunoglobulin (Ig) G_1 and IgG_3 isotypes, which are considered to be most important for protection against meningococcal disease[2]. The recombinant hexavalent vaccine (HexaMen) exists of two OMVs each containing 3 different PorA types [3, 4]. Immunogenicity studies with different immunisation schedules were carried out in English infants and Dutch toddlers and schoolchildren[5, 6]. HexaMen was immunogenic in infants, toddlers and schoolchildren. However, multiple doses of vaccine were required to induce a significant serum bactericidal activity (SBA) and differences were found in the magnitudes of SBA responses to different PorAs. This variability is probably caused by differences in immunogenic properties of the various PorA antigens and not due to the hexavalent presentation form[7]. HexaMen has been shown to elicit both higher SBA titres and a higher percentage of responders with a fourfold increase in SBA in UK infants (after four doses) than in Dutch toddlers and schoolchildren (after three doses). Furthermore, the SBA levels were higher in toddlers compared to schoolchildren[6]. The differences in SBA levels between toddlers and schoolchildren could not be explained by differences in IgG isotype distribution between these two age groups[8]. Furthermore, differences in antibody levels against the various PorA subtypes were not caused by differences in the IgG isotype distribution of those antibodies[9].
Generation of antibody avidity maturation has now been accepted as indicative of the induction of immunological memory and a T-cell dependent immune response[10-12]. It is shown that high avidity antibodies have better functional capacities in bacterial killing and inducing protection against experimental infection than antibodies of low avidity[13, 14]. Previously, a significant antibody avidity maturation against P1.7-2,4 was found during the course of vaccination with MonoMen, even when IgG titers fell after the primary series. The avidity index (AI) of antibodies increased significantly during the primary series of vaccinations and after the booster. Furthermore, an increase in AI of antibodies was associated with increased SBA[2]. Avidity maturation was also shown for antibodies against P1.7,16, one out of six PorA subtypes present in HexaMen following vaccination in UK infants[15].
The aim of the present study was to test whether the differences in SBA against the

various PorAs in HexaMen could be explained by a difference in subtype-specific antibody avidity maturation. Specific antibody titres were measured by a standard enzyme-linked immunosorbent assay (ELISA) and by an ELISA method measuring only high-avidity antibodies. We compared the antibody avidity maturation in toddlers to that in older schoolchildren immunised with HexaMen to detect possible age-related differences in avidity maturation.

Materials and methods

Subjects and vaccinations

In this study, serum samples were used from a previously performed open randomised, placebo controlled phase II study in toddlers (2-3 years old) and schoolchildren (7-8 years old) in Rotterdam, the Netherlands using a hexavalent PorA OMV vaccine (HexaMen)[6]. HexaMen was produced using two different trivalent *Neisseria meningitidis* strains, PL16215 (P1.7,16; 5-1,2-2; 19,15-1) and PL10124 (P1.5-2,10; 12-1,13; 7-2,4). PorA represents 90% of the vaccine protein content. The vaccine OMVs express no class 2/3 proteins and only low amounts of class 4 and 5 proteins[3]. Furthermore, a low amount of galE LPS is present in the vaccine vesicles. All children received 3 vaccinations at timepoint 0 and 2 and 8 months later. Serum samples were drawn before the start of the trial, after 2 vaccinations, before and after the booster vaccination. Sera from 60 toddlers and 61 schoolchildren who received 100 μg total protein (15 μg of each PorA; lot E 9281) per vaccination were available.

ELISA and avidity ELISA

To assess total anti-OMV IgG titres and antibody avidity an enzyme-linked immunosorbent assay (ELISA) was performed on all samples as described earlier[2]. Briefly, Immulon 2 (Dynex Technologies, Inc.) 96-well plates were coated overnight with 100 μl/well monovalent OMVs from the isogenic class3 and 4 negative vaccine strain H44/76 expressing either P1.5-2,10; P1.12-1,13 or P1.7-2,4 as subtype at a protein concentration of 4 μg/ml. Sera were diluted 1:100 in PBS-0.1% Tween 80 and a threefold serial dilution of serum samples was incubated for 90 minutes at 37°C. One plate contained all samples from 2 children in duplicate. After three washes, 100 μl of a 1.5 M NaSCN solution (dissolved in PBS) was added to one half of each plate and plain PBS was added to the other half. After incubation for 15 minutes at room temperature all wells were washed three times and for detection of bound antibody the plates were incubated with rabbit-anti-human IgG 1:5000 conjugated with horseradish peroxidase conjugate for 90 minutes at 37°C. Subsequently after washing, TMB substrate was added, the reaction was allowed to proceed for 10 minutes and was stopped by adding 100 μl of 2 M H_2SO_4 per well. The absorbance at 450 nm was read

using an EL312e Bio-Kinetics reader. IgG antibody titres were determined as the dilution yielding 50% of the maximal OD as was obtained for that day. Samples with antibody titres below the assay's detection limit were assigned a value of 50. Titres obtained after treatment with NaSCN were called "avidity titres". An Avidity Index (AI) was expressed as the percentage of antibodies that remained bound to coated antigen after treatment with NaSCN: AI = titre (NaSCN+)/titre (NaSCN-) x 100^2. Geometric Mean Titres (GMT), Geometric Mean Avidity Titres (GMAT) and the Geometric Mean Avidity Index (GMAI) were calculated.

Serum bactericidal assay

The SBA was measured as described in previous studies[2, 6]. Results of the bactericidal activity against 3 isogenic PorA strains (TR1213, TR4 and TR10) and H1.5, a PorA deficient strain of H44/76 [16, 17] of 60 toddlers and 61 schoolchildren were used . From a subset of 23 toddlers, SBA titres measured against 3 wildtype P1.7-2,4 strains 91/40, 97/181 and 92/53 obtained from New Zealand were used as well. The serum bactericidal titre (SBA titre) is reported as the reciprocal of the lowest serum dilution yielding ≥ 90% killing.

Statistical analysis

Antibody titres were log-transformed and geometric mean titres (GMT), avidity titres (GMAT) and SBA titres (GMSBA) were calculated with 95% confidence intervals. Differences in GMTs, GMATs and mean avidity indices (AIs) between toddlers and schoolchildren or between different subtypes were assessed using independent or paired sample t-tests on the log-transformed values. Spearman's rho correlation coefficient was used for calculation of correlations between the different IgG antibody titres, AI and SBA results, using SPSS software.

Results

IgG antibody titres and IgG avidity titre

The GMT and GMAT of the sera from toddlers and schoolchildren taken at the various time points, measured against the OMV preparations with PorA serosubtype P1.7-2,4, P1.12-1,13 and P1.5-2,10, are shown in table 1. Very low IgG titres were found in the pre-bleeds of all children. After the first two immunisations the GMT and GMAT increased significantly against all 3 PorAs. However, in toddlers the GMT as well as the GMAT against P1.5-2,10 were significantly higher compared to the other 2 PorAs ($p<0.001$). In schoolchildren, the GMT and GMAT were lowest against subtype P1.7-2,4 and significantly higher against P1.12-1,13 ($p<0.001$ and $p=0.002$ respectively). The highest GMT and GMAT in schoolchildren after two immunisations were found

against P1.5-2,10 (p<0.001). After the booster vaccination the GMTs and GMATs against P1.7-2,4 and P1.12-1,13 in toddlers were similar whereas a higher GMT and GMAT were found against P1.5-2,10 (p<0.001). In schoolchildren, the GMT and GMAT were lowest against subtype P1.7-2,4, significantly higher against P1.12-1,13 (p=0.001 and p=0.045 respectively) and the highest GMT and GMAT in schoolchildren were found against P1.5-2,10 (p<0.001).
Post-booster GMTs in general were higher in toddlers than in schoolchildren, but the difference was significant only for the GMT against P1.7-2,4 (p=0.003). After the booster GMATs against P1.7-2,4 and P1.12-1,13 were significantly higher in toddlers than in schoolchildren (p=0.011 and p=0.022 respectively), whereas the GMAT against P1.5-2,10 did not differ significantly between the two groups (p=0.078).

Avidity index
In most pre-bleeds, antibody titres were below the detection limit. Therefore, AIs could not be calculated in those samples (table 1). After 2 vaccinations the mean AI in toddlers was similar for P1.7-2,4 and P1.12-1,13 but significantly higher for P1.5-2,10 (p<0.001). After the booster immunisation a similar increase in AI was seen for all subtypes, up to 61.3% (95% CI 54.6-67.9) for P1.7-2,4; 65.9% (95% CI 60.4-71.5) for P1.12-1,13 and 70.9% (95 % CI 64.6-77.2) for P1.5-2,10 (p=0.023, P1.5-2,10 compared to P1.7-2,4).
After the booster the mean AI in schoolchildren was 65.5% (95% CI 60.9-70.1) for P1.7-2,4 and 61.7% (95% CI 57.2-66.3) for P1.12-1,13 and significantly higher for P1.5-2,10: 73.4% (95% CI 68.3-78.5) (p=0.01 and p<0.001 respectively). There were no significant differences in mean AIs between toddlers and schoolchildren for any of the subtypes at any time point.

Correlations between SBA and ELISA
SBA levels for the sera were reported previously[6]. We used these results to correlate avidity ELISA results and SBA for the sera obtained from toddlers and schoolchildren. These calculations included pre- and post vaccination sera, therefore covering the whole range of SBA titres. The results are described in table 2. In general, correlations between the avidity titre and SBA tended to be highest. Correlations between the IgG titre assessed by standard ELISA and SBA were also high, but correlations between AI and SBA were significantly lower. Correlation coefficients in general are highest for subtype P1.5-2,10; the most immunogenic subtype of the three tested. Correlations between SBA and ELISA results for P1.7-2,4 were remarkably low. We hypothesised that this could be explained by underestimation of the immunogenicity of P1.7-2,4 in HexaMen measured by SBA against the isogenic TR4 P1.7-2,4 strain, as we showed in an earlier study[18]. Therefore, SBA titres of a subset of 23 serum samples measured

Table 1. Geometric mean titer (GMT), avidity titer (GMAT) and Mean Avidity Index (AI) in percentages and Geometric Mean SBA titer (GMSBA) of sera from toddlers and school children after vaccination with HexaMen.

Group	Sample*	P1.7-2,4				P1.12-1,13				P1.5-2,10		
Toddlers		GMT (95% CI)	GMAT (95% CI)	Mean AI (95% CI)	GMSBA (95% CI)	GMT (95% CI)	GMAT (95% CI)	Mean AI (95% CI)	GMBSA (95% CI)	GMT (95% CI)	GMAT (95% CI)	Mean AI (95% CI)	GMSBA (95% CI)
	1	83 (67-104)	57 (50-65)	NA#	1.0 -	96 (75-122)	62 (52-73)	NA#	1.1 (0.9-1.2)	75 (58-97)	63 (51-78)	NA#	1.1 (0.9-1.4)
	2	2934 (2370-3632)	1364 (1097-1696)	46.0 (42.8-49.3)	1.4 (1.1-1.7)	2795 (2300-3397)	1162 (910-1484)	44.4 (40.2-48.5)	6.0 (3.9-9.2)	5067 (4281-5998)	2804 (2264-3474)	56.1 (51.7-60.6)	126.3 (90.9-175.4)
	3	422 (335-531)	200 (152-264)	51.5 (47.0-55.9)	1.1 (0.9-1.4)	467 (376-579)	178 (139-228)	42.4 (38.6-46.3)	1.2 (1.0-1.5)	569 (448-724)	303 (236-387)	55.3 (51.1-59.4)	9.9 (6.5-15.0)
	4	6752 (5702-7996)	4038 (3375-4830)	61.3 (54.6-67.9)	2.0 (1.5-2.8)	6836 (5803-8052)	4432 (3659-5368)	65.9 (60.4-71.5)	7.9 (5.4-11.6)	9456 (8101-11039)	6827 (5668-8224)	70.9 (64.6-77.2)	194.5 (164.9-229.4)
School children	1	69 (56-85)	60 (52-71)	NA#	1.0 (1.0-1.1)	103 (85-127)	59 (51-67)	NA#	1.1 (1.0-1.2)	73 (61-88)	61 (53-71)	NA#	1.1 (1.0-1.3)
	2	1089 (846-1401)	497 (387-636)	49.1 (44.1-54.2)	1.5 (1.1-2.0)	1344 (1054-1713)	625 (481-813)	49.3 (44.6-54.1)	2.8 (2.0-4.0)	2141 (1653-2772)	1226 (959-1568)	59.4 (53.3-65.6)	32.7 (22.5-47.5)
	3	261 (203-336)	121 (92-158)	52.7 (45.5-60.0)	1.2 (1.0-1.4)	456 (336-509)	125 (96-162)	40.8 (35.6-45.9)	1.2 (1.0-1.5)	426 (314-579)	210 (162-271)	54.2 (47.3-61.2)	5.5 (3.7-8.1)
	4	4542 (3778-5461)	2861 (2375-3447)	65.5 (60.9-70.1)	2.2 (1.5-3.2)	5428 (4541-6488)	3220 (2664-3893)	61.7 (57.2-66.3)	4.5 (3.0-6.6)	7584 (6479-8877)	5392 (4510-6447)	73.4 (68.3-78.5)	112.5 (84.4-150.0)

* Samples: 1: Pre priming; 2: post priming; 3: pre booster; 4: post booster

NA: not applicable; specific IgG titres too low to calculate avidity index

against three wild type P1.7-2,4 strains were used in correlation analysis as well. Correlation coefficients increased significantly for two out of three wild type meningococcal strains, especially the correlation between SBA and avidity titre (table 3).

Discussion

In the present study, the avidity index of antibodies after vaccination with HexaMen was studied as a possible explanation for differences in SBA between PorA subtypes present in the vaccine. Longworth *et al.* showed that HexaMen induced a significant avidity maturation against P1.7,16, one of the other subtypes present in HexaMen, in infants[15]. We analysed the avidity maturation against three out of six HexaMen subtypes in toddlers and schoolchildren, enabling us to study PorA-specific and age-related differences in the induction of avidity maturation. Highest SBA titres were found against subtype P1.5-2,10. In both age groups, mean AI against P1.5-2,10 was already significantly higher after 2 vaccinations compared to P1.7-2,4 and P1.12-1,13, for which similar AIs were found. The booster induced an additional increase in AI which was comparable with that fort P1.7-2,4 and P1.12-1,13, resulting in the highest AIs for P1.5-2,10 post-booster. Intrinsic immunogenic properties of this PorA subtype seem to be involved[7], leading to the induction of higher IgG antibodies with higher AIs. Similarly, serotype-dependent differences in IgG titre and AI induction were seen after vaccination with a multivalent pneumococcal conjugate vaccine[19].
In both age groups we found that the AI of PorA- specific antibodies increased during the course of vaccination with HexaMen. No differences in AIs were found between the two studied age groups at any timepoint. This is in contrast with recent findings about avidity maturation after vaccination with meningococcal group C conjugate vaccines. Anticapsular antibody avidity was lower in children with a mean age of 2.6 years than in children aged 4.6 years[20]. This difference might be explained by the different immunological response against polysaccharide antigens presented in a conjugate vaccine compared to protein antigens in an OMV formulation containing low levels of LPS as an immuno-stimulator[21]. Longworth's results and ours indicate that avidity maturation was induced after vaccination with HexaMen for all subtypes and in all age groups, which might be beneficial for the overall vaccine efficacy.
Measuring antibody avidity as a surrogate marker of vaccine immunogenicity and immunological memory induction would be less complicated than measuring SBA, which is still presumed to be the golden standard. Therefore we correlated AI and IgG titres and avidity titres with SBA titres. Generally, the avidity titre correlates best with SBA, however, correlation coefficients between SBA and avidity titre were only slightly higher than correlation coefficients between SBA and IgG antibody titres measured by

Table 2. Spearman's rho correlation coefficients between SBA and specific IgG antibody titres, avidity titres and avidity index.

SBA TR4	SBA TR1213 P1.7-2,4	SBA TR10 P1.12-1,13	P1.5-2,10
Toddlers			
IgG titer	0.42	0.72	0.87
Avidity titer	0.45	0.72	0.89
AI	0.17	0.38	0.52
School children			
IgG titer	0.41	0.62	0.83
Avidity titer	0.44	0.64	0.85
AI	0.28	0.38	0.36

standard ELISA. Burrage *et al.*, who measured avidity titres and SBA after vaccination with meningococcal C conjugate vaccines, found similar marginal differences, although in their study slightly higher correlations for the standard ELISA and SBA than for the avidity ELISA were found[22]. The avidity titre is a combination of IgG level and avidity

Table 3. Geometric mean SBA titres measured against 3 wild type meningococcal isolates with serosubtype P1.7-2,4 in 23 toddlers after 3 vaccinations with HexaMen and Spearman's rho correlations with specific IgG titres, avidity titres and avidity index.

strains	TR4	91/40	97/181	92/53
GM SBA	2.0	1,9	9,3	4,25
(95% CI)	(1.1-3.5)	(1.0-3.5)	(5.1-16.9)	(2.2-8.2)
SBA-IgG titer	0.25	0.33	0.44*	0.20
SBA-avidity titer	0.34	0.39	0.58**	0.44*
SBA-AI	0.16	0.11	0.25	0.22

* Correlation is significant at the p=0.01 level

** Correlation is significant at the p=0.05 level

index and is therefore probably the best indicator of functional antibody levels. The AI, being independent of the absolute IgG titre, allows comparison of antibody avidity during a vaccination trial and thus presumably the induction of immunological memory, but is probably less suitable as a sole marker of immunogenicity. However, it should be noted that the use of AI or GMAT as a useful measure of vaccine immunogenicity can only be proven in an efficacy trial.

The GMT after the booster against P1.7-2,4 was significantly higher in toddlers compared to schoolchildren, as well as GMATs against this subtype and against P1.12-1,13. It has been shown earlier that HexaMen induces highest IgG titres in infants and young children although titres before the priming series are consistently low in all age groups. Isotype distribution and AI of these antibodies do not differ between the age groups, thus the difference is restricted to the absolute IgG titre. The reason for the higher immunogenicity of this vaccine in younger children is still unknown.
The correlation between SBA against P1.7-2,4 and avidity titre was very poor using the isogenic TR4 strain in SBA. We showed earlier that the use of this strain in SBA probably underestimates the immunogenicity of P1.7-2,4[18]. Higher SBA titres were found when two out of three wild type meningococcal strains with serosubtype P1.7-2,4 were used as target strains in SBA. The correlation coefficients between SBA and ELISA results improved significantly when these SBA titres were used in correlation analysis.
The AI for P1.7-2,4 after vaccination with HexaMen is significantly lower than after vaccination with MonoMen (mean post-booster AI for toddlers and schoolchildren combined: 61 % vs. 76% in toddlers vaccinated with MonoMen)[2]. Furthermore, the increase in AI against P1.7-2,4 after a MonoMen booster is much higher than after a booster vaccination with HexaMen: 32% vs. 15%. This corresponds with the finding that MonoMen induced higher SBA titres and a higher number of vaccine responders.
In conclusion, there is a significant avidity maturation induced by the hexavalent vaccine for three PorA subtypes. This maturation is most pronounced for the most immunogenic PorA subtype P1.5-2,10, indicated by the highest AI after the primary series as well as after the booster vaccination. Differences in SBA between subtypes P1.7-2,4 and P1.12-1,13 can not be explained by differences in AI. No difference in AI between toddlers and schoolchildren was found after immunisation with HexaMen, indicating that avidity maturation induced by this vaccine is not age-dependent.

Acknowledgements

We thank Kees van Limpt for measuring SBA against the isogenic and wild type strains and dr. P.G. M. Mulder for help with statistics.

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Chapter 3.5

Cross-reactivity of antibodies against PorA after vaccination with a Men B outer membrane vesicle vaccine

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Infection and Immunity 2003 Apr; 71(4): 1650-1655

Abstract

The cross-reactivity of PorA-specific antibodies induced by a monovalent P1.7-2,4 (MonoMen) and/or a hexavalent (HexaMen) meningococcal B outer membrane vesicle vaccine (OMV) in toddlers and school children was studied by serum bactericidal assays (SBA). First, isogenic vaccine strains and PorA-identical patient isolates were compared as a target in SBA, to ensure that the vaccine strains are representative for patient isolates.
Geometric Mean Titers (GMT) in SBA against patient isolates with subtypes P1.5-2,10 and P1.5-1,2-2 after vaccination with HexaMen were generally lower than those against vaccine strains with the same subtype, although the percentage of vaccine responders (=> 4-fold increase in SBA after vaccination) was not affected. Using various P1.7-2,4 patient isolates, GMTs as well as the number of vaccine responders were higher than for the P1.7-2,4 vaccine strain, indicating that the use of the P1.7-2,4 vaccine strain may have underestimated the immunogenicity of this subtype in HexaMen.
Secondly, the cross-reactivity of antibodies induced by MonoMen and HexaMen was studied using several patient isolates that differed from the vaccine subtypes by having minor antigenic variants of one variable region (VR), by having a completely different VR or by having a different combination of VRs. MonoMen induced P1.4 specific antibodies that were cross-reactive with P1.4 variants P1.4-1 and P1.4-3. HexaMen induced a broader cross-reactive antibody response against various patient isolates with one VR identical to a vaccine-subtype or a combination of VRs included in HexaMen. Cross-reactivity, measured by a 4-fold increase in SBA after vaccination, against these strains ranged from 23 to 92% depending on the subtype of the tested strain and was directed against both VR1 and VR2. The extended cross-reactivity of vaccinee sera induced by HexaMen against antigenic variants has important favorable implications for meningococcal B OMV vaccine coverage.

Introduction

Neisseria meningitidis remains an important cause of bacterial meningitis and septicemia in humans, with peak occurrences in toddlers and adolescents. Despite optimal treatment, mortality rates are relatively high. Therefore, prevention of this disease is of great importance. Nowadays, effective vaccines are commercially available against serogroups A, C, W135 and Y of *N. meningitidis,* but not against serogroup B. As the group B capsular polysaccharide is not a suitable vaccine component, attention is focused on outer membrane proteins. The National Institute of Public Health and the Environment (RIVM) of the Netherlands has developed two vaccines based on outer membrane vesicles (OMV) of *N. meningitidis* serogroup B.

Table 1. Phase II clinical trials in The Netherlands using RIVM meningococcal B OMV vaccines in children. $Al(OH)_3$: aluminium hydroxide, $AlPO_4$: aluminium phosphate; 2+1: two primary vaccinations followed by a booster vaccination, 3+1: three primary vaccinations followed by a booster vaccination.

Trial number (reference)	Vaccine	Immunization schedule	Population and adjuvant
I[3]	hexavalent	2+1, $AlPO_4$	toddlers (n=172) and schoolchildren (n=165)
II[4]	monovalent	1 booster, $AlPO_4$	toddlers and schoolchildren from trial I, 2.5 years after HexaMen (n=177)
III[5]	monovalen	2+1 vs. 3+1, $AlPO_4$ vs. $Al(OH)_3$	toddlers (n=134)

Table 1 shows all phase II trials that have been performed with these vaccines, in which the most important antigen is the porin A (PorA). This transmembrane protein consists of 8 loops, of which the surface-exposed loops 1 and 4 contain one variable region each (VR1 and VR2 respectively). The variation in these two loops determines the subtype. First, a recombinant, hexavalent vaccine consisting of two OMVs each containing three different PorA subtypes was constructed including the subtypes P1.5-2,10; P1.12-1,13; P1.7-2,4; P1.19,15-1; P1.7,16 and P1.5-1,2-2 (HexaMen). In clinical phase II trials in infants, toddlers and school children, subtype antigens P1.5-2,10 and P1.5-1,2-2 were most immunogenic, whereas the most prevalent subtype in the Netherlands P1.7-2,4 induced the lowest bactericidal activity assessed by the

corresponding vaccine strains in SBA. The geometric mean titers (GMTs) measured by serum bactericidal assay (SBA) against the P1.5-2,10 and the P1.5-1,2-2 vaccine strains were at least 4-6 times higher than the GMT against the P1.7-2,4 strain[2, 3]. A second OMV vaccine was constructed expressing only the P1.7-2,4 subtype (MonoMen). This vaccine was highly immunogenic in toddlers and induced a booster response in children previously vaccinated with HexaMen[4, 5]. The booster response was not only observed for the P1.7-2,4 subtype but to a lesser extent also for other subtypes included in HexaMen. This suggests that cross-reactivity could contribute to the immune response and hence protection from disease.

The theoretically predicted coverage of MonoMen and HexaMen based on the exact match of vaccine subtypes is 39 % and 50 % of group B meningococci in the Netherlands respectively[10]. In 2000, up to 72 different subtypes of *N. meningitidis* were isolated in the Netherlands[10]. It is impossible to include all of these variants in an outer membrane protein based vaccine. Little is known about the cross-reactivity of antibodies induced against a specific PorA subtype present in either a monovalent or a hexavalent OMV vaccine. Theoretically, four kinds of cross-reactivity could be found: (i) cross-reactivity against strains having minor antigenic variants of one VR, (ii) cross-reactivity with strains sharing only one VR1 or VR2 with a vaccine subtype (iii) cross-reactivity against strains having a different combination of VRs compared to vaccine subtypes and (iv) cross-reactivity against strains having VR combinations not included in the vaccine. Only data on cross-reactivity against minor VR variants of the P1.5-2,10 subtype in HexaMen are available, showing a reduced killing of strains with minor sequence variants of the P1.10 epitope[9]. Presence of cross-reactive antibodies of any kind would have great implications on vaccine efficacy.

We studied the specificity of antibodies in serum samples of toddlers or school children vaccinated with HexaMen, MonoMen or both. First, to investigate whether vaccine strains used in SBA are representative for patient isolates of the same subtype, we collected a set of patient isolates with subtypes P1.5-2,10; P1.5-1,2-2 and P1.7-2,4. These strains were used as a target in SBA with sera collected in phase II trials and titers were compared with titers obtained when the vaccine strains were used in SBA. Secondly, we collected several patient isolates which frequently cause meningococcal disease in the Netherlands, differing from vaccine subtypes either in minor VR variants, having a completely different VR, or having a different combination of VRs to study the cross-reactivity of the antibodies induced by HexaMen and MonoMen in SBA.

Materials and Methods

Vaccine and serum samples

Available sera from three different clinical phase II trials in children were used. These trials have been described earlier and are summarized in table 1[3-5]. Only the pre-vaccination serum samples and the samples drawn post booster were used for analysis.

Bacterial strains

The vaccine strains used in our experiments are all isogenic variants of strain H44/76 P1.7,16, each expressing one of the vaccine subtypes (TR52, TR10, TR1213 and TR4)(12). The *N. meningitidis* patient isolates used to study the cross-reactivity of the immune response induced by the two RIVM vaccines are listed in table 2. These strains were obtained from the Netherlands Reference Laboratory for Bacterial Meningitis (AMC/RIVM) and from the RIVM collection. Serosubtyping of the strains was based on the sequence of PorA variable regions and classified according to the recently changed nomenclature[7, 8] .

Strain characterization

Expression of PorA, Opa, Opc and LPS phenotype was determined using semi-quantitative whole cell enzyme-linked immunosorbent assay (ELISA). The following monoclonal antibodies were used: MN 14C11.6 (anti-P1.7), B306 (anti-Opc), MN43F8.10 (anti-L8), 4A8B2 (anti-L3), MN42F12.32 (anti-L2), 17-1-L1 (anti-L1), MN5A10F (anti-P1.9), MN5C11G (anti-P1.16), MN20B9.34 (anti-P1.4) and 2 monoclonals against Opa proteins: 15-1-P5.5 (anti-P5.5) and MN20E12.70 (anti-B128).

Colony blot for antigen expression

The strains used for vaccine production express a low amount of Opa and Opc proteins. When ELISA results of the patient isolates used in SBA were positive for Opa and/or Opc, a negative colony was selected using double-staining colony blotting to exclude possible cross-reactivity with class 5 proteins. This method has been described by Kuipers *et al.*[6]. For the double staining the appropriate monoclonal against Opa or Opc together with a monoclonal of another IgG isotype against a common antigen that should be present on all colonies (e.g. against the PorA subtype or LPS phenotype) were used. The colony blot procedure was repeated on the picked colonies as a control and the selected strains were checked in ELISA for expression of several proteins.

Table 2. Characterization of patient isolates used in cross-reactivity experiments. Right columns: reaction with monoclonal antibodies (Mab) in whole cell ELISA against P1.4 (MN20B9.34) and/or P1.7 (MN14C11.6). nt= non-typable.

Strain	Serologic classification	PorA	VR 1	VR 2	Reaction with P1.4 Mab	Reaction with P1.7 Mab
N405/94	B:19,7,1:nt	P1.18,4-1	PPSKGQTGNKVTKG	HVVVNNNVATHVP	-	-
200487	B:4:nt	P1.7-2,del1	AQAANGGASGQVKVTKA	HVVVNNKV	-	-
MI/97027	B:4:nt	P1.7-2,del2	AQAANGGASGQVKVTKA	HVV	-	-
200483	B:4:15	P1.7-2,del3	AQAANGGASGQVKVTKA	H	-	-
60/96	Y:1:4	P1.21-5,4-3	QPQVPNSVQGNQVKVTKA	HVVVNNKVTTHVP	++	-
MC 50	C:nt:16	P1.21,16	QPQVTNGVQGNQVKVTKA	YYTKDTNNNLTLVP	-	-
M1080	B:19,7,1:7,1	P1.7-1,1	AQAANGGAGASGQVKVTKVTKA	YVAVENGVAKKVA	-	++
200840	B:4:nt	P1.7-2,13-2	AQAANGGASGQVKVTKA	YWTTVNTGSATTTFVP	-	
2001642	B:4:nt	P1.7-2,13-2	AQAANGGASGQVKVTKA	YWTTVNTGSATTTFVP	-	-
2001825	B:1,4:nt	P1.7-2,13-1	AQAANGGASGQVKVTKA	YWTTVNTGSATTTTFVP	-	+/++
2001395	B:nt:nt	P1.7-2,13	AQAANGGASGQVKVTKA	YWTTVNTGSATTTTTFVP	-	-
200486	B:4:nt	P1.7,13-2	AQAANGGASGQVKVTKVTKA	YWTTVNTGSATTTFVP	-	-
2001624	B:1:16	P1.7-2,16	AQAANGGASGQVKVTKA	YYTKDTNNNLTLVP	-	-
200408	B:4:2	P1.7-2,2	AQAANGGASGQVKVTKA	HFVQQTPKSQPTLVP	-	-
200488	B:nt:nt	P1.7-2,9	AQAANGGASGQVKVTKA	YVDEQSKYHA	-	-
200041	B:4:4	P1.7-11,4	AQAANGGASGQVKVTKVTKVTKA	HVVVNNKVATHVP	++	-
2001738	B:4:7	P1.7-8,4-1	AQAANGGAGASGQVKVTKVTKVTKA	HVVVNNNVATHVP	-	++
2001795	B:nt:12,4	P1.12-1,4	KPSSTNAKTGNKVEVTKA	HVVVNNKVATHVP	++	-

Immunoblot

The amount of PorA expressed by each strain was determined by semiquantitative immunoblotting[6], using a monoclonal antibody against loop 3 of PorA (MN23G2.38) which is a shared epitope of all PorA subtypes. The molecular weight marker was stained separately with AuroDye to visualize the protein bands.

Selection of a PorA negative strain

Strain 2000488, subtype P1.7-2,9 was grown on GC agar plates overnight for 18-20 hours. Four µl of the plasmid pTZ19R, which contains a PorA gene with a kanamycin resistance box (G.Vidarsson, personal communication), was cut in the presence of 1 µl of the restriction enzyme EcoRI, 3 µl of buffer H 10x and 22 µl water during 1 hour at 37°C. Bacteria were removed from the plate with a sterile swab and resuspended in 10 ml Müller- Hinton Broth (MHB) (SVM, Bilthoven, the Netherlands)/ 10 mM $CaCl_2$. A dilution of 1:5 was made in 5 ml of MHB/10 mM $CaCl_2$ and this was divided into 2 tubes each containing 2.5 ml of the suspension. Thirty µl of restricted plasmid was added to 1 tube whereas nothing was added to the other tube. After 2 hours of incubation in a 37°C/5% CO_2 incubator, 250 µl of each tube was plated on a GC plate containing kanamycin (100 µg/ml) in triplicate. These plates were incubated overnight. The next day, plates on which the suspension without any DNA was plated, showed no bacterial growth, whereas the other plates contained several colonies. These colonies were picked and checked for PorA expression using immunoblot.

Serum bactericidal assay

SBA was measured as described earlier[16.] The serum bactericidal titer was reported as the reciprocal of the lowest serum dilution yielding ≥ 90% killing. A vaccine responder was defined as having a 4-fold or greater increase in SBA titer compared with the pre-vaccination titer. For the patient isolates of subtype P1.5-1,2-2 and P1.5-2,10 SBA was performed using 3-fold dilutions of sera, because of expected high titers against these subtypes.

Statistics

Geometric mean titers (GMTs) and 95% confidence intervals (CI) were calculated. Wilcoxon signed rank tests were used to evaluate the significance of differences between titers. A p-value < 0.05 was considered significant.

Table 3. Geometric Mean Titers (GMTs) and 95% Confidence Intervals (CIs) for SBA titers andthe percentage of children with a 4-fold increase in bactericidal activity after vaccination against patient isolates of the same subtype as the P1.7-2,4 vaccine strain.

		isogenic H44/76 P1.7-2,4	strain 91/40 B:4:P1.7-2,4	strain 92/53 B:4:P1.7-2,4	strain 97/181 B:14:P1.7-2,4
trial I post booster (n=46)	GMT (± 95% CI)	2.9 (1.9-4.3)	2.5 (1.7-3.8)	5.6* (3.5-8.8)	11.3* (7.5-17.1)
	% 4-fold increase	37.0	26.0	65.2	84.8
trial II post booster (n=130)	GMT (± 95% CI)	4.0 (3.0-5.1)	3.8 (2.7-5.3)	10.8* (7.3-16.0)	11.0* (7.8-15.4)
	% 4-fold increase	48.5	31.2	51.2	61.9
trial III post booster (n=60)	GMT (95% CI)	57.0 (41.3-78.8)	27.4* (19.0-39.6)	86.5* 54.5-137.3)	123.5* (88.0-173.3)
	% 4-fold increase	100	100	100	100

* significantly higher or lower than GMT against the isogenic strain in SBA (Wilcoxon signed rank test)

Results

Vaccine strains versus patient isolates with identical subtypes

Three P1.7-2,4 isolates (strain 91/40 B:4:P1.7-2,4, strain 92/53 B:4:P1.7-2,4 and strain 97/181 B:14:P1.7-2,4) from New Zealand patients were used as target strains in SBA with 46 post-booster samples from trial I, all post-booster sera from trial II and 60 post-booster serum samples with a measurable SBA titer from trial III. Results are shown in table 3. In all tested sera higher GMTs and a significant increase in vaccine responders were observed for two out of three P1.7-2,4 patient isolates compared to the P1.7-2,4 vaccine strain. The other isolate gave a significantly lower GMT only in children vaccinated with MonoMen although the percentage vaccine responders was similar. These results indicate that the SBA titers obtained with the P1.7-2,4 vaccine strain may have underestimated the immunogenicity of P1.7-2,4 in HexaMen. To confirm this result, post-booster sera of a subgroup of 11 children from trial I with a high bactericidal GMT of 52.7 (95% CI 30.8-90.1) were tested against 6 other P1.7-2,4 patient isolates. For only 1 out of the 6 isolates a significantly lower GMT was observed, whereas the percentage responders did not vary significantly (range 64 to 91%) whereas for the other 5 isolates GMTs were similar or even higher (data not shown).

Sera from children vaccinated in trial I with HexaMen were also tested using patient isolates with VR1/VR2 combinations P1.5-1,2-2 (2 strains) and P1.5-2,10 (3 strains). For one P1.5-1,2-2 patient isolate a significantly lower GMT was measured than for the P1.5-1,2-2 vaccine strain, whereas the GMT using the other patient isolate did not differ significantly (table 4).However, the percentage vaccine responders in SBA was very similar for each strain. For P1.5-2,10, three patient isolates showed significantly lower GMTs than the vaccine strain but the percentage of children with a 4-fold increase in SBA after vaccination was 100% for all three P1.5-2,10 strains.

Table 4. Geometric Mea Titers (GMTs) and 95% Confidence Intervals (CIs) for SBA titers and the percentage of children with a 4-fold increase in bactericidal activity after vaccination against patient isolates of the same subtype as the vaccine strains with subtype P1.5-1,2-2 and P1.5-2,10.

		isogenic H44/76 P1.5-1,2-2	strain 126E P1.5-1,2-2	strain 5372 P1.5-1,2-2
trial I post booster (n=46)	GMT (95 % CI)	53.2 (35.8-79.1)	37.0 (24.1-56.5)	20.8* (13.7-31.7)
	% 4-fold increase	97.8	95.6	95.6

		isogenic H44/76 P1.5-2,10	strain B40 P1.5-2,10	strain 21/51 P1.5-2,10	strain 870227 P1.5-2,10
trial I post booster (n=46)	GMT (95 % CI)	383.0 (288.3-508.6)	29.7* (21.8-40.5)	70.3* (52.7-93.7)	25.8* (19.4-34.2)
	% 4-fold increase	100	100	100	100

* significantly higher or lower than GMT against the vaccinec strain in SBA (Wilcoxon signed rank test)

Cross-reactivity against VR1 and VR2

Serum samples drawn before vaccination and after the booster vaccination of a subgroup of 50 toddlers and school children immunized with HexaMen (trial I) were used to study cross-reactivity. The focus was on patient isolates with the P1.7 and the P1.13 epitopes, since strains with these epitopes are highly prominent in the Netherlands. In addition, we included 2 patient isolates with P1.7-2,13-2 as a VR1/VR2 combination in this study, since this subtype of *N.meningitidis* was the second most important cause of meningococcal sepsis or meningitis in the Netherlands in 2000. Table 5 shows a great variation in GMT, ranging from 2.2 (95% CI 1.4-3.5) against P1.7-1,1 to 59.6 (95% CI 33.8-105.0) against P1.7-2,13-1. The percentage of children with at least a 4-fold increase in SBA titers varied from 23% for P1.7-1,1 to 92% for

Table 5. Geometric Mean Titers (GMTs) and 95% Confidence Intervals (CIs) for SBA titers and the percentage of children with a 4-fold increase in bactericidal activity against a panel of vaccine variant strains after vaccination with a hexavalent meningococcal OMV vaccine. NC = new combination of vaccine VRs, i = identical to vaccine VR, v= variant of vaccine VR, u = unrelated to any of the vaccine VRs.

		P1.7-2,13,2 I	P1.7-2,13-2 II	P1.7-2,13-1	P1.7,13-2	P1.7-1,1	P1.21,16	P1.7-2,9	P1.7-2,16	P1.7-2,2	P1.7-2,13	P1.7,16 H44/76	P1.7-2,4 TR4	P1.12-1,13 TR1212
Relation to vaccine VRs		NC, VR 1 = i VR 2 = v	NC, VR1 = i VR2 = v	NC, VR1 = ii VR2 = v	NC, VR1 = i VR2 = v	VR1 = v VR2 = u	VR1 = u VR2 = i	VR1 = i VR2 = u	NC, VR1 = i VR2 = i	NC, VR1 = i VR2 = v	NC, VR1 = i VR2 = i	vaccine strain	vaccine strain	vaccine strain
Trial I post booster (n=50)	GMT (95% CI)	8.0 (4.6-13.9)	2.6 (1.5-4.5)	59.6 (33.8-105.0)	3.2 (1.8-5.8)	2.2 (1.4-3.5)	6.7 (3.8-11.9)	4.3 (2.6-7.2)	7.1 (4.4-11.4)	8.8 (5.2-15.0)	5.3 (3.6-7.7)	15.8 *10.1-24.5)	5.7 (3.8-8.4)	15.6 (10.1-24.0)
	% 4-fold increase	56	23	92	28	23	58	36	54	60	40	84	64	88

Table 6. GMTs and 95 % CIs for SBA titers and the percentage of children with a 4-fold increase in bactericidal activity against P1.4 variant strains after vaccination with a monovalent and/or hexavalent OMV vaccine. ND = not done

		P1.7-2,del1	P1.7-2,del3	P1.7-2,del2	P1.7-11,4	P1.12-1,4	P1.7-8,4-1	P1.18,4-1	P1.21-5,4-3	P1.7-2,4 vaccine strain
trial I (n=20)	GMT (95% CI)	168.9 (105.9-269.3)	68.6 (36.8-127.8)	5.7 (3.1-10.3)	19.0 (9.9-36.6)	ND	2.8 (1.9-4.1)	ND	ND	13.0 (7.9-21.3)
	% 4-fold increase	100	90	30	80	ND	15	ND	ND	100
trial II (n=20)	GMT (95% CI)	128.0 (69.2-236.9)	52.0 (25.0-108.0)	6.7 (3.9-11.5)	107.6 (56.0-206.8)	ND	3.7 (2.0-6.8)	ND	ND	28.8 (16.2-51.4)
	% 4-fold increase	95	80	50	100	ND	6	ND	ND	100
trial III (n=20)	GMT (95% CI)	21.1 (9.0-49.1)	16.6 (7.1-38.5)	2.5 (2.2-3.0)	163.1 (103.5-257.0)	3.2 (2.0-5.2)	5.5 (3.1-9.7)	8.0 (4.8-13.2)	512	71.0 (43.7-115.4)
	% 4-fold increase	68	60	0	100	20	40	60	95	100

P1.7-2, 13-1 (table 5). Unexpectedly, the GMT and percentage of vaccine responders differed significantly between two strains with the identical subtype P1.7-2,13-2. Furthermore, the response against the patient isolate with subtype P1.7-2,9 was ratherhigh although P1.9 is not included in the vaccine and P1.7-2 is thought to be a hidden epitope in the vaccine. Nevertheless, 36 % of all children had a significant increase in SBA titer against this strain. To ensure that the response against the P1.7-2,9 strain was directed against PorA, we created a PorA negative mutant of this strain and used this mutant as a target in SBA. The GMT was considerably lower for the mutant than for the PorA positive strain and the percentage vaccine responders decreased from 36 to 5% (data not shown). The response against 2 patient isolates with P1.16 as VR2 was rather similar: 54 and 58% for P1.7-2,16 and P1.21,16 respectively. Since P1.21 is not present in the vaccine, this shows that the presence of the VR1/VR2 combination P1.7,16 in HexaMen induced bactericidal antibodies specific for VR2.
To determine the cross-reactivity induced by vaccination with MonoMen, a subgroup of sera from 20 toddlers vaccinated with MonoMen (trial III) with a high titer against P1.7-2,4 was also tested against patient isolates having a different VR2. None of the toddlers had a significant response against strains with VR1/VR2 combinations P1.7-2,16; P1.7-2,13; P1.7-2,2 or P1.7-2,13-2 and only 1 (5%) had a response against the P1.7-2,9 isolate. This indicated that the majority of antibodies induced by MonoMen were directed against VR2 P1.4. However, 100% of toddlers had a 4-fold increase against a patient isolate with VR1/VR2 combination P1.7-11,4; and only 20% against a P1.12-1,4 patient isolate, indicating an effect of VR1 on the accessibility for bactericidal antibodies directed against VR2.

Cross-reactivity against P1.4 variants
As bactericidal antibodies induced by MonoMen were mainly directed against VR2, we tested cross-reactivity against P1.4 variants. We were unable to obtain patient isolates with VR1/VR2 combinations P1.7-2,4-1; P1.7-2,4-3 or any isolate with P1.4-2 as VR2 epitope. Results are shown in table 6. In 40% of the children vaccinated with MonoMen a 4-fold increase in SBA titer was found against a patient isolate with VR1/VR2 combination P1.7-8,4-1. Furthermore, 60% of the sera from trial III had a significant response to a P1.18,4-1 patient isolate. Against a serogroup Y strain with VR1/VR2 combination P1.21-5,4-3 a very high GMT of 512 and 95% vaccine responders in trial III was measured. This response was induced by MonoMen and not by any cross-reacting antibodies against serogroup Y capsular polysaccharide, since pre-vaccination titers were all low. Thus, vaccination with MonoMen induced bactericidal antibodies against P1.4 that can at least cross-react with P1.4-1 and P1.4-3. In sera from the trials I and II only 15% and 6% respectively of children had a

significant response against the P1.7-8,4-1 patient isolate. To study the cross-reactive antibody response against VR2 more closely, we used deletion mutants obtained from patients. The response against the deletion mutant which still had most of its VR2 conserved (P1.7-2,del1) showed very high GMTs in children vaccinated in trial I and II. Against strain P1.7-2,del3, in which only one amino acid of the P1.4 epitope was present, higher GMTs than against the vaccine strain were observed, but a lower percentage of vaccine responders. Strain P1.7-2,del2 showed lowest GMTs and lowest number of responders. Children from trial III in general showed lower GMTs against all deletion strains, indicating that the specific anti-P1.4 antibodies were less able to kill these strains, compared to antibodies evoked by HexaMen.

Discussion

The RIVM PorA-based OMV vaccines have been studied in clinical phase II trials in infants, toddlers and school children. So far, only isogenic vaccine strains were used as indicators to assess the immunogenicity of MonoMen and HexaMen in SBA against patient isolates of the same subtype. HexaMen induced a poor SBA response against subtype P1.7-2,4 as measured with the P1.7-2,4 vaccine strain[3]. This was of great concern, since this is the most prominent subtype of meningococcus B from patients in the Netherlands as well as in New Zealand. In this study we showed that GMTs as well as percentage of vaccine responders were higher when P1.7-2,4 patient isolates in stead of the P1.7-2,4 vaccine strain were used in SBA, indicating that the immunogenicity of P1.7-2,4, especially in the HexaMen vaccine may have been underestimated. A reason for this finding might be a higher expression of capsular polysaccharide or lipopolysaccharide in the P1.7-2,4 vaccine strain which hampers the accessibility of the PorA epitopes. Alternatively, the level of PorA expression could be much lower in the vaccine strain. However, on immunoblot, PorA expression was comparable for vaccine strains and patient isolates (data not shown). The number of vaccine responders when patient isolates with subtype P1.5-2,10 or P1.5-1,2-2 were used as target strains in SBA was consistently 100%. The bactericidal GMTs were significantly lower compared to the vaccine strains but the significance of this finding is unclear since the level of SBA titers required for protection against meningococcal serogroup B disease is unknown.

To assess the probable effectiveness of a vaccine based on a protein as variable as PorA in *N. meningitidis*, the cross-reactivity of antibodies induced by such a vaccine is an important issue. There is some evidence that the monovalent Norwegian (P1.7,16) and Cuban (P1.19,15) meningococcal OMV vaccines induce cross-reactive bactericidal immune responses, since toddlers showed a cross-reactive immune response ranging from 24 to 41% vaccine responders against heterologous strains[15]. Antibodies induced

by class 5 proteins, which are present in the Norwegian vaccine and can induce bactericidal antibodies, may have caused this cross-reactivity[14]. However, infants did not show any cross-reactive antibodies after vaccination with the Norwegian or the Cuban vaccine in SBA[15]. Using a whole blood assay, Morley *et al.* found very limited cross-reactive antibodies against one out of four tested heterologous serogroup B strains after immunizing infants with the Cuban vaccine but SBA did not show any cross-reactive immune response either[11]. Apparently, opsonophagocytosis measured by WBA also contributes to cross-reactivity of serum antibodies. A clinical phase III study in young adults suggested that these monovalent vaccines were not efficacious against non-vaccine type strains. In order to obtain protection against more subtypes, multivalent vaccines are desirable[13].

We showed that HexaMen, containing 6 different combinations of VR1 and VR2 epitopes in one vaccine, induced a broad response against various patient isolates sharing one VR with a vaccine-subtype and to minor VR variants of subtypes included in HexaMen. Cross-reactivity measured by a 4-fold increase in SBA after vaccination against a variety of patient isolates ranged from 23 to 92%. Just like the difference in SBA when vaccine strains are compared with some of the patient isolates with the same VR1/VR2 combination, the difference in percentage vaccine responders against the 2 patient isolates with VR1/VR2 combination P1.7-2,13-2 partly depended on so far unknown strain characteristics. LPS phenotype, Opc and Opa expression and the amount of PorA were all similar on immunoblot, yet twice as many vaccinees showed a significant antibody response against one strain compared to the other. In an earlier study, a subset of sera from the same vaccinees was tested against the PorA deficient mutant vaccine strain H1.5. There was no increase in SBA against this strain[3]. The vaccine did not contain any class 3 or 4 proteins. This indicated that the induced antibody response by HexaMen was mainly directed against PorA and that apparently other variable parts besides VR1 and VR2 of the PorA protein also contributed to a significant immune response.

After vaccination with MonoMen a strong bactericidal antibody response was observed against the P1.7-2,4 patient isolates. Patient isolates having P1.7-2 as VR1 together with a completely different VR2 like P1.9 or P1.16 were not killed by these sera, indicating that this monovalent vaccine induced in general VR2-specific antibodies. These antibodies were mainly directed against P1.4, but also showed bactericidal activity against P1.4-1 and P1.4-3. Thus, even though a monoclonal antibody against P1.4 does not cross-react with P1.4-1, sera from vaccinees show cross-reactivity with patient isolates having minor varieties in their VR2 epitopes. Children vaccinated with HexaMen and (older) children vaccinated with HexaMen and MonoMen did not respond to the P1.7-8,4-1 patient isolate. This indicates that they had fewer antibodies that were specifically directed against P1.4 and therefore are less able to kill minor

P1.4 variants. Martin *et al.* also showed that antibodies against the most immunogenic subtype of HexaMen, P1.5-2,10, showed significantly lower GMTs against variants P1.5-2,10-1 and P1.5-2,10-6, although the percentage of infants with an SBA titer >4 after vaccination with HexaMen was still reasonably high[9].
Only 20% of the toddlers responded with a 4-fold increase in SBA titer against a patient isolate with subtype P1.12-1,4. This indicates that the presence of another VR1 besides P1.7-2 in combination with P1.4 as VR2 probably results in differences in the conformation of PorA, changing the accessibility of the VR2 epitope for P1.4-specific antibodies. Furthermore, cross-reactivity against subtype P1.7-2,9 was induced by HexaMen, but not by MonoMen. We hypothesized that the response against this strain evoked by HexaMen is probably caused by cross-reactivity of antibodies directed against P1.7,16. The short size of loop 4 encoding the P1.9 epitope (sequence: see table 2) could make VR1 more accessible for antibodies induced by the P1.7 epitope of P1.7,16 [1]. The results in table 6 using several patient isolates with second loops shortened by naturally occurring deletions indeed show that antibodies induced by HexaMen were better able to kill patient isolates in which most of the P1.4 epitope was deleted compared to antibodies induced by MonoMen. The phenomenon that some of the deletion mutants showed higher bactericidal titers than the vaccine strain has been shown earlier for mutants in which one VR was totally deleted. Other (unknown) epitopes may become more accessible. As a consequence, deletion mutants are not ideal tools in studying epitope specificity of serum antibodies[9, 16].
We conclude that sera from children vaccinated with a monovalent as well as a hexavalent vaccine created by the RIVM killed disease-causing strains of the same subtypes as present in the vaccine and that the immunogenicity of P1.7-2,4 in HexaMen was probably underestimated. When children were immunized with MonoMen, most antibodies were directed against VR2 of PorA. These antibodies were cross-reactive with minor variants of P1.4 but not with strains having a different VR2 together with P1.7-2 as a VR1. HexaMen induced cross-reactive antibodies against all tested patient isolates with minor VR variant strains and strains containing (combinations of) VRs not present in the vaccine, although the number of vaccine responders was rather unpredictable. Antibodies were directed against VR1 as well as VR2 of PorA but the accessibility of VR epitopes for these bactericidal antibodies depended on the specific VR1/VR2 combination. As a consequence, protection as predicted from SBA induced by a multivalent PorA-based vaccine is probably not restricted to the vaccine VR1/VR2 combinations, but much broader. The extended cross-reactivity of vaccinee sera induced by a hexavalent vaccine against antigenic variants has important favorable implications for meningococcal B vaccine coverage, thereby limiting the number of VR combinations required in a multivalent PorA-based vaccine.

Acknowledgements

Dr. Sacchi, Dr P.Krizova and dr. D. Martin for donating strains for the RIVM collection. Dr. Zollinger and dr. Achtman for kindly providing monoclonal antibodies against Opa proteins.

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Chapter 4

General discussion and future perspectives

Introduction

Invasive meningococcal disease is one of the most life-threatening infectious diseases in children and adolescents and is feared by health care workers because of its fulminant course. Progress in pediatric intensive care management, timely recognition of the disease by parents and general practitioners and the early administration of antibiotics have improved the outcome of disease, especially in countries with a high standard of health care. Nevertheless, mortality rates of 10% or higher are still common among children with meningococcal sepsis. The absolute disease burden of meningococcal infections is highest in sub-Saharan African countries where the infection is usually caused by meningococcal serogroup A. However, in Western Europe, the United States, Australia and New Zealand, meningococcal disease is also one of the major infectious causes of childhood mortality. To control this disease three major strategies can be applied: 1) development and optimization of disease-specific treatment and supportive care, 2) disease prevention by antibiotic prophylaxis of family members and other close contacts of patients and 3) disease prevention by the development of an effective and broadly protective meningococcal vaccine.
Identification of patients who are at highest risk of severe morbidity or mortality by meningococcal infection may be helpful in the prediction of disease outcome and may facilitate the choice for an optimal therapeutic strategy for the individual patient. Similarly, in the search for a highly immunogenic and broadly protective vaccine, accurate laboratory predictors of protection need to be developed to evaluate the immune response induced by experimental meningococcal vaccines.
The principal aims of the studies presented in this thesis are to explore the nature and quality of the host response in children with meningococcal disease (**chapter 2**) or after vaccination with experimental meningococcal vaccines (**chapter 3**). The results of these studies and suggestions on future directions of research are discussed in this chapter.

Meningococcal sepsis

One of the most intriguing questions concerning meningococcal disease is why so many children and adults are carriers of *N. meningitidis* at any moment in their life, whereas so little of them become ill. Furthermore, it is unknown why some of patients with meningococcal infections develop benign meningococcemia whereas others develop meningitis and again others develop meningococcal sepsis with or without septic shock. Sørensen et al.[1] were the first to provide evidence for the importance of the genetic background in relation to susceptibility to infectious diseases in general. Adult adoptees, of whom a biologic parent had died from an infection before the age of 50, had a 5-fold higher risk of dying from an infectious cause than control subjects. Genetic polymorphisms are stable genetic variants not following simple patterns of heredity, such as the deletion or replacement of a single nucleotide (single nucleotide polymorphisms, SNPs). The presence of such genetic polymorphisms might very well explain the large variability in the clinical picture of *N. meningitidis* infections and its wide distribution without causing apparent disease in many human beings. It is well recognized that non-survivors of meningococcal infection die as a result of a deleterious host response rather than from direct invasion of *N. meningitidis* itself. SNPs in the genes encoding pattern recognition molecules of microbial antigens, innate immune response genes and coagulation regulatory pathways have already been implicated in alternations in the host response and ultimately meningococcal disease outcome[2]. In **chapter 2.1**, the current knowledge about genetic polymorphisms and their relation to meningococcal disease susceptibility, severity and outcome is reviewed. Recently, Haralambous et al.[3] showed that the risk ratio of siblings of meningococcal disease patients to contract meningococcal disease is 30.3. After correction for exposure, it was estimated that host genetic factors contribute to one third of this increased hazard risk. This again emphasizes the influence of host genetic components on meningococcal disease susceptibility. Certain variant alleles of the gene encoding mannose-binding lectin (MBL), a plasma opsonin involved in activation of the complement pathway, resulting in low plasma MBL levels are commonly found in meningococcal disease patients[4]. These, and other genetic polymorphisms involved in meningococcal disease susceptibility, theoretically offer the possibility to screen individuals on their relative risk to contract meningococcal disease. Genetic polymorphisms can also influence the disease severity and affect disease outcome. Most studies on genetic polymorphisms focus on the role of a single polymorphism. However, the scenario that combinations of polymorphisms will convey a higher susceptibility for or an adverse outcome of meningococcal disease is much more likely. Studies of combinations of many polymorphisms in relevant host immune response genes can be performed using human genome microarrays and large

numbers of DNA samples from patients. Since meningococcal disease is relatively rare and the costs of such techniques are very high, collaborative trials should be designed. Our group is currently studying this subject in collaboration with British colleagues. These investigations may provide a better insight in the pathogenesis of meningococcal disease by the elucidation of novel, relevant, functionally important genetic variants in human host response genes. Furthermore, when the expression of host response genes during meningococcal disease is known, a microarray containing those human host response genes which are relevant to meningococcal disease could be used to test the effect of experimental therapeutic strategies on the gene expression profile. Eventually, the major challenge will be to translate the knowledge on the genetic background of meningococcal infections into the development of new, effective therapies.

The innate immune system is responsible for the recognition of invading pathogens or their components. The Toll-like receptors (TLRs) are the principle signaling molecules through which infections are sensed. TLRs consist of at least ten members, each involved in recognizing a variety of pathogen-associated molecular patterns (PAMPs). These are conserved motifs, unique to the microorganism and essential for their metabolism and thus survival[5]. Host-pathogen recognition of *N. meningitidis* is initiated by LPS-binding protein (LBP), a specific lipid transfer protein, which catalyzes the transfer of bacterial membrane LPS to CD14 present on the surface of mononuclear phagocytes. Membrane-bound CD14 is thought to facilitate the transfer of LPS to the Toll-like receptor (TLR)-4, which is associated with MD2. The MD2 molecule is involved in bringing TLR4 to the cell surface and association of MD2 with TLR4 is crucial for the recognition of LPS. Together, the TLR4/MD2 complex triggers the intracellular signaling which eventually leads to the inflammatory response and production of antimicrobial products as well as various proinflammatory cytokines[6-9].

The innate immune system provides several mechanisms to combat meningococci once they have entered into the blood stream. Endotoxin, released in large amounts by meningococci, is bound to LPS-binding protein, bactericidal/permeability-increasing protein, alkaline phosphatase and other molecules. Theoretically, clearance of LPS would prevent many of the devastating effects of meningococci and could provide an attractive treatment against meningococcal disease. LBP not only facilitates the LPS response, but also LPS clearance by transferring LPS from the meningococcal outer membrane directly to high-density lipoproteins (HDL). During this process, LPS loses its biological function. Furthermore, HDL is thought to possess anti-inflammatory properties itself as well[10]. Recombinant HDL has therefore been investigated as

adjunctive treatment in patients with meningococcal disease. Serum cholesterol and low-density lipoprotein (LDL) also mediate LPS clearance by detoxification forming complexes and neutralization of its toxic effects[11-15]. We therefore studied the levels of cholesterol and lipoproteins in the initial phase sera of children with meningococcal sepsis or septic shock (**chapter 2.2**) and found extremely low levels of total cholesterol, HDL and LDL. Absolute levels of total cholesterol were significantly lower in nonsurvivors than in survivors. However, this difference disappeared after correction for age. LDL concentrations were extremely low and were even undetectable in some patients. Levels of total cholesterol and lipoproteins were inversely correlated with disease severity parameters. Together these results suggest that young children, who have lower concentrations of total cholesterol and lipoproteins, are less capable of LPS clearance, which may lead to a more severe disease course. However administration of rHDL does not correct the extremely low levels of LDL. It is therefore uncertain whether rHDL would be an effective therapy in children with meningococcal sepsis. Another role of cholesterol is its function as a substrate for the production of cortisol in the adrenal gland. Cortisol levels in children with severe meningococcal disease are inappropriately low with regard to the enormous amount of stress. Yet adrenocorticotrophic hormone (ACTH) levels are very high in these children. We propose that a shortage of cholesterol as a substrate may explain the low levels of cortisol. To support this hypothesis, further studies on the production of cortisol precursors are needed.

High levels of pro-inflammatory mediators, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1 and IL-6 can be found in patients with meningococcal disease[16-18] and are associated with disease fatality. Chemokines are small peptides that are similarly involved in the innate immune system against infectious diseases and are produced by a variety of cells such as monocytes, lymphocytes, neutrophils and endothelial cells. We studied their role and their association with disease severity and mortality in children with meningococcal sepsis. Levels of CXC and CC chemokines in the initial phase of meningococcal sepsis predicted disease severity and outcome (**chapter 2.3**). Chemokines may be suitable candidates for implementation in prognostic scores based on laboratory parameters. Furthermore, they may be useful parameters to predict bacteremia or sepsis in patients at the emergency department[19]. The results of our study suggest that chemokines may play a key role in the pathophysiology of meningococcal disease and that chemokines as well as their receptors are potentially new targets for therapeutic approaches. Chemokine receptor antagonists, anti-chemokine antibodies and broad-spectrum chemokine inhibitors are currently under development[20, 21]. Future research will have to focus on their applicability in meningococcal disease. However, the expectations for major progress should be tempered by the knowledge that preceding studies on anti-cytokine

monoclonal antibodies or antagonists (e.g. anti-TNF-α, IL-1RA) did not have a beneficial effect in the treatment of meningococcal disease[22, 23]. In addition of the activity of a single cyto- or chemokine will almost certainly not be enough to prevent the devastating effects of invasive meningococcal infections.

Studies in adults show that severe infection frequently results in a refractory state of monocytes and neutrophils, also referred to as 'endotoxin tolerance' or 'immunoparalysis'. This indicates that these cells become hyporesponsive after being exposed to small amounts of endotoxin. Interleukin-10 may play a role in this hyporesponsive state[24, 25]. Persistence of the impaired immunological function of monocytes and neutrophils is thought to contribute to mortality and morbidity by sepsis[26]. However, it is also known that excessive inflammation is harmful to the host. Meningococcal sepsis involves large amounts of LPS. We therefore hypothesized that peripheral blood mononuclear cells (PBMCs) from patients with meningococcal sepsis would be tolerant to LPS either on admission or in a later stage of disease. We thought that the presence of LPS tolerance might differentiate between survivors and nonsurvivors of meningococcal disease. Furthermore, we wanted to study the contribution of LPS on chemokine induction, since there is a lack of knowledge in this field. PBMCs from 20 children were isolated on consecutive days after admission to our PICU. Production of TNF-α, MIP-1α, MCP-1, GRO-α and IL-8 was measured and compared to the production of PBMCs from healthy young adults. We concluded that PBMCs from children with severe meningococcal disease are still highly responsive to stimulation by meningococcal OMC and that endotoxin tolerance is not present in the acute phase of survivors from meningococcal sepsis patients. The production of IL-8, MCP-1 and GRO-α was even higher than in control PBMCs. However, the production of TNF-α, MIP-1α and IL-8 is inversely related to PRISM scores, indicating that PBMC production capacity diminishes in the most severely ill patients.

The contribution of LPS to chemokine production differed per chemokine, being most important in the upregulation of TNF-α and MIP-1α. The CXC chemokines IL-8 and GRO-α are less dependent on LPS whereas MCP-1 production is independent of the presence of LPS. This emphasizes the role of other immunomodulators besides LPS, which are present in the outer membrane of *N. meningitidis*. Previously it has been shown that LPS-negative meningococci induce pro-inflammatory activity independently from the TLR4/MD2 receptor, probably through a TLR2 signaling pathway[27, 28]. However, multiple restrictions apply to these studies. We chose to isolate and freeze PBMCs for practical reasons: it is difficult to perform PBMC or whole blood stimulation assays directly after admission of a patient. Furthermore, carrying out a single

stimulation assay at a time may introduce considerable inter-assay variability. Ideally, whole blood stimulation assays would render a more representative impression whether LPS tolerance is present or not. In such an assay, not only PBMCs but also neutrophil production would be taken in consideration. Although a fair amount of PBMCs is probably lost due to the cryopreservation procedure, it has been shown that function of frozen PBMCs is very well restored after thawing[29-31].
Secondly, we used PBMCs from healthy adult volunteers since we could not obtain PBMCs from age-matched controls. In future studies on PBMC stimulation in children, a separate study on PBMC stimulation capacity of healthy children should be performed as well.
Thirdly, in our patient group, we were allowed to draw blood only when an arterial catheter was present to minimize patient discomfort. This meant that when the patient's condition improved and presence of an arterial catheter was no longer necessary, the catheter was removed and the patient left our study protocol. Therefore, the longitudinal study of PBMC stimulation capacity was hampered.

Acquired immunity against *N. meningitidis*

The acquired immune response after invasive meningococcal disease is thought to be protective. Study of the immune response in children who survive meningococcal sepsis is useful in order to establish goals for the immunity that should be induced by vaccination. OMV vaccines, in which the predominant antigen is PorA, are the only vaccines against serogroup B that have been studied in large trials. Knowledge of the immune response following meningococcal infection may provide important additional information, which can be of use in vaccine development. In **chapter 2.6** the specific immune response against PorA after invasive meningococcal sepsis in children was studied. A PorA-specific antibody avidity maturation was seen. IgG antibody titers were low and SBA could not be shown in these samples, as was reported earlier[32]. Despite these apparently low antibody titers, the presence of higher antibody avidity against the PorA subtype of the disease-causing subtype compared to a heterologous PorA subtype indicates a PorA-specific memory response. The avidity index of antibodies induced by invasive meningococcal disease was lower than that of antibodies induced by MonoMen vaccination (**chapter 3.3**), indicating that the immune response induced by this vaccine is relevant for protection against disease.

Vaccination against *N. meningitidis* in children

It is clear that, in view of the significant mortality and morbidity caused by meningococcal disease, the best solution to combat disease would be to prevent it. In

the Netherlands, serogroup C disease incidence increased significantly in recent years. In June 2002 a catch-up vaccination program using group C conjugate vaccines started. All infants born after September 2002 are routinely immunized against serogroup C disease. Since then, group C disease incidence has declined dramatically, as was also observed in Britain after introduction of this vaccine. However, a disadvantage of the C capsular conjugate vaccine is the possibility of capsular switch[33]. Intensive surveillance of meningococcal colonization and invasive isolates should be continued to be able to recognize this adverse effect.
An efficacious vaccine against serogroup B meningococci is not yet available. In **chapter 3.1**, recent developments in search for a serogroup B vaccine are reviewed. Traditionally, the focus of vaccine development against serogroup B has been on outer membrane vesicle vaccines. Alternative approaches include the use of multivalent OMV vaccines, modified capsular or lipopolysaccharide vaccines and the use of commensal *Neisseria* species as widely protective vaccines. Recently, the availability of the complete genome sequence of *N. meningitidis* has improved the opportunities to develop novel vaccines and has facilitated the efficiency and rapidity of their development. Several newly discovered vaccine antigens induce functional antibody responses in animals and passive protection in infection models. None of these have yet been investigated in humans. In the future, a combination of gene up-regulation and expression of these proteins in a meningococcal OMV vaccine is a more promising approach to combine the effectivity of PorA in OMVs with the potential new candidates.

The development of an experimental meningococcal vaccine will eventually lead to phase II safety and immunogenicity trials in humans. The Netherlands Vaccine Institute (NVI, former part of the RIVM) OMV vaccines both have been proven to be safe and immunogenic. However, the protective efficacy of the vaccines has to be proven as well. Since meningococcal disease is relatively rare, a conventional phase III efficacy trial would need large numbers of vaccinees and would take several years to conduct. The establishment of well-defined correlates of protection could greatly enhance the possibility for rapid introduction of new vaccines, without the need for large-scale efficacy trials for each new product[34]. As an example, the introduction of serogroup C conjugate vaccines in the UK was based solely on serologic data measured in an extensive clinical trial research program. Induction of functional antibodies was mainly measured by SBA using rabbit complement. A reevaluation by Borrow *et al.* [35] shows that in addition to measuring rSBA, a fourfold rise in SBA titer from pre- to postvaccination, a significant booster response and the presence of avidity maturation are all indicative of protection. For meningococcal serogroup A and C, Goldschneider *et al.*[36, 37] convincingly proved that SBA titers >4 after vaccination with polysaccharide

vaccine correlated with clinical protection. However, for serogroup B this has not been proven yet. To measure immunity induced by meningococcal serogroup B vaccination, several laboratory methods have been developed. These are reviewed in **chapter 3.2**. So far, SBA has always been considered to be the best, but not ideal, method. Other assays seem to be suitable as additional measurements of protection, but it remains uncertain whether these correlate better with vaccine efficacy. The combination of several assays is recommended for evaluation of the immune response after vaccination, in order to assess the quality of a meningococcal serogroup B vaccine candidate. Animal models mimicking meningococcal disease can provide much valuable information in that respect.

All available models have shortcomings in comparison with the natural course of disease in humans. One of the proposed additional assays to evaluate immunological memory induction is the assessment of antibody avidity. There is growing evidence that successful induction of immunological memory can be obtained by measuring antibody avidity maturation after vaccination with several conjugate vaccines[38, 39]. **Chapters 3.3 and 3.4** of this thesis clearly show the presence of avidity maturation after vaccination with the NVI OMV vaccines. We assume that immunological memory against at least three out of the six subtypes present in HexaMen is induced. However, whether presence of immunological memory is enough for protection against invasive meningococcal disease when absolute SBA titers are low, remains to be proven. The interval between nasopharyngeal colonization and invasion is presumably short. Results with Hib conjugate vaccines suggest that the presence of immunological memory leads to a more rapid immune response with antibody levels detectable after 4 to 5 days while in naive individuals the response takes seven days[40, 41]. The kinetics of antibody response following the NVI OMV vaccine has to be studied in more detail. Furthermore, mucosal immunity should also be studied since a significant reduction of nasopharyngeal carriage was shown after meningococcal C vaccination[42]. The superiority of high-avidity antibodies induced by vaccination in providing SBA should be studied in appropriate animal models, as has been done for pneumococcal conjugate vaccines[43].

The major concern about OMV vaccines is the high variability of their main component PorA. The NVI hexavalent OMV vaccine (HexaMen) is the only candidate vaccine combining several PorA subtypes in order to induce a much broader protection. The six PorA subtypes present in HexaMen have been shown to differ in immunogenicity. One of the least immunogenic subtypes, P1.7-2,4, is the most prevalent subtype in the Netherlands. A monovalent P1.7-2,4 OMV vaccine (MonoMen) was developed.

However, it will be impossible to include all disease-causing subtypes in one vaccine, since PorA is highly variable. Furthermore, the PorA subtype distribution of disease-causing isolates may evolve to have minor or major variations in time and the use of a vaccine covering only a subset of disease-causing isolates, may select for non-vaccine subtypes to cause disease. Therefore, cross-reactivity of antibodies induced by MonoMen or HexaMen against major or minor PorA variants would greatly improve the possible vaccine efficacy. In **chapter 3.5** we show that the monovalent P1.7-2,4 OMV vaccine induces antibodies mainly against VR2 which were cross-reactive with all tested variants of P1.4. HexaMen induced cross-reactive antibodies against patient isolates with exact-matched vaccine subtypes as well as against patient isolates with VR variants and patient isolates containing (combinations of) VRs not present in the vaccine. Antibodies elicited by both vaccines were mainly directed against VR2 but also against VR1. The extended cross-reactivity of vaccinee sera induced by HexaMen against antigenic variants has important implications for meningococcal B OMV vaccine coverage. However, the need remains to be able to rapidly adjust the vaccine content to newly emerging subtypes if necessary.

Future perspectives

The studies presented in the first part of this thesis focussed on the host response against *N. meningitidis* in children in relationship to disease severity and outcome. The ultimate goal of these studies was to use this information to predict disease severity and/or outcome and to develop new, effective therapies against meningococcal disease. Serum CC and CXC chemokine levels are strong predictors of meningococcal disease severity as well as outcome, and might serve as new therapeutic targets. Future studies using antibodies against chemokines or chemokine inhibitors are needed to assess their applicability in meningococcal disease. The development of an animal model or an ex vivo whole blood model of meningococcal disease would be enormously helpful in the study of these new treatments. The studies on chemokine production by patient PBMCs and the role of LPS in this process indicate that results obtained in studies in adult sepsis patients or in healthy humans, do not always correlate with results in children.
The development and application of a multivalent meningococcal OMV vaccine based on PorA is a promising approach in the prevention of serogroup B infections. However, optimization of this vaccine is needed. Extension of the hexavalent vaccine to a nonavalent vaccine, including three additional subtypes, would enlarge coverage of disease-causing subtypes and probably induce additional cross-reactivity. For the introduction of a meningococcal B vaccine, serologic correlates of protection need to be established. Besides the induction of an SBA titer >4 or a fourfold increase in SBA

titer, we propose the use of avidity ELISA, since it is a relatively simple measure and highly informative on the quality of the immune response. Introduction of a meningococcal B vaccine could then be based on safety and immunogenicity data from phase II trials and will provide observational data to show efficacy and allow confirmation of immunological correlates of protection. Furthermore, the development of a representative animal model for meningococcal disease, mimicking the natural course of infections in human through the nasopharyngeal route, requires the highest attention. In a representative animal model, high-avidity antibodies could be compared with low-avidity antibodies in their ability to protect against invasive disease. Combination of a Men B vaccine with the now routinely used Men C vaccine and perhaps with the pneumococcal conjugate vaccine as well, should be an important focus of study. Since all of these vaccines are preferably given to infants at a very early age, combining them provides a great advantage.

Ideally, in the future a globally covering meningococcal vaccine will be developed, protecting humans from serogroup A, B, C, W135 and Y disease. Such a vaccine could prevent 1.2 million disease cases each year. The completion of the genome sequence of *N. meningitidis* has improved the opportunities for developing novel and improved vaccine antigens and facilitated the efficiency and rapidity of their development. Together with the extensive knowledge on OMV vaccines this could lead to a promising new experimental vaccine against *N. meningitidis.*

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Chapter 5

Summary

Chapter 5.1

Summary

Summary

This thesis describes several studies on laboratory predictors of severity and outcome of meningococcal disease as well as laboratory correlates of protection after vaccination with an experimental vaccine against *N. meningitidis* in children. Several aspects of the host immune response, such as the role of cholesterol, lipoproteins and chemokines in the innate immune response of children with severe meningococcal disease, the function of peripheral blood mononuclear cells in response to meningococcal OMVs with or without LPS as well as the avidity, isotype distribution and cross-reactivity of antibodies induced by vaccination were studied. In this chapter, a comprehensive summary of the results are presented.

In **chapter 1** a short introduction and the specific aims of the thesis are stated.

Chapter 2 presents the results of the clinical studies in children with meningococcal sepsis. First, in **chapter 2.1**, a comprehensive review of the influence of genetic polymorphisms on the susceptibility for and severity of meningococcal disease is given. Host genetic polymorphisms in antibody receptors, LPS binding receptors or proteins, innate complement proteins as well as cytokines and hemostatic proteins can play an important role in the various stages of meningococcal infections, but much is still unknown about the genetic background of contracting meningococcal disease. Possibly, gene polymorphisms also play a role at the important level of colonization and invasion of meningococci, which would make the identification of individuals at high risk possible. Also, it is most likely that individuals with certain combinations of several polymorphisms have the highest overall risk of dying from meningococcal disease. Therefore, we propose the use of molecular-genetic techniques such as micro-arrays in future studies on the interaction of multiple polymorphisms in severe meningococcal infection.
Chapter 2.2 describes the presence of extremely low levels of total serum cholesterol, HDL and LDL in the initial phase sera of children with severe meningococcal disease. Standard deviation (SD) scores of these parameters were calculated to correct for age-related differences. Absolute serum cholesterol levels were significantly lower in non-survivors, but SD scores were similar between survivors and nonsurvivors. Total cholesterol, HDL and LDL levels on admission were inversely associated with disease severity, as indicated by disease severity scores such as PRISM, SOFA and DIC as well as by cytokine concentrations. The concentrations of total cholesterol and lipoproteins steadily increase after 24 hours in recovering patients and were normalized one to three months after PICU admission. The low total serum cholesterol level in young children may be associated with an impaired capacity of LPS clearance.

Furthermore, a positive correlation between total serum cholesterol and cortisol was found. Cholesterol is a substrate for cortisol and its low serum concentration might explain the low cortisol levels in the most severely ill patients. However, further study on this aspect is needed.
In **chapter 2.3** it is shown that initial phase serum levels of chemokines in patients with meningococcal sepsis accurately predict mortality and correlate strongly with disease severity. This suggests that chemokines play a key role in the pathophysiology of meningococcal disease. Chemokines are potentially new targets for therapeutic approaches.
Chapter 2.4 and 2.5 describe a study on the response of PBMCs obtained from children suffering from meningococcal sepsis. In chapter 2.4 we studied the presence of endotoxin tolerance of PBMCs in children during the initial phase of meningococcal disease and the persistence of this phenomenon throughout the period of manifest sepsis. We concluded that PBMCs from children with severe meningococcal disease are still highly responsive to a relevant stimulus of meningococcal OMC and that the endotoxin tolerance phenomenon is not present in the acute phase of surviving meningococcal sepsis patients. However, production of TNF-α, MIP-1α and IL-8 are inversely related to PRISM scores, indicating that PBMC production capacity diminishes in the most severely ill patients. In chapter 2.5 we show that the contribution of LPS to chemokine production differed per chemokine. LPS is most important in the induction of TNF-α and MIP-1α. IL-8 and GRO-α production are less dependent on LPS and MCP-1 production is completely independent of the presence of LPS. This study emphasizes the role of other immunomodulators besides LPS present in *N. meningitidis* outer membranes.
In **chapter 2.6** the avidity of antibodies against PorA subtype P1.7-2,4 induced by invasive meningococcal disease is studied. We found a PorA-specific antibody avidity maturation response in meningococcal sepsis patients after convalescence. The GMAI against subtype P1.7-2,4 was significantly higher than against subtype P1.7,16 or a PorA-negative meningococcal mutant. The GMAI after invasive meningococcal disease was lower than after vaccination with a monovalent P1.7-2,4 OMV vaccine (studied in chapter 3.3), indicating that the immune response induced by this vaccine is relevant for protection against disease.

Chapter 3 reports the results of studies on antibody avidity as a predictor of protection after vaccination with experimental meningococcal OMV vaccines as well as on the cross-reactivity induced by these vaccines. First, in **chapter 3.1** a review on the present status of developments in the field of meningococcal serogroup B vaccines is presented.
In **chapter 3.2** currently available laboratory correlates of protection are summarized.

Chapter 3.3 describes the study of antibody avidity and IgG isotype distribution after vaccination with a monovalent meningococcal B OMV vaccine (MonoMen). It was shown that avidity maturation occurs after vaccination with MonoMen, especially after boosting, indicated by a significant increase of the AI. Vaccination with MonoMen induced mainly IgG_1 and IgG_3 antibody isotypes, which are considered to be most important for protection against meningococcal disease. An increase in AI of antibodies is associated with increased SBA, independent of the level of specific IgG and the IgG isotype distribution. We concluded that the measurement of the AI and IgG isotype distribution of antibodies after vaccination can be a supplementary method for predicting protective immunity to be evaluated in future phase III trials with meningococcal serogroup B vaccines.
A similar study was done in sera of children immunized with a hexavalent OMV vaccine (HexaMen) and is described in **chapter 3.4**. Antibody avidity was measured against three out of the six subtypes present in the vaccine in 2 age-groups: toddlers, aged 2-3 years, and schoolchildren who were 7-8 years of age. Again, a significant avidity maturation was induced by HexaMen for the three studied PorA subtypes. This maturation was most pronounced for the most immunogenic PorA subtype P1.5-2,10, indicated by the highest AI after the primary series as well as after the booster vaccination. Differences in SBA between subtypes P1.7-2,4 and P1.12-1,13 were not explained by differences in AI. No difference in AI between toddlers and schoolchildren was found after immunisation with HexaMen, indicating that avidity maturation induced by this vaccine is not age-dependent.
There are many different circulating subtypes of *N. meningitidis* which can all cause invasive meningococcal disease. It will be impossible to include all of these variants in an outer membrane protein based vaccine. Therefore, presence of cross-reactive antibodies of any kind against subtypes which are not present in the vaccine could have great implications on vaccine efficacy. In **chapter 3.5** we studied the specificity of antibodies in serum samples of toddlers or school children vaccinated with HexaMen, MonoMen or both. Cross-reactivity against strains having minor antigenic variants of one VR, against strains sharing only one VR1 or VR2 with a vaccine subtype, against strains having a different combination of VRs compared to vaccine subtypes and cross-reactivity against strains having VR combinations not included in the vaccine was studied. We concluded that sera from children vaccinated with a MonoMen and HexaMen killed disease-causing strains of the same subtypes as present in the vaccine and that the immunogenicity of P1.7-2,4 in HexaMen was probably underestimated. When children were immunized with MonoMen, most antibodies were directed against VR2 (P1.4) of PorA. These antibodies were cross-reactive with minor variants of P1.4 but not with strains having a different VR2 together with P1.7-2 as a VR1. HexaMen induced cross-reactive antibodies against all tested patient isolates

with minor VR variant strains and strains containing (combinations of) VRs not present in the vaccine, although the number of vaccine responders was rather unpredictable. Antibodies were directed against VR1 as well as VR2 of PorA but the accessibility of VR epitopes for these bactericidal antibodies depended on the specific VR1/VR2 combination. As a consequence, protection as predicted from SBA induced by a multivalent PorA-based vaccine is probably not restricted to the vaccine VR1/VR2 combinations, but much broader. The extended cross-reactivity of vaccinee sera induced by a hexavalent vaccine against antigenic variants has important favorable implications for meningococcal B vaccine coverage, thereby limiting the number of VR combinations required in a multivalent PorA-based vaccine.

Finally, in **chapter 4** the results of this thesis are discussed and suggestions for future research are given.

Chapter 5.2

Nederlandse Samenvatting

Neisseria meningitidis, ook wel de meningokok genoemd, is een bacterie die bij minstens 10% van de mensen op enig moment in hun leven de neus/keel holte bewoont zonder dat men daar iets van merkt. Soms veroorzaakt deze bacterie ernstige ziektes zoals hersenvliesontsteking of bloedvergiftiging (sepsis), die snel dodelijk kunnen verlopen. Dit gebeurt voornamelijk bij jonge kinderen onder de 2 jaar en bij kinderen in de tienerleeftijd. Het is onbekend waardoor deze relatief veel voorkomende bacterie slechts in zeldzame gevallen tot ziekte lijdt, en waarom bij sommige kinderen de ziekte vervolgens zo desastreus verloopt.
De meningokokken worden onderverdeeld in verschillende categorieën de zogenaamde serogroepen. Recent is in Nederland een vaccin ingevoerd tegen de meningokok serogroep C. Deze subgroep van meningokokken veroorzaakt echter minder dan de helft van de ziektegevallen, en beschermt niet tegen andere subgroepen van de meningokok. Serogroep B is in meer dan de helft van de gevallen verantwoordelijk voor meningokokkenziekte. Daarom wordt in het Nederlands Vaccin Instituut in Bilthoven gewerkt aan de ontwikkeling van een vaccin wat tegen deze serogroep bescherming biedt.
In dit proefschrift worden verschillende studies naar laboratorium parameters beschreven die de ernst van ziekte weergeven. Hierbij wordt gekeken naar verschillende aspecten van de aangeboren menselijke afweerreactie, zoals de rol van cholesterol en bepaalde eiwitten, genaamd chemokines tijdens ernstige meningokokkenziekte. De reactie van witte bloedcellen op de meningokok met of zonder zijn meest toxische component lipopolysaccharide (LPS) is ook onderzocht. Daarnaast is bekeken hoe door middel van laboratorium onderzoek de mate van bescherming die door het experimentele vaccin tegen meningokokken serogroep B wordt opgewekt kan worden beoordeeld. Zo werd onderzocht hoe goed de antistoffen opgewekt door dit vaccin zich kunnen binden aan de bacterie en of deze antistoffen kunnen kruisreageren met andere typen meningokokken, zodat de dekking van het vaccin zo optimaal mogelijk is.

In **hoofdstuk 1** wordt een korte introductie gegeven van de doelstellingen van de onderzoeken zoals die zijn beschreven in dit proefschrift.

Hoofdstuk 2 beschrijft de resultaten van verschillende klinische studies bij kinderen met meningokokkensepsis. Allereerst, in **hoofdstuk 2.1**, wordt een kort overzicht gegeven van de literatuur over de invloed van genetische varianten op de vatbaarheid voor en de ernst van meningokokkenziekte. Bepaalde genetische varianten in genen van de mens voor antistofreceptors, LPS receptor, afweerrespons-eiwitten zoals cytokines en eiwitten die betrokken zijn bij de bloedstolling blijken een verhoogde kans

op het overlijden aan eenmaal verkregen meningokokkenziekte met zich mee te brengen. Er is echter nog veel onduidelijk over de genetische factoren die van invloed zijn op de gevoeligheid voor het verkrijgen van meningokokkenziekte. Misschien zijn er ook genetische varianten die een rol spelen bij de kolonisatie of bewoning van de meningokok in de neus/keel holte en vervolgens bij het binnendringen van de bacterie in de bloedbaan. Verder is het waarschijnlijk dat een bepaalde combinatie van verschillende “verkeerde” genenvarianten uiteindelijk het meest bepalend is voor het krijgen van ernstige meningokokkenziekte. Dit moet onderzocht worden met speciale moleculair-genetische technieken waarbij de interactie van vele genenvarianten tegelijk kan worden onderzocht.

Cholesterol en de eiwitten HDL en LDL zijn stoffen die van nature de eigenschap bezitten om LPS, één van de meest toxische componenten van de meningokok, te kunnen binden. Derhalve zouden deze stoffen van toepassing kunnen zijn bij de behandeling van meningokokkenziekte. In **hoofstuk 2.2** beschrijven wij dat bij kinderen met meningokokkensepsis extreem lage waardes van totaal cholesterol, HDL en LDL worden gevonden. Standaard-deviatie scores werden berekend om te kunnen corrigeren voor leeftijd, aangezien bekend is dat jongere kinderen van nature al lagere cholesterol waardes hebben. Hoewel de absolute cholesterol waardes significant lager waren bij kinderen die de ziekte niet overleefden vergeleken met de overlevenden, verdween dit verschil na leeftijdscorrectie. De hoeveelheid cholesterol, HDL en LDL waren negatief gecorreleerd met de ernst van ziekte, zoals uitgedrukt door verschillende ziekte-scores en cytokine concentraties. Vierentwintig uur na opname zijn de waardes van cholesterol, HDL en LDL al gestegen bij de herstellende patienten en 1 tot 3 maanden later zijn alle waardes genormaliseerd. Wij concludeerden dat het lage gehalte van cholesterol mogelijk geassocieerd is met de slechtere capaciteit van jonge kinderen om LPS te kunnen wegvangen. Daarnaast was werd er een verband gevonden tussen de hoeveelheid cholesterol en cortisol, een stresshormoon waarvan cholesterol één van de bouwstenen is. Deze positieve correlatie zou een verklaring kunnen zijn voor de lage hoeveelheden cortisol die vaak gevonden wordt bij kinderen met meningokokkenziekte. Dit aspect dient echter nog nader te worden onderzocht.
Hoofdstuk 2.3 toont aan dat chemokines; eiwitten die onder andere betrokken zijn bij de migratie van witte bloedcellen, heel precies de mortaliteit kunnen voorspellen. Verder vertonen deze eiwitten ook zeer sterke correlaties met ziekte-ernst parameters. Dit lijkt erop te wijzen dat chemokines een centrale rol spelen in de pathofysiologie van meningokokkenziekte, en daarom een mogelijk nieuw doelwit zijn voor therapeutische interventies.
In **hoofdstukken 2.4** en **2.5** wordt een studie beschreven naar de respons van perifeer bloed mononucleaire cellen (PBMC’s); een subgroep van witte bloedcellen,

van kinderen met meningokokkensepsis. Dit onderzoek werd verricht omdat in de literatuur gesuggereerd wordt dat blootstelling aan LPS de witte bloedcellen 'ongevoelig' maakt, zodat zij niet meer adequaat zouden reageren. Dit fenomeen wordt 'LPS tolerantie' genoemd. In hoofdstuk 2.4 wordt beschreven dat de PBMC's van kinderen in de acute fase van meningokokkensepsis nog steeds adequaat responderen op een stimulus, en dat dit zo blijft tijdens de herstelfase. Het LPS tolerantie fenomeen lijkt bij deze kinderen dus niet aanwezig te zijn. Wel vonden wij dat de respons van een aantal eiwitten negatief gecorreleerd was met ziekte-ernst, wat aangeeft dat in de meest zieke kinderen de capaciteit van de PBMC's om te responderen wat verminderd is. Meningokokken die genetisch zodanig veranderd zijn dat ze geen LPS meer bevatten, blijken toch nog in staat te zijn om de productie van ontstekingseiwitten door PBMC's tot stand te brengen. Het belang van LPS voor deze eiwitproductie verschilt sterk per chemokine; bij sommigen lijkt de productie ervan zelfs volledig LPS-onafhankelijk tot stand te komen. Deze bevindingen benadrukken het feit dat naast LPS de meningokok nog andere bestanddelen bezit die de afweerrespons van de mens in werking doen zetten (hoofdstuk 2.5).

De antistoffen tegen meningokokken die gevormd worden wanneer kinderen meningokokkenziekte doormaken zijn waarschijnlijk van groot belang om ervoor te zorgen dat je deze ziekte niet nog een keer krijgt. Het is belangrijk om de karakteristieken van deze antistoffen goed in kaart te brengen, omdat dit de antistoffen zijn die men wil induceren door vaccinatie tegen meningococcen. Immers, met vaccinatie wil men tevens liefst levenslange bescherming tegen meningokokken induceren. Eén van de eigenschappen van antistoffen in het serum van kinderen die men kan testen is de aviditeit: de kracht waarmee antistoffen kunnen binden aan het antigeen waartegen de antistof gericht is. Het PorA eiwit is een antigeen van de meningokok wat tevens gebruikt wordt als antigeen in een experimenteel vaccin ontwikkeld door het NVI tegen meningokokken. In **hoofdstuk 2.6** beschrijven we de aviditeit van antistoffen tegen het PorA eiwit zoals die gevonden wordt bij kinderen 1 tot 3 maanden na het doormaken van de ziekte. Er werd aangetoond dat de aviditeit van anti-PorA antistoffen met name gericht is tegen het PorA-type van de meningokok die de ziekte heeft veroorzaakt. Gemiddeld was 60% van de antistoffen hoog avide, wat een lager percentage is dan werd gevonden bij antistoffen geïnduceerd door vaccinatie met het experimentele NVI-vaccin. Dit kan er op duiden dat vaccinatie met dit vaccin een goede antistofrespons opwekt.

Hoofdstuk 3 beschrijft de resultaten van onderzoek naar aviditeit als voorspeller van de bescherming die geïnduceerd wordt door vaccinatie tegen meningococcen. Tevens

werd bestudeerd of vaccinatie met het NVI-vaccin kruisbeschermende antistoffen opwekt tegen meningokokken die niet in het vaccin zitten. Allereerst wordt in **hoofdstuk 3.1** een review gegeven van de huidige stand van zaken op het gebied van de ontwikkeling van een vaccin tegen serogroep B meningokokken. In **hoofdstuk 3.2** wordt vervolgens beschreven welke laboratorium voorspellers nu verricht worden of in ontwikkeling zijn en mogelijk in de toekomst kunnen helpen bij het snel en efficiënt bepalen van de effectiviteit van een experimenteel vaccin.
Eén van de twee vaccins die ontwikkeld worden in het NVI is MonoMen. Dit vaccin bestaat uit buitenmembraan blaasjes van de meningokok die uitsluitend P1.7-2,4 als PorA type (het antigeen) bevatten. Dit is het PorA type wat het meest gevonden wordt bij meningokokken die ziekte veroorzaken in Nederland, maar ook in Nieuw Zeeland. In een eerder beschreven onderzoek werd dit vaccin gegeven aan peuters van 2 tot 3 jaar in een schema van 2 of 3 vaccinaties met een maand ertussen waarna na ongeveer 8 maanden een booster vaccinatie werd gegeven. In **hoofdstuk 3.3** wordt beschreven dat de aviditeit van antistoffen steeds sterker wordt naarmate meer vaccinaties gegeven zijn. Vooral na de boostervaccinatie stijgt de aviditeit sterk. Deze sterke stijging gaat gepaard met een sterke stijging aan bacterie-dodende (bactericide) antistoffen in het serum en is onafhankelijk van de hoeveelheid antistoffen. Er werden met name IgG1 en IgG3 antistoffen gezien na vaccinatie, type antistoffen waarvan wordt gedacht dat deze het meest belangrijk zijn voor bescherming tegen vaccinatie. Een zelfde soort onderzoek werd verricht bij kinderen die gevaccineerd waren met het andere vaccin wat ontwikkeld wordt in het NVI: HexaMen (**hoofdstuk 3.4**). Dit vaccin bevat de 6 meest voorkomende PorA types van de meningokok. Bij dit onderzoek werd gekeken naar kinderen die 2 tot 3 jaar oud waren en kinderen die 7 tot 8 jaar oud waren ten tijde van vaccinatie. De aviditeit van antistoffen tegen drie van de zes PorA types werd bepaald. Opnieuw zagen we dat de aviditeit sterk stijgt tijdens de vaccinatiestudie, het meest uitgesproken tegen PorA type P1.5-2,10. Dit type PorA is geassocieerd met de hoogste serum bactericide titer. De aviditeit van antistoffen tegen de andere 2 PorA types was vergelijkbaar. Er was geen leeftijdsafhankelijk verschil in aviditeit. Wij concluderen dat het meten van de aviditeit van antistoffen na vaccinatie een goede aanvullende methode is om de effectiviteit van het vaccin te kunnen voorspellen en dat deze methode geëvalueerd zou moeten worden in grote effectiviteitsstudies met vaccins tegen serogroep B meningokokken in de toekomst.

PorA, het eiwit wat verantwoordelijk is voor de verdere onderverdeling, of subtypering, van de meningokokken is een heel variabel eiwit. Er bestaan 2 variabele regio's (VR) binnen het eiwit die in het bijzonder zeer variabel zijn: VR1 en VR2. De naamgeving van het PorA type is gebaseerd op de variatie in deze 2 variabele regio's. Jaarlijks worden er in Nederland alleen al meer dan 70 verschillende PorA types gevonden bij

meningokokken die hersenvliesontsteking of sepsis hebben veroorzaakt. Het is haast onmogelijk om al die verschillende PorA types in een vaccin te verwerken. Het is mogelijk dat antistoffen tegen een bepaald PorA type kunnen kruisreageren met PorA types met kleine of grote verschillen. Zo zou een vaccin wat gericht is tegen enkele PorA types mogelijkerwijs toch bescherming kunnen bieden tegen een veel groter aantal verschillende PorA types. Daarom hebben wij de specificiteit van de antistoffen opgewekt door MonoMen en HexaMen onderzocht (**hoofdstuk 3.5**). Het MonoMen vaccin induceerde antistoffen die met name gericht waren tegen VR2 van het PorA eiwit en niet tegen VR1. Meningokokken met exact hetzelfde VR2 PorA type worden door deze antistoffen redelijk goed herkend en gedood, maar meningokokken met hetzelfde VR1 maar een andere VR2 worden niet meer herkend door deze antistoffen. HexaMen bleek antistoffen op te wekken die ook kruisbescherming gaven tegen meningokokken met PorA types die kleine variaties hadden in vergelijking met het PorA in het vaccin. Het bleek moeilijk voorspelbaar welke meningokok wel of niet herkend en gedood zou worden door de antistoffen. Het lijkt erop dat met name de combinatie van VR1 en VR2 van invloed is op de toegankelijkheid van PorA voor de antistoffen. In elk geval lijkt de dekkingsgraad van deze vaccins niet strikt beperkt te zijn tot meningokokken met exact dezelfde PorA types als in het vaccin aanwezig, maar is er een redelijke kruisreactiviteit van antistoffen en dus mogelijk een aanzienlijke bredere dekking. Dit zou het aantal PorA types wat in ieder geval opgenomen moet worden in een toekomstig vaccin drastisch kunnen inperken.

Tenslotte worden in **hoofdstuk 4** de resultaten van dit proefschrift samengevat en bediscussieerd.

Dankwoord

Dit proefschrift kwam tot stand met de hulp van velen, die ik hierbij graag wil bedanken.

Allereerst de patiënten met meningokokkensepsis en hun ouders, die op de ICP toestemming gaven voor wetenschappelijk onderzoek op onmiskenbaar één van de moeilijkste momenten in hun leven. Mijn grote bewondering en dank hiervoor.

Mijn promotor, prof.dr. R. de Groot, beste Ronald. Jou wil ik heel graag bedanken voor de vele kansen die je mij geboden hebt in de afgelopen 8 (!) jaar, van afstudeeronderzoek, via Seattle, tot en met een promotieonderzoek aan toe. Op de momenten dat er dingen mis liepen stond jij vierkant achter mij en dat heb ik erg gewaardeerd.

De leden van de kleine commissie: prof. dr. T. van der Poll, Prof. dr. J.J.M. van Dongen, Prof. dr. H.A. Verbrugh; hartelijk dank voor het beoordelen van mijn proefschrift.

Prof. dr. H. Hooijkaas, Prof. dr. ir. H.F.J. Savelkoul, Prof. dr. J.W. van der Meer wil ik hartelijk danken voor hun bereidheid plaats te nemen in de grote commissie. Prof. dr A.J. Pollard, I'm very honored that you will visit Rotterdam to participate in the discussion on my thesis.
Dr. J.A. Hazelzet, beste Jan, altijd paraat en geïnteresseerd. Ik hoop ooit net zo'n goeie dokter te worden als jij, heel erg bedankt voor alles.

Dr.ir. G.P.J.M. van den Dobbelsteen, mijn co-promotor Germie, zonder wie ik het allang had opgegeven om ooit te promoveren. Jouw praktische aanpak, altijd bereid en bereikbaar voor advies waren echt broodnodig voor mij. Daarnaast ben je gewoon een fantastisch mens en ben ik best trots dat ik jouw eerste promovendus ben!

Alle artsen en verpleegkundigen van de ICP wil ik bedanken voor de hulp bij de inclusie van patiënten, ook al leverde dat voor jullie alleen maar extra werk op, op toch al drukke momenten…

Alle collega's van het Laboratorium voor Vaccin Research van het NVI. Het is even wennen, zo'n dokter in het lab, maar dankzij jullie heb ik een hele goede tijd in Bilthoven gehad! Met name wil ik de volgende mensen bedanken: Betsy, voor het leren van de turbo-ELISA, en nee, platen worden niet gesmeerd…., Loek, voor veel

hulp met het schrijven van artikelen en pragmatische tips, Kees van LPO voor je zoektochten naar sera en titers. En natuurlijk Harry, de beste 'doktersassistent' die je je kan wensen, hoeveel plaatjes heb jij wel niet voor me aangeënt of gecoat? Jouw flinke dosis humor sleepte ons door mega-experimenten heen. Geweldig dat jij mijn paranimf bent, dik verdiend en erg gezellig! Ook jouw spontane fotografeeractie op 5 april 2002 zal ik nooit vergeten. Ook Hans en Wilma nog erg bedankt hiervoor! En Wilma, zonder jouw lay-outen had ik het echt niet op tijd gered. Vele AIO's zullen jaloers op me zijn, wat een geweldige hulp ben jij geweest!

Collega's van de subafdeling infectieziekten en immunologie: Bedankt voor alles wat promoveren bij de infectieziekten zo leuk maakt! Met name wil ik bedanken: Edwin, Pieter (wie weet wordt het nog eens Fraaij Rietveld Vermont & Zonen..?!), Gwenda, Annemarie, Debby, Linda, Anneke, Marieke (E.). We hebben veel lief en gelukkig weinig leed gedeeld, en altijd stonden jullie voor me klaar. Edwin, Pieter, Gwenda: de vanzelfsprekendheid waarmee jullie meningokokkendiensten op jullie namen en daardoor in weekenden of nachten voor mij stonden te ficollen vond ik echt fantastisch. Jullie zijn geweldige collega's en ik hoop dat we dat nog heel lang blijven. Later kwamen Marieke, Debby, Sander en Vishal mij ook nog te hulp hierbij, ook jullie ontzettend bedankt hiervoor. Annemarie, je bent een geweldige steun en toeverlaat! Debby, naast een hele goede collega ben jij ook een geweldige vriendin geworden. Thanks voor heel veel steun op vele fronten en op naar een toekomst vol theemomenten…en ik ben supertrots met jou als paranimf! Conne, ontzettend bedankt voor al je hulp en je bereidheid om alles zo soepel mogelijk te laten lopen.

De buurvrouwen José en Karen, bedankt voor de gezelligheid en het mee-moederen. Marieke (B.), bedankt voor de geslaagde samenwerking bij de acute stress studie, het liep soepel en gezellig mede dankzij jouw grote inzet. Veel succes met het afronden van jouw proefschrift.

Ester, mijn illustere voorganger! Je stond al tijdens mijn afstudeeronderzoek klaar met raad en daad en dat ben je blijven doen. In de loop van mijn eigen promotieonderzoek heb ik me vaak verwonderd over de enorme hoeveelheden patiëntensera die jij hebt verzameld; zonder jouw vele werk had ik nooit kunnen promoveren.

Nimet wil ik graag bedanken voor de hulp bij het bepalen van de ziektescores en het schrijven van het cholesterol-artikel.

Wendy Keijzers en Arie van der Ende van het Referentielaboratorium in Amsterdam. Het is heerlijk werken als mensen zo behulpzaam en mee-denkend zijn, dank voor jullie onmisbare hulp bij het kruisreactiviteit-stuk.

Mijn geweldige vriendinnen: Wieke en Annoek, wat heerlijk en ook bijzonder om met zijn drieën in hetzelfde promotieschuitje te zitten! Zet ‘m op met jullie proefschriften, kanjers als jullie zijn worden die vast prachtig. Lieve Wiek, zo sterk als jij bent, ook dit ga jij redden. Hee Annoek, hoeveel Blood papers volgen er nog?! Sabine, die plas water tussen ons staat onze vriendschap niet in de weg hè! Bedankt voor honderden mailtjes vol steun en gezelligheid, ik had het vaak hard nodig…Miriam, als niet-dokter ben je altijd verbazingwekkend goed in het geven van de beste adviezen omtrent mijn werk! Al bijna 30 jaar (slik) leef jij mee, en ook dit was weer niet zo mooi geworden zonder jouw creativiteit. Simone, Leiden is toch mooi een stukje dichter bij Amsterdam…’Bergambacht en Schoonhoven’: ook jullie vergeet ik niet hoor!
I’d like to thank Meg for a wonderful stay and probably the best sight-seeing tour one could have in your beautiful city of Seattle! I hope to return many times; it’s great to have you as a friend.

Lieve pa, ma, Ingeborg, Marcel, Bengt en Febe, jullie zijn voor mij onmisbaar. Pa, één van jouw vele wijsheden diende als motto voor dit proefschrift en slaat niet alleen op het schrijven ervan, maar ook op de volhardendheid die nodig zal zijn in de pogingen om meningokokkeninfecties voorgoed te bestrijden. En dan heb ik ook nog eens een geweldige schoonfamilie: Jan Willem, Bep, Floris, Vincent, Marjolein en Mees! Jullie allemaal bedankt voor alle steun.

Een werkende moeder kan niet zonder oppas! Dank aan alle mensen van het Steigertje die zo geweldig voor onze zoon zorgen en aan de lieve opa’s, oma’s, ooms & tantes (al dan niet familie) die voor de noodopvang zorgen.

Steven, lieve Steven, bedankt voor je relativeringsvermogen, cursussen “interne rust”, je kookkunsten na een lange dag, je levenslust, en vooral voor oneindig veel liefde… En Finn, mijn kleine grote Finn, lieve prachtige zoon! Sinds jij er bent is de wereld net een beetje mooier. En weet ik elke dag weer waar het allemaal om draait.

Curriculum vitae

Clementien Liesbeth Vermont was born in Goes on March 30, 1975. In 1993, she graduated from the Goese Lyceum, after which she studied medicine at the Erasmus University in Rotterdam. During her study she worked as a medical student at the department of psychiatry and performed a clinical internship at the department of Internal Medicine at the Cerrahpa?a University Hospital in Istanbul, Turkey. Furthermore, she passed the propedeutical exam of psychology at the University of Leiden in 1997. She participated in a research project on the prevalence of coagulase-negative staphylococci on the Neonatal Intensive Care Unit of the Sophia Children's Hospital in Rotterdam and the Wilhelmina Children's Hospital in Utrecht under supervision of dr. N.G. Hartwig from the division of pediatric infectious diseases and immunology and prof.dr. A. van Belkum from the department of Medical Microbiology, both at the Erasmus MC. In 1998, she worked for 3 months at the department of Infectious Diseases at the Children's Hospital and Medical Center in Seattle, USA (head: prof. Craig Rubens) on the role of a serine protease in the pathogenesis of group B streptococci.

After obtaining her medical degree in April 2000, she started as a research-physician at the department of pediatrics, division of infectious diseases and immunology (head: Prof. dr. R de Groot) on the research projects presented in this thesis. She collaborated with the Netherlands Vaccine Institute (former part of the National Institute of Public Health and the Environment) in Bilthoven under supervision of dr.ir. G.P.J.M. van den Dobbelsteen.

From February till July 2004 she worked as a pediatric resident (AGNIO) at the department of pediatrics at the Medical Center Rijnmond Zuid (head: dr. A.M. Oudesluys-Murphy). On July 1 2004 she started her pediatric training (AGIO) at the Leiden University Medical Center (prof.dr. J.M. Wit and dr. R. Holl).

She is married to Steven Roosen and together they have a son, Finn (March 2003).

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