"Meningococcal infections"

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Enhanced understanding of pathogenesis leading to novel approaches in therapy and prevention

Ester Doret de Kleijn

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Enhanced understanding of pathogenesis leading to novel approaches in therapy and prevention

"Meningokokken infecties"

Nieuwe mogelijkheden voor preventie en behandeling door verbeterd inzicht in de pathogenese

PROEFSCHRIFT

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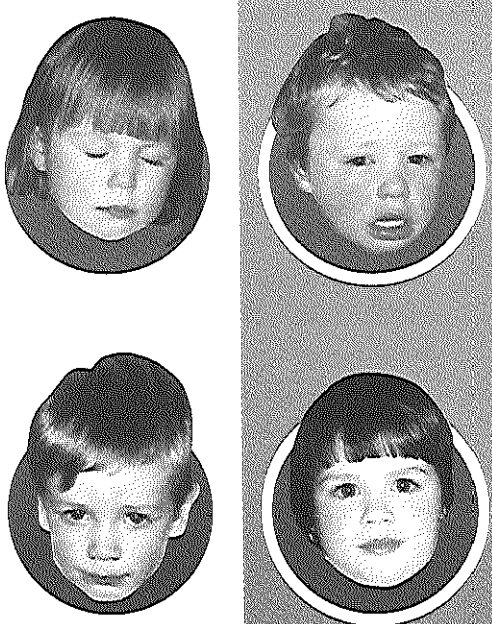
Contents

Prevention of meningococcal infections

Chapter 1

General Introduction



General Introduction

Neisseria meningitidis forms the most common cause of bacterial meningitis in the Western World since the eradication of infection by *Haemophilus influenza* type b (Hib) through vaccination. In addition *N. meningitidis* is a major cause of sepsis frequently resulting in death or disability. Various factors contribute to the continuing interest for meningococcal disease by health care professionals and the general public. The rapid progression of a substantial number of meningococcal infections, with high mortality and morbidity in previously healthy, young children and adolescents attracts attention from the media, which has also been a driving force in countries such as the Netherlands and Britain to increase the efforts to treat and prevent these diseases. Outbreaks and epidemics of meningococcal disease occur throughout the world. In the Netherlands, the number of patients with infection by *Neisseria meningitidis* has increased from 175 cases in the beginning of the 1980s to 600 cases in 1999. In the Netherlands, each year approximately 45 children and adults die as a result of meningococcal infection (Centraal Bureau voor de Statistiek).

Pathogenesis

Environmental, microbial and genetic factors play a role in the development of meningococcal disease. The nasopharynx is the natural habitat of the meningococcus in approximately 10% of the population. Transmission from person to person takes place by airborne spread during prolonged close contact. Although many children become colonised in the nasopharyngeal niche with *Neisseria meningitidis*, only a few cases develop invasive disease. The nature of the specific interactions between host defences and the invading pathogen plays a major part in the variability of the spectrum and severity of clinical disease. Meningococcal carriage is a natural immunising process resulting in the induction of systemic protective antibody responses. The presence of bactericidal antibodies confers protection to systemic meningococcal disease. The host defence against serogroup B meningococci, whose capsular polysaccharides are poorly immunogenic, may also utilise phagocytosis as a means of bacterial killing. Poorly defined defects of the local immune system may lead to translocation of bacteria through the epithelial layers and access to the bloodstream. Once meningococci and their toxic products are present in the circulation a plethora of inflammatory reactions may be encountered. The intravascular inflammatory response consists of 1. activation of the cascades of procoagulation, anticoagulation, fibrinolysis, complement and the kallikrein/kinin systems, 2. the release of intercellular mediators by the cytokine network and 3. the altered function of various cells in the vascular wall. This may result in severe capillary leak and a decreased circulatory volume, vasodilatation in some vascular beds (long, liver) and vasoconstriction in others (extremities), intravascular thrombosis and depression of the myocardial function. The haemorrhagic diathesis is the most obvious clinical sign in meningococcal disease, which is caused by activation of the coagulation system, inhibition

of fibrinolysis and localised vessel wall damage. Severe meningococcal disease is associated with severe disseminated intravascular coagulation (DIC) consisting of deposition of fibrin and microvascular thrombi and depletion of platelets and coagulation factors. A profound fall in circulating levels of the natural anticoagulant protein correlated to the severity of thrombotic lesions and outcome.

Treatment and prevention

Neisseria meningitidis strains are extremely susceptible for antibiotics, although increasingly intermediate level resistance to penicillin is reported. Unfortunately, despite the availability of an increasing array of potent antibiotics and advances in intensive care management, there is still a high mortality and morbidity. The overall mortality of meningococcal sepsis remains at 10%. Interventions to correct the disordered physiology may improve the prognosis. These new strategies include among others endotoxin neutralising agents such as recombinant bactericidal/ permeability-increasing protein (rBPI), immunomodulating agents such as anti-TNFα and agents influencing the coagulopathy by inhibition of coagulation and induction of fibrinolysis (protein C, activated protein C, antithrombin III, tissue factor pathway inhibitor). A discussion was restarted whether corticosteroid treatment may improve outcome in septic shock.

Prevention of meningococcal disease by the development of a vaccine against *Neisseria meningitidis* has a high priority. Currently, polysaccharide vaccines against meningococci of serogroup A, C, W135 and Y are available. The polysaccharide of serogroup B, which forms 85% of the meningococcal strains in the Netherlands, is poorly immunogenic. Alternative vaccine candidates, including the outer membrane proteins, are under investigation as vaccine candidates to prevent infection by meningococci of serogroup B.

Aims of the study

In this thesis we studied the pathogenesis of meningococcal sepsis and new therapeutic approaches to this disease. Also different steps are studied in the development of a vaccine to prevent meningococcal disease. Specific aims of our studies were as follows:

1. Analysis of the hormonal and metabolic changes in severe meningococcal disease.
2. Validation of a prognostic scoring system "the Rotterdam score" in patients with meningococcal septic shock.
3. Evaluation of the efficacy and safety of protein C concentrate in children with meningococcal septic shock.
4. Analysis of the safety and immunogenicity of different outer membrane protein based meningococcal vaccines in children.
5. Evaluation of the IgG subclass distribution in different age groups after vaccination with hexavalent meningococcal vaccine.
6. Analysis of the presence of immunological memory 2.5 years after vaccination with hexavalent meningococcal vaccine.

Outline of this thesis

Part one of this thesis contains selected aspects of the pathogenesis of meningococcal sepsis. **Chapter 2** reviews the pathophysiology of meningococcal sepsis in children and provides an overview on recent progress in the major areas of research on meningococcal sepsis. In **Chapter 3** the time course and variability of endocrine and metabolic parameters in children with meningococcal sepsis is studied. The role of serum cortisol and ACTH levels is analysed in a larger group of children with meningococcal sepsis. The results of this study are described in **chapter 4**.

Part two contains studies of experimental therapy and prognosis in children with severe meningococcal disease. In **chapter 5** a prognostic scoringssystem is validated for children with meningococcal septic shock. This score is based on four simple laboratory tests. In **Chapter 6** selected pharmacological, laboratory and clinical parameters are measured in a placebo-controlled dose-finding study with protein C concentrate in a group of children with meningococcal septic shock.

In part three the immunogenicity and side-effects of a newly developed meningococcal outer membrane vesicle vaccine are presented. **Chapter 7** provides an overview of vaccine candidates against *Neisseria meningitidis* serogroup B. **Chapter 8** reports the immunogenicity and safety of a hexavalent OMV meningococcal vaccine in 2-3 and 7-8 years old children. To explain the age dependent differences in immunogenicity IgG subclass levels after hexavalent vaccination were investigated and analysed in **chapter 9**. The immunogenicity and safety of a monovalent P1.4 meningococcal OMV vaccine are reported in **chapter 10**. This part ends with the presence of immunological memory in children 2.5 years after vaccination with hexavalent meningococcal vaccine **(Chapter 11)**.

The final chapters summarise the previous studies and end in a concluding chapter with future perspectives.

Chapter 2

Pathophysiology of Meningococcal Sepsis in Children

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Abstract

Septic shock with purpura is a syndrome frequently diagnosed in children and predominantly caused by *Neisseria meningitidis*. Despite improvements in management and therapy the mortality and morbidity in these patients are still high. During the last years much effort has been put into understanding of the systemic host response during this acute infectious disease. This host response can be divided in: process of recognition of endotoxin, the cascade of pro- and counter inflammatory mediaters, the endothelial damage resulting in capillary leakage and inappropriate vascular tone, and the procoagulant state.

Conclusion: This paper reviews the recent insights in the pathophysiology of the host response and their possible consequences for novel therapies in meningococcal sepsis.

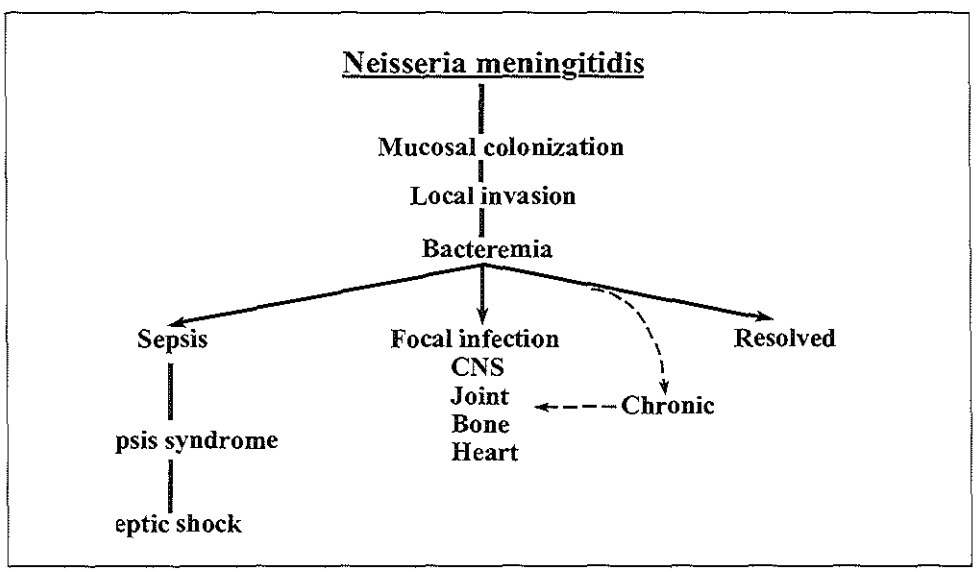

Figure 1 Clinical evolution of infections by *Neisseria meningitidis*.

Bacteremia: presence of viable bacteria in the circulating blood confirmed by culture

Sepsis: clinical suspicion of a severe infection and evidence of systemic response to infection (tachycardia, tachypnea, hyperthermia or hypothermia)

Sepsis syndrome: sepsis plus evidence of altered end-organ perfusion with at least one of the following: acute changes in mental status, oliguria, elevated lactate and hypoxemia

Septic shock: sepsis syndrome with hypotension requiring fluid resuscitation and/or vasopressor support

CNS: central nervous system

Introduction

Meningococcal disease, first described by Vieusseux in 1805 as epidemic cerebrospinal fever, remains a major health problem in many countries [126]. Clinical manifestations vary from self-limiting bacteremia to meningitis or fulminant sepsis (Figure 1). Meningococcal sepsis is characterized by a rapid onset of disease, fever, purpura and ultimately shock. Non-specific presenting symptoms such as a flu-like picture and a rash are commonly observed. The diagnosis of meningococcal disease becomes obvious when petechiae develop. Meningococcal disease is predominantly seen in children with a peak incidence around 2 years of age. A second peak is noted among teenagers. The overall mortality rate of meningococcal septic shock varies between 20% and 50%, is higher in infants than in older children and has not changed significantly over the past three decades despite improvements in management and therapy. [12, 40, 50, 58, 91, 102, 122, 138].

The systemic inflammatory response in patients with meningococcal disease aims to neutralize microorganisms and their toxic products, but it may also overreact and thus induce serious tissue damage to the host. Three pathways characterize the intravascular inflammatory response: 1) activation of cascade systems, 2) the release of intercellular pro- and anti-inflammatory mediators and 3) altered function of endothelial cells in the vascular wall. In meningococcal infection coagulation, fibrinolysis, complement and kallikrein-kinin systems, cytokine production, and activation of neutrophils and platelets, are all apparently upregulated by native lipopolysaccharides (LPS) in a dose-dependent manner [14] (figure 2). This review provides an overview of the systems involved in the pathophysiology of meningococcal sepsis and of possible therapeutic interventions.

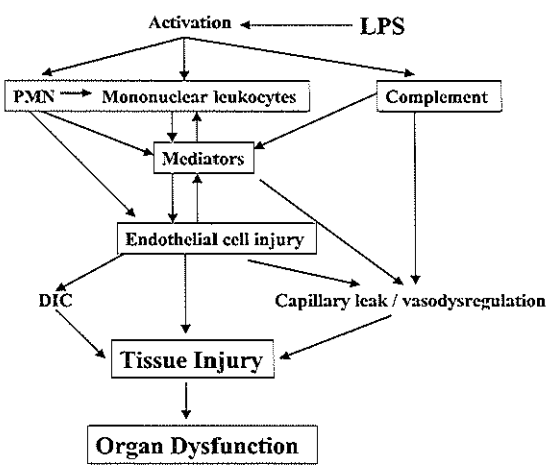

Figure 2 Pathophysiology of meningococcal sepsis.
LPS: Lipopolysaccharide, PMN: Polymorphonuclear leukocytes, DIC: disseminated intravascular coagulation.

Role of LPS in meningococcal disease

Meningococci enter and replicate in the bloodstream, after crossing the epithelial barrier without causing an intense local inflammatory response. They liberate various amounts of endotoxin (lipopolysaccharides)-containing outer cell wall fragments partly in the form of blebs [27]. Blebs bind antibodies that would otherwise attach to whole bacteria and probably play a crucial role in the pathogenesis of this form of septic shock. High levels of circulating endotoxins correlate with fatal outcome and with severity of disease [13, 14, 129, 130]. Low levels of bactericidal antibodies and immaturity of the T-cell system may play an important role in the development of meningococcal disease in young children. The presence of bactericidal antibodies is crucial. The levels of "natural" bactericidal antibodies are influenced by carriage of meningococci or colonization by nonpathogenic bacteria such as *Neisseria lactamica* [125]. High density lipoproteins, complement factors, antibodies, albumin, transferrin and lipopolysaccharide binding protein (LBP) have the ability to complex with LPS. Several of these proteins appear to have a detoxifying effect [53, 85, 116]. LBP however, amplifies the effect of LPS. After complexation with LBP, LPS is presented to the CD14 receptor of macrophages and PMN's (Figure 3). This interaction leads to cellular activation at a much lower concentration of LPS than without LBP [85]. High levels of LPS can also directly activate CD14 receptors. Bactericidal/permeability-increasing protein (BPI) is a potent bactericidal protein produced by polymorphonuclear leukocytes (PMN). It is stored in the azurophilic granules and also expressed on the cell surface. The bactericidal activity of BPI is caused by the strong affinity of BPI for LPS [134]. In addition to bactericidal capacity, BPI also neutralizes LPS activities in vitro and vivo [84].

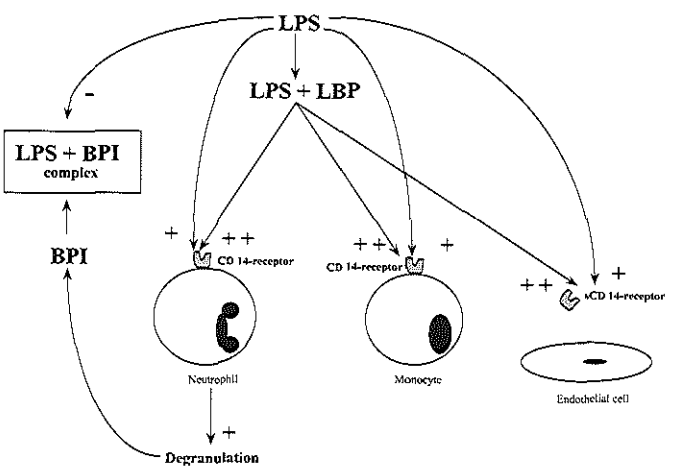

Figure 3 Competition between BPI and LBP in the binding of LPS.
LPS: lipopolysaccharides, BPI: bactericidal/permeability-increasing protein, LBP: lipopolysaccharide binding protein, CD14: CD14 membrane bound antigen, sCD14: soluble CD14 antigen, +: activation, -: neutralization

Pro- and counterinflammatory mediators

Lipopolysaccharides induce a release of proinflammatory mediators in gram-negative sepsis. These mediators are synthesized and released by macrophages, monocytes, and endothelial cells. Cytokines are paracrine agents: they act locally on a variety of tissues signaling information to adjacent cells. Experimental and clinical data have shown that tumor necrosis factor (TNF)-α and interleukin (IL)-1ß are the key mediators in meningococcal sepsis. TNF-α and IL-1ß exert their effects by different mechanisms including the induction of other cytokines, activation of neutrophils and leukocytes, enhancement of adherence of PMN and monocytes to endothelium, generation of prostaglandins and production of nitric oxide. The release of other mediators (IL-6, IL-8, LIF, IL-12 and IFN-γ) is triggered by LPS, TNF-α, and IL-1ß. These cytokines are elevated in meningococcal sepsis and their levels correlate with severity of disease. Interleukin (IL)-6 is a major pyrogen, stimulates the synthesis of acute-phase proteins [59] and has the ability to induce proliferation and antibody production by B-cells. Interleukin (IL)-8 is a potent chemoattractant, activates neutrophils and is thought to be involved in neutrophil-mediated vessel-wall injury [3, 29]. Leukemia inhibitory factor (LIF) has multiple actions, many of which are shared with TNF-α, IL-1 and IL-6 [4, 133]. IL-12 is a recently described proinflammatory cytokine, which seems to play a key role in the differentiation of Th1 cells and induces the production of interferon (IFN)-γ by T-cells and natural killer (NK)-cells [19, 61, 65, 118-120, 139]. The levels of IL-12 in meningococcal septic shock are related to outcome and severity of disease [55]. Interferon-γ (IFN-γ) activates other cytokines. Plasma levels of IFN-γ are increased in sepsis, although not consistently [16, 17, 44, 55, 60, 70, 129].

The short peak of pro-inflammatory cytokines is directly followed by an increase of counterregulatory cytokines like IL-1 receptor antagonist (IL-1Ra), IL-10, soluble TNF receptors (sTNFRs) and soluble IL-6 receptor (sIL-6R). These mediators except sIL-6R are considered to be anti-inflammatory because they reduce mortality in experimental endotoxemia [41, 95, 123]. IL-1Ra inhibits the proinflammatory actions of IL-1 by competitive binding to the IL-1 receptor. IL-1Ra and also sTNFRs are present in the circulation during early meningococcal infection [122]. The role of sTNFR is complex. It is believed that sTNFR is released in the circulation after binding of TNF-α on the target cell. This shedding may protect the cell against ongoing stimulation of TNF-α [76]. In contrast sIL-6R seems to stimulate the biologic activity of IL-6. Septic patients had significantly lower concentrations of sIL-6R compared to healthy volunteers [36, 37, 140]. Interleukin 10 is released in massive amounts into the systemic circulation during the initial phase of fulminant meningococcal septic shock and high serum levels of IL-10 are related to outcome in children with this disease [26, 80, 104]. IL-10 is a potent inhibitor of cytokine production [22, 24, 41, 65]. It also suppresses the procoagulant activity induced by LPS at the surface of human monocytes [104]. IL-10 stimulates the production of IL-1Ra and induces the release of sTNFRs by monocytes [78].

Generally, serum levels of proinflammatory cytokines are significantly increased at the onset of disease in patients with meningococcal sepsis and these levels are associated with outcome

and severity of disease [14, 40, 59, 129, 133, 136]. These cytokines are cleared from plasma rapidly. A negative correlation has been reported between the initial levels of cytokines and the time between first appearance of petechiae and admission [59, 71]. This shorter duration of petechiae in non-survivors suggests a shorter disease course and associated higher levels of cytokines. The earlier admission of non-survivors may indicate a higher production of LPS per time span, thus triggering mediator systems more intensively or may be explained by a higher responsiveness to lipopolysaccharides or to proinflammatory cytokines [71]. Westendorp et al. found a genetically encoded anti-inflammatory cytokine profile during the initial phase of infection, which decreased the non-specific host response and favored growth of microorganisms. Families from meningococcal disease patients characterized by low TNF production in an ex vivo whole blood stimulation setup, had a tenfold increased risk for fatal outcome, whereas high IL-10 production increased the risk 20-fold. Families with both characteristics had the highest risk [137]. However, others have studied the genotype of both surviving and non-surviving patients with severe sepsis and found support for the hypothesis that individuals with a genotype for high TNF production have a worse outcome [8, 90, 113]. Their results showed an association between a polymorphic variation in the TNF-α gene promotor region and death from meningococcal disease [90].

Endothelial damage and capillary leakage

Endothelial cells are not merely a selective permeability barrier between blood and underlying tissues, but actively play an important role in maintaining homeostasis. During sepsis, endotoxin and several other mediators activate vascular endothelial cells and initiate a rapid alteration of structure and function of these cells (figure 4). Finally endothelial damage is leading to a severe capillary leak syndrome. Capillary leakage and subsequently edema are the result of: destructive changes of endothelium (glycosaminoglycan component [68]), active separation of tight junctions between endothelial cells [75] and high molecular protein leakage. These processes are partly induced by circulating mediators (TNF-α, IL-1, IL-8, platelet activating factor (PAF), leukotrienes, thromboxane A_2, thrombin, vascular permeability factor, complement factors, kinins [7, 21]), and the adherence of neutrophils and platelets [46, 124, 132]. Activated leukocytes are primed to initiate phagocytosis and microbial killing by degranulation and release of proteolytic enzymes and toxic oxygen radicals, but in this way they also play a role in endothelial damage [23, 46, 124, 132]. Elastase is one of these degranulation products of activated neutrophils. Elastase-α 1-antitrypsin complexes in plasma appear to be of prognostic significance: levels are higher in non-survivors [92]. Ultimately, increased vascular permeability leads to profound interstitial edema with diffuse parenchymal cell injury and persistent hypovolemia followed by organ dysfunction.

Complement

The complement system plays a key role in host defense mechanisms, resulting in lysis of bacteria, enhancement of phagocytosis by monocytes or polymorphonuclear leukocytes or

neutralization of endotoxin [34, 35]. This system is an essential element in the maintenance of homeostasis not only of several immunological functions, but also of the coagulation and fibrinolysis system, vascular permeability and vascular tonus. Fulminant meningococcal sepsis is associated with excessive complement activation [13, 34]. Complement peptides generated during activation, have pro-inflammatory effects such as stimulation, aggregation and degranulation of neutrophils and induction of expression of selectins on the endothelial surface. However, overstimulation or inadequate inhibition of the complement system may lead to an inappropriate reaction and ultimately to tissue injury [48]. The complement system can be activated through the classical and the alternative pathway. The classic pathway requires recognition and binding of bacterial antigens by specific antibodies. In vitro studies indicate that lipid A and the polysaccharide side chain may complex with C1q and factor B to initiate activation of the classical pathway without involvement of antibodies. The alternative complement pathway can be activated by a variety of substances, including polysaccharides, bacterial endotoxins, cytokines and immune complexes [23, 111]. Normally, intravascular clearance of bacteria is mediated through the deposition of complement components: C3b for phagocytic clearance [109]; the membrane

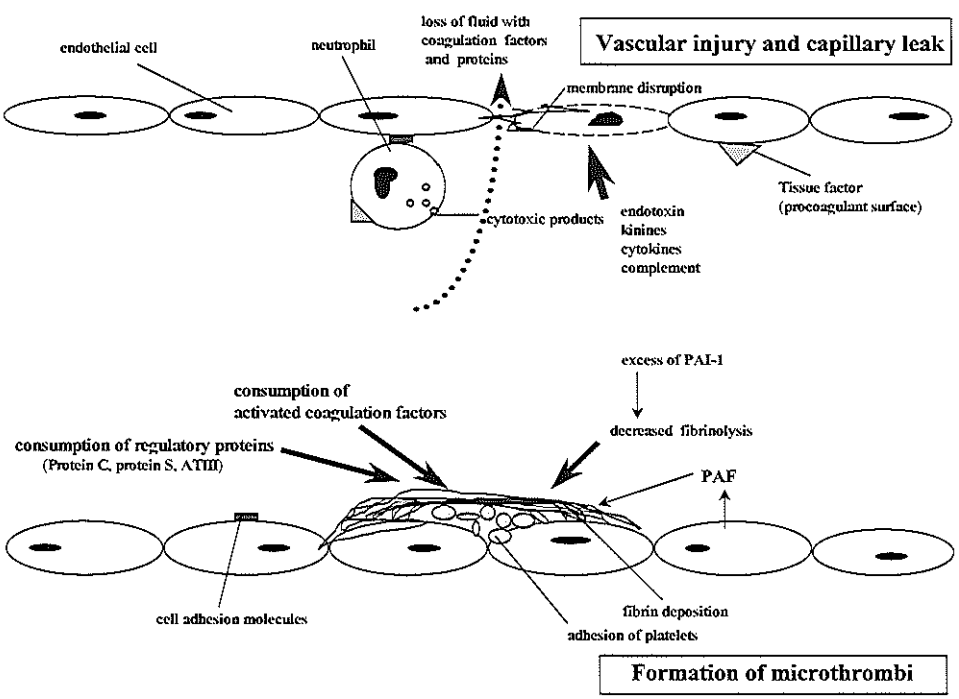

Figure 4 Disseminated intravascular coagulation, characterized by microvascular thrombosis and bleeding diathesis. AI: plasminogen activator inhibitor; PAF: platelet activating factor.

attack complex or C5b-C9 for lysis [34, 35]. Previous studies indicate that the degree of complement activation in sepsis is related to the amount of circulating native lipopolysaccharides [39, 81, 89]. In meningococcal septic shock increased levels of complement factors C3, C4, C5, and terminal complement complex and decreased levels of prekallikrein are related to outcome [13]. The presence of C4bc, C4bd and Bd point to activation of both the classical and alternative pathways. Brandtzaeg et al. suggest that complement activation is predominantly caused by alternative pathway activation [10]. In contrast, we could prove an important contribution of the classical pathway in the activation of the complement system in patients with meningococcal sepsis. This activation was related to outcome and severity of disease [56]. Brandtzaeg and colleagues showed that complement activation may persist during the first 12-24 hours of disease, when production of other inflammatory mediators is already downregulated [13]. Complement deficient individuals point to the importance of the complementsystem in meningococcal disease. Meningococci cause 80% of all systemic bacterial infections in these individuals, although in late-complement-component deficient individuals mortality is five to ten fold lower than in complement sufficient individuals [25]. The complement and the coagulation system share the same protein as their inhibitor in plasma, i.e. C1-esterase inhibitor (C1-INH). C1-INH levels may increase up to two-fold during uncomplicated infections [49]. During sepsis C1-INH levels were found to be normal or even decreased especially in non-survivors [93]. In our patients we also found decreased levels of C1-INH [56, 57]. Hack et al. hypothesized that increased degradation of C1-INH in sepsis may result in an insufficient control of the complement and coagulation systems [49].

Coagulation and fibrinolysis

Coagulation disorders and abnormalities of fibrinolysis are common in patients with meningococcal sepsis. The most severe manifestation is disseminated intravascular coagulation (DIC), characterized by microvascular thrombosis and bleeding diathesis (table 1 [83]). Widespread microvascular thrombosis does contribute substantially to organ dysfunction and survival. The production of thrombin and the conversion of fibrinogen to fibrin, may be activated by the intrinsic pathway (through factor XII) or by the extrinsic pathway (through factor VII/tissue factor) activation (figure 5). Activation of the coagulation system in sepsis occurs predominantly through the extrinsic route. The importance of the extrinsic route of coagulation was shown in an experimental model of baboons in which infusion of monoclonal antibodies against tissue-factor protects against lethal shock by *E. coli.* and attenuates coagulopathy [115]. In contrast, inhibition of the intrinsic pathway by administration of monoclonal antibodies against factor XII has no effect on the coagulopathy in the same model [100].

Endotoxin and TNF-α induce the expression of tissue factor by monocytes, macrophages and endothelial cells which activate factor VII. Of interest, increased levels of tissue factor are present in circulating monocytes isolated from blood of patients with meningococcal sepsis. The highest values were found in nonsurvivors [97]. Through kallikrein, activation of factor XII results

in the generation of bradykinin, complement, plasmin and elastase. In this way, factor XII mediates inflammation, fibrinolysis, and tissue damage as well as clot formation. Hypotension is probably partly mediated by generation of kinins such as bradykinin. Factor XII levels are lowest in patients with septic shock [62, 94].

Levels of natural inhibitors of coagulation are markedly altered during meningococcal sepsis. Several studies confirmed that antithrombin III (AT-III) levels [15, 31-33, 77], protein S, and notably protein C [15, 28, 31, 33, 77, 102, 103], are decreased in meningococcal septic shock. The decline in protein C levels is more pronounced than the decrease in ATIII and protein S levels. The decrease in ATIII, protein C and protein S levels is associated with the presence of DIC and poor outcome. The higher mortality of infants with meningococcal septic shock is probably related to immaturity of the protein C system [58, 102]. Elevated initial levels of the extrinsic pathway inhibitor (EPI), another inhibitor of coagulation, were found in patients with fulminant meningococcemia [15]. This is in contrast to the levels of ATIII and protein C. The levels of EPI were significantly higher in nonsurvivors

- Deposition of microthrombi
  - expression of procoagulant surface
  - increased turnover of activated clotting factors
  - consumption of regulatory proteins
  - release of excess fibrinolytic inhibitors
  - release of platelet activating factor
- Bleeding diathesis
  - increased fibrinolysis
  - consumption of coagulation factors/platelets
  - interference with platelet aggregation and fibrin polymerization by FDP's
- Vascular injury and capillary leak
  - direct toxic effect of endotoxin
  - activation of kinins, complement and cytokines
  - neutrophil adhesion and release of cytotoxic products
  - loss of clotting factors by capillary leak

(Adapted from Manco-Johnson83)

Table 1 Factors involved in the pathophysiology of DIC

in comparison to survivors. Furthermore, levels of EPI increased during the course of disease [15].

The fibrinolytic system becomes activated by tissue plasminogen activator (tPA) during the early course of meningococcal sepsis. Subsequently, fibrinolysis is inhibited by increased levels of plasminogen activator inhibitor (PAI-1) [11, 58, 71]. In patients with sepsis and septic shock tPA levels are increased and related to outcome and severity of disease [99, 128]. However, we could not detect significant differences in the initial levels of tPA in survivors and nonsurvivors with meningococcal septic shock [58, 71]. In adult patients with non-meningococcal septic shock, levels

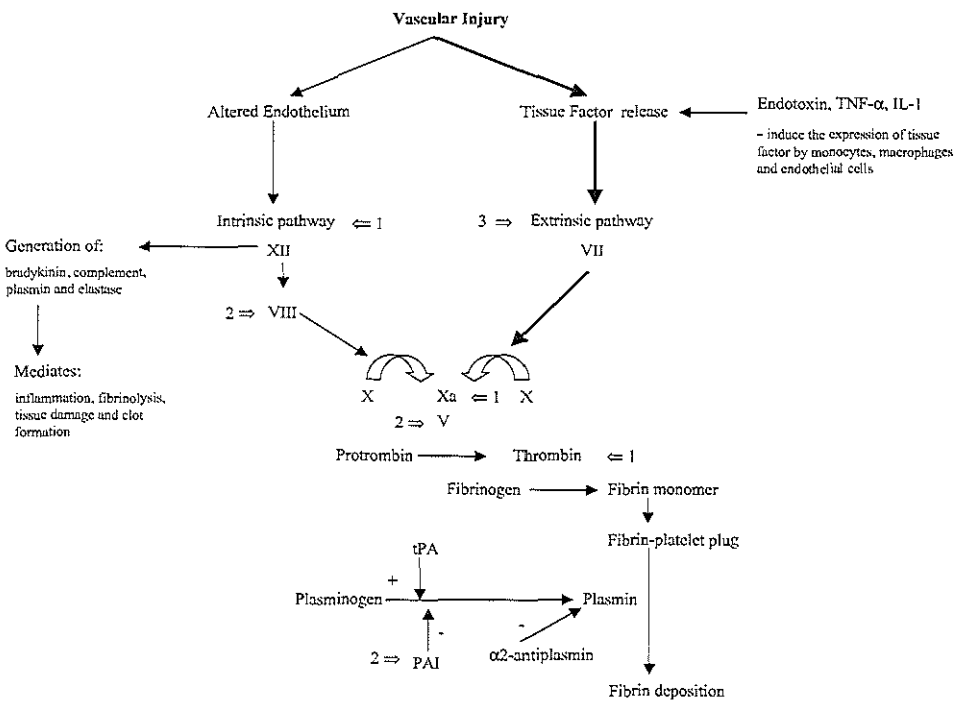

Figure 5 Hemostasis in meningococcal sepsis.

TNF-α: tumor necrosis factor ; IL-1: interleukin-1

V: coagulation factor V; X: coagulation factor X and Xa, when activated; tPA: tissue plasminogen activator; PAI: plasminogen activator inhibitor.

The intrinsic pathway involves the activation of coagulation factor XII, and VIII. The extrinsic pathway involves the activation of factor VII by tissue factor.

Natural inhibitors: ⇒

1: antithrombin III

2: activated Protein C

3: tissue factor pathway inhibitor

of plasminogen and alpha-2-antiplasmin are low in septic shock but not related to outcome [62, 128]. In contrast in children with meningococcal septic shock, alpha-2-antiplasmin levels as well as the ratio PAI-1/tPA were related to outcome in patients with meningococcal septic shock [11, 28, 58]. These changes in fibrinolytic parameters result in an ineffective fibrinolysis. Of interest, a genetic polymorphism in the promotor of the PAI-1 gene is suggested to explain higher PAI-1 levels in non-survivors at a similar TNF stimulus [71].

The massive consumption coagulopathy is characterized by low levels of coagulation factors VII, X, V, prothrombin, fibrinogen, and platelets. Because of the massive demand of anticoagulation factors due to widespread activation of the anticoagulant pathway, the host's endogenous anticoagulants are depleted causing purpura fulminans [102]. This depletion is possibly age-related [58, 102]. Platelets also interact with neutrophils through a selectin-mediated mechanism. These interactions may bring activated platelets close to the endothelium [51]. Even without adherence to the endothelium, platelets can profoundly affect endothelial function by the release of vasoactive substances from platelet storage granules. Such substances include adenosine diphosphate (ADP), serotonin, PAF and lipoxin A4, which may mediate vasodilatation [64]. Platelet adhesion to vascular endothelium is mediated by coagulation factor VIII (von Willebrand factor), which is found in plasma, endothelial cells and in the storage granules of platelets. Platelets also form and release thromboxane A2 and leukotriene C1, which may mediate vasoconstriction [108]. The result is a procoagulant state which is reflected in formation of microthrombi in the skin, the extremities and the adrenals. The excessive consumption of coagulation factors results in a hemorrhagic diathesis and local tissue hemorrhages.

Circulatory dysfunction

Shock or circulatory collapse in patients with fulminant meningococcal sepsis are caused by a combination of an inappropriate vascular tone, myocardial dysfunction, capillary leakage and intravascular microthrombosis. Initially, patients with meningococcal sepsis present with intense vasoconstriction. Subsequently, the systemic vascular resistance falls due to vasodilatation in the course of the treatment requiring volume suppletion and vasopressors. A dysbalance between forces causing vasodilatation and those causing vasoconstriction of the blood vessels results in generalized vasodilatation and hypotension, but in some capillary beds like the skin and the pulmonary circulation it leads to vasoconstriction. Vasoconstrictor substances that are elevated in patients with shock include catecholamines, renin, aldosterone, thromboxane A2 and endothelin [7, 69, 114, 127]. A deficiency of PGI_2 synthesis by the endothelium is also involved in vasoconstriction during meningococcal disease [63]. On the other hand, bradykinin and nitric oxide are potent vasodilator compounds leading to hypotension [82, 110, 117].

The major mechanism of death in meningococcal sepsis is circulatory collapse resulting from a combination of capillary leak, intravascular volume depletion, myocardial failure and vasodilatation [87]. As compensatory mechanisms fail, hypotension ensues, perfusion of vital

organs becomes inadequate and the resulting hypoxia and acidosis contribute to myocardial dysfunction. There is only a limited number of hemodynamic studies in pediatric patients with meningococcal sepsis [9, 87, 88]. Probably the most detailed is the one of Mercier et al. The results of this study suggest a different cardiovascular pattern in children with meningococcal sepsis than in adult patients with sepsis. All of the non-survivors had a decreased cardiac index (CI) and nearly all of the survivors had a normal CI in stead of the increased CI which can be encountered in adult patients with sepsis [87]. The timespan in which the circulatory failure takes place is different in meningococcal sepsis in comparison with other forms of sepsis. Mortality happens in the first 48 hours, while recovery seemed much longer in adults (7-10 days) than in children (1-3 days) [9]. The mechanism of the myocardial failure in human sepsis remains partly speculative. Circulating myocardial substances like TNF-α, IL-1 and endotoxin itself and not myocardial hypoperfusion is probably responsible for this depression [72, 73, 79, 98]. However, also other aspects of the systemic inflammatory response including circulating cytokines, tissue damaging, leukocyte-endothelial cell- myocite interaction leading to severe myocardial edema and mismatch of oxygen supply and demand in cardiac microvasculature will cause this myocardial depression [87, 131]. To what extent these mechanisms are also applicable to meningococcal sepsis is not clear yet.

Therapeutic interventions

The most important complication requiring urgent intervention is the development of shock in the presence of a profound capillary leak. The aim of the whole treatment is to provide sufficient fluid to maintain the intravascular volume and electrolyte balance, while minimizing the accumulation of extravascular fluid. When shock continues despite aggressive correction of the volume deficit, inotropic support should be given [67]. Recently, extracorporeal membrane oxygenation (ECMO), is suggested as a support therapy for patients with severe cardiorespiratory failure. Venoarterial ECMO provides both circulatory and respiratory support and could assist septic patients if conventional medical managment is failing. The potential risk of haemorrhage is of concern, because of the necessity for anticoagulation during ECMO. The little experience of ECMO for meningococcal disease is encouraging with two-thirds of the patients surviving and most survivors leading functionally normal lives [5, 18, 47]. The question remains whether these patients would have survived without ECMO.

New therapies directed towards modulating the systems involved in the pathophysiology, have so far shown little benefit in clinical practice (Table 2[135]). To neutralize circulating endotoxin, antibodies against the lipid A moiety of endotoxin (HA-1A) have been studied. HA-1A has been evaluated in adults [86, 143] and recently in children with meningococcal septic shock. In a European multicenter trial in children with meningococcal septic shock, the morbidity and mortality were not statistical significant different between children in the HA-1A and in the placebogroup (manuscript in preparation). New studies to evaluate the efficacy of recombinant-BPI and high density lipoproteins (HDL) in children with meningococcal sepsis, are recently started. A phase 2 trial of rBPI-21 showed a mortality of 4% in patients with a predicted mortality range of 20-50% [45]. Early haemofiltration with plasma or whole blood exchange may be useful in the management of meningococcal sepsis in children [6, 101, 105, 121]. However, Frieling et al. showed that plasma or whole blood exchange did not significantly influence IL-6 concentrations but as a possible side effect, did increase the soluble IL-6 receptor concentration directly after an exchange session followed by a rapid decrease [38]. Although, several authors underlined the possible value of this treatment in meningococcal sepsis, no controlled studies are available. Inhibition of cytokines by anti-TNF-antibodies or IL-1-receptor antagonist showed no efficacy in patients with sepsis or septic shock [1, 20, 30, 96, 106]. As agents to control disseminated intravascular coagulation in patients with fulminant meningococcemia heparin, antithrombin III concentrate, fresh frozen plasma and protein C concentrate have been studied [32, 33, 42, 43, 52, 54, 74, 107, 112]. However with the exception of fresh frozen plasma and protein C, which have some fibrinolytic properties, these drugs cannot add to the dissolution of fibrin clots. Recombinant tissue plasminogen activator (rt-PA) is a new fibrinolytic drug. It induces a clot-selective fibrinolysis that is associated with only a little decrease of fibrinogen. Preliminary experience with rt-PA in

author	experimental treatment	type of study	number of patients	conclusion
Beca J [5]	ECMO	retrospective, descriptive study	n=9 children (5 survivors)	ECMO supported the circulation succesfully.
Goldman AP [47]	ECMO	reetrospective, descriptive study	n=12 children (8 survivors)	ECMO might be considered to support patients with meningococcal disease.
Champion MP [18]	ECMO	descriptive	n=2 (both survivors)	ECMO may have a role in the managment of children with refractory shock due to meningococcal disease.
van Deuren M [121]	plasma or whole blood exchange (PEBE)	descriptive study with historical controls	n=15 children and young adults (12 survivors)	early initiation of PEBE may improve the rate of survival.
Reeves JH [105]	haemofiltration plasmafiltration	retrospective case notes	n=18 children (5 survivors) n= 9 children (3 survivors)	haemofiltration or plasmafiltration is safe, but their effect on outcome remains unknown.
Best C [6]	early heamo-diafiltration	descriptive	n=4 children (all survivors)	use for treatment is speculative
Frieling JTM [38]	plasma or whole blood exchange	prospective, descriptive patient study	n=9 children (6 survivors)	plasma or whole blood exchange increase the concentration of soluble IL-6 receptors.
Westendorp RGJ [126]	leukaplasmapheresis	open prospective study with historical controls	n=13 children (10 survivors)	leukaplasmapheresis might be of value
Gerard, P [42]	heparin	descriptive	n=19 children (17 survivors)	heparin must be considered as an adjunctive therapeutic agent
Haneberg B [52]	heparin	randomized therapeutic trial	n=26 heparin n=11 (9 survivors) no heparin n=15 (13 survivors)	heparin has no influence on outcome
Kuppermann N [74]	heparin	retrospective, descriptive study	heparin n=6 (3 survivors) no heparin n=18 (10 survivors)	heparin may limit digit and extremity necrosis
Gerson WT [43]	protein C	descriptive study	n=1 child (survived)	could be instrumental in survival.
Rivard GE [107]	protein C	pilot study	n=4 children (all survivors)	safe, possible contribution in decreasing morbidity and mortality rates.
Smith OP [112]	protein C.heparin and haemodiafiltration	pilot study	n=12 children and young adults (all survivors)	decrease in mortality and morbidity, call for a doubleblind randomised controlled trial.
Fourrier F [32]	antithrombin III	descriptive	n=5 young adults (all survivors)	could contribute in decreasing morbidity and mortality.
Keeley SR[66]	tissue plasminogen activator	descriptive	n=1 child (survived)	possiblility to decrease the morbidity of amputations.
Zenz, W [141]	recombinant tissue plasminogen activator	descriptive	n=2 children (both survivors)	rt-PAmay be an additional approach in patients with life threatening meningococcal septic shock.
Zenz W [142]	recombinant tissue plasminogen activator	descriptive	n=2 children (all survivors)	should be considered as an investigational adjuvant therapeutic option.
Aiuto LT [2]	recombinant tissue plasminogen activator	descriptive	n=1 child (survived)	improvement of organ perfusion and cardiac performance.
Giroir BP [45]	recombinant bac-tericidal /permeability increasing protein	open-label, dose escalation study with historical controls	n=26 children (25 survivors)	marked biological effect of rBPI.

Table 2 Experimental treatment in meningococcal septic patients

two patients suggests that rt-PA should be considered as an investigational therapeutic option in patients with lifethreatening disease and no response to conventional treatment [2, 66, 141, 142]. However, severe bleeding can be a serious side effect, because titration of the dosage is difficult in the acute phase since PAI-1 levels are high in the initial phase, but rapidly decrease in time. A new trial to evaluate the efficacy of protein C in the treatment of children with meningococcal septic shock has recently started. Possible future treatment modalities include r-BPI (phase 3 trial has started), C1 esterase inhibitor, protein C, rt-PA and tissue factor pathway inhibitor.

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Chapter 3

Endocrine and metabolic responses in children with meningococcal sepsis: striking differences between survivors and non-survivors

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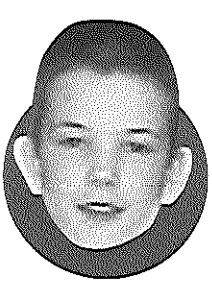

Abstract

To get insight in the endocrine and metabolic responses in children with meningococcal sepsis 26 children were studied the first 28 hours after admission. On admission there was a significant difference in cortisol/ACTH levels between non-survivors (n=8) and survivors (n=18). Non-survivors showed an inadequate cortisol stress response in combination to very high ACTH levels whereas survivors showed a normal stress response with significantly higher cortisol levels (0.62 vs 0.89 μmol/l) in combination with moderately increased ACTH levels (1234 vs 231 ng/l). Furthermore there was a significant difference between non-survivors and survivors regarding pediatric risk of mortality score (31 vs 17), TSH (0.97 vs 0.29 mE/l), T_3 (0.53 vs 0.38 nmol/l), reverseT_3 (rT_3) (0.75 vs 1.44 nmol/l), C-reactive protein (34 vs 78 mg/l), non-esterified fatty acids (0.32 vs 0.95 mmol/l) and lactate (7.3 vs 3.2 mmol/l). In those who survived most important changes within 48 hours were seen in a normalization of cortisol and ACTH levels but without a circadian rhythm; a decrease of rT_3 and an increase in the ratio T_3/rT_3; a decrease in the levels of the non-esterified free fatty acids and an unaltered high urinary nitrogen excretion. At this moment it is yet unknown whether the hormonal abnormalities are determining factors in outcome of acute meningococcal sepsis or merely represent secondary effects. Understanding the metabolic and endocrine alterations is required in order to design possible therapeutic approaches. The striking difference between non-survivors and survivors calls for reconsideration of corticosteroid treatment in children with meningococcal sepsis.

Introduction

Septic shock with purpura is a life threatening clinical syndrome predominantly caused by *Neisseria meningitidis* and characterized by a sudden onset and rapid progression of disease. The physiological changes that constitute the process of sepsis are induced by microbial agents during bloodstream infection or by the toxic products of pathogens that are released from sites of focal infection. This process involves changes generated by the immune system in which hormones, cytokines and enzymes are involved.

In adult patients it has been shown, that sepsis may lead to pronounced neuro-endocrine and metabolic alterations including increased serum cortisol concentrations, low thyroid hormones, insulin resistance, elevations of plasma glucose, lactate and free fatty acid concentrations, and increased muscle protein breakdown [1-4]. During the time course of sepsis an ebb and flow phase can be detected. Main features in the ebb phase are a decrease in metabolic rate and temperature and in the flow phase there is an increase in metabolic rate and urinary nitrogen excretion [5, 6]. There are several differences between the host response of young children compared to adults during meningococcal sepsis [7]. Little is known about the neuro-endocrine changes in critically ill infants and children. Previous studies in critically ill infants and children showed an altered thyroid function at the onset of acute diseases called "the euthyroid sick syndrome" [8-12]. Dopamine infusion induces or aggravates partial hypopituitarism in newborn infants resulting in inhibited prolactin and growth hormone secretion [8]. The present study was undertaken to evaluate the time course of the endocrine and metabolic responses of children with meningococcal sepsis during the first 48 hours of admission in the pediatric intensive care unit.

Material and methods

Study protocol

Children above 3 months and below 18 years of age with septic shock and petechiae/purpura requiring intensive care treatment were enrolled in this study. The group consisted of children primary admitted or referred to the pediatric intensive care unit (PICU) of the Sophia Children's Hospital between October 1997 and October 1998. Patients were eligible for inclusion when they met the following criteria: 1) presence of petechia/purpura; 2) presence of shock for less than 6 hours defined as persistent hypotension (systolic blood pressure <75 mm Hg for children between 3-12 months, <80 mm Hg for 1-5 years, <85 mm Hg for 6-12 years, <100 mm Hg for children older than 12 years), or evidence of poor end-organ perfusion, defined as at least two of the following: a) unexplained metabolic acidosis (pH < 7.3 or base excess < -5 mmol/l or plasma lactate levels > 2.0 mmol/l); b) arterial hypoxia (PO_2 < 75 mmHg, a PO_2/FiO_2 ratio < 250 or transcutaneous oxygen saturation < 96%) in patients without overt cardiopulmonary disease; c) acute renal failure (diuresis < 0.5 ml/kg/h for at least one hour despite acute volume loading or evidence of adequate intravascular volume without pre-existing renal disease); or d) sudden deterioration of the baseline mental status. The patients participated in a randomized, double-blinded, dose-finding study of Protein C concentrate (Human), Immuno-Baxter. Because Protein C is assumed not to influence the endocrine and metabolic assays and did not influence mortality we did not account for it in further analysis. The Medical Ethics Committee of the Erasmus University Rotterdam approved the study protocol. Informed consent was obtained from the parents or legal representatives.

Clinical parameters

The pediatric risk of mortality (PRISM II) score was calculated based on the most abnormal values regarding 14 physiological variables during the first 6 hours of admission. A higher score means a higher risk of mortality [13-15]. The interval between appearance of petechiae and admission to the PICU, length of stay on the PICU and duration of inotropic support were recorded. In order to distinguish non-survivors from survivors established parameters to monitor the severity of disease such as PRISM, lactate and C-reactive protein were analyzed [14, 15].

Collection of blood

Arterial blood samples were collected within two hours after admission (T=0), after 24 hours (T=24) and 48 hours (T=48) for determination of thyroid hormones, insulin, glucose, pre-albumin, C-reactive protein (CRP), non-esterified free fatty acids (NEFA) and lactate. Bloodsamples for cortisol and ACTH were taken at T=0 and 12 hours (T=12) after admission. An ACTH test was not performed as all children had severe stress due to the life-threatening disease on admission. A diurnal rhythm for cortisol and ACTH was estimated by sampling blood on the second day of admission at 8.00 am and subsequently at 14.00 pm and 20.00 pm.

Hormonal assays

Cortisol/ACTH

The plasma concentrations of cortisol were measured by competitive luminescence immunoassay (LIA). Detection limits: 0.03-1.38 μmol/l. The plasma concentrations of ACTH were measured by immunoradiometric assay (IRMA, ELSA-ACTH CIS bio international), using two monoclonal antibodies. The within-run coefficient of variation was 6.1% at 22 pg/ml, 2.9% at 59 pg/ml and 2.1% at 778 pg/ml. The between-run coefficient of variation was 5.3% at 40 pg/ml, 4.8% at 203 pg/ml and 1.3% at 1055 pg/ml. Reference values for cortisol: 8.00 h: 0.2-0.6 μmol/l, 14.00 h: 0.1-0.5 μmol/l, 20.00 h: 0.05-0.3 μmol/l. Reference values for ACTH: 20-100 ng/l. From the values of cortisol and ACTH the ratio cortisol/ACTH was calculated.

Thyroid hormones

Plasma T_4, T_3 and reverse T_3 (rT_3) were measured by established radioimmunoassay procedures as previously described [16, 17]. Reference values of the laboratory for T_4: 64-132 nmol/l, T_3: 1.1-2.6 nmol/l, rT_3: 0.15-0.43 nmol/l. From the values of T_3 and rT_3 the ratio T_3/rT_3 was calculated. The plasma concentrations of free T_4 (fT_4) were measured by a direct, labeled antibody, competitive immunoassay technique (Amerlite MAB fT_4 Assay). The within-assay coefficient of variation was 7.6% at 5.43 pmol/l, 4.3% at 16.1 pmol/l and 3.5% at 52.8 pmol/l. The between-assay coefficient of variation was 9.0% at 5.67 pmol/l, 5.6% at 17.4 pmol/l and 4.2% at 49.2 pmol/l. Reference value for fT_4: 11-25 pmol/l.

The plasma concentrations of *TSH* were measured by an ultrasensitive immunometric assay (Amerlite TSH-30, Ortho-Clinical Diagnostics), using one monoclonal antibody. The within-assay coefficient of variation was 8.0% at 0.087 mIU/l, 4.2% at 4.22 mIU/l and 4.1% at 21.5mIU/l. The between-assay coefficient of variation was 11.7% at 0.077mIU/l, 6.6% at 4.25mIU/l and 5.1% at 21.4 mIU/l. Reference value for TSH: <4.5 mE/l.

Insulin/glucose

Insulin was measured in serum with an immunoradiometric assay (IRMA). Detection limits: 5-400 mU/l. Glucose measurements were determined on the routine clinical chemistry analyzers (Dimension ES, Dupont Medical Products, Wilmington, Delaware, USA) (reference values hypoglycemia < 2.6 mmol/l, hyperglycemia > 11 mmol/l). From the values of insulin and glucose the ratio insulin/glucose was calculated.

Metabolic assays

Lactate was measured by enzymatic endpoint determination (Hitachi 911, Boehringer Mannheim, Mannheim, Germany) (normal < 2.0 mmol/l). C-reactive protein was determined by an immuno-nephelometric assay (normal: < 5 mg/l) [18]. Plasma non-esterified fatty acids (NEFA) concentrations were determined by enzymatic method (Nefac-kit, Wako, Instruchemie BV).

Reference values for NEFA for children between 4 months - 10 year: 0.3 – 1.1 mmol/l, children > 10 year: 0.2 – 0.8 mmol/l.

Urinary nitrogen excretion

Urine was collected daily for 24 hours and analyzed for urinary nitrogen. Total urinary nitrogen excretion was defined as 1.25 x urinary urea nitrogen, in order to adjust for the urinary nitrogen loss as ammonia, creatinine, and uric and amino acids [19]. No correction was made for nitrogen losses through stools, skin, wounds, nasogastric suction, or blood sampling.

Caloric intake

The patients were fed enteral and/or parenteral according to a standard feeding protocol. During stay on the PICU glucose was administered at a rate of approximately 4-6 mg/kg/min. If enteral feeding could not be started on the second day, parenteral feeding was started. Initial dose of proteins was 1.0 g/kg/day (Aminovenös N-paed 10%, Fresenius, Holland) and lipids (in case the body temperature was <38.5 °C) 1.0 g/kg/day (Intralipid 20%, Pharmacia, Upjohn, Holland). If clinically possible enteral and/or parenteral nutrition was adjusted on day 3 and 4 to normal needs for healthy children. The total caloric intake was recorded and calculated daily. The amount of caloric intake was corrected for extra protein calories such as plasma and/or albumin infusions. To estimate the adequacy of caloric intake the amount of energy intake was compared with calculated values for resting energy expenditure for healthy children according to the formula of Schofield for age, sex and weight [20].

Statistics

Statistical analysis was performed with a statistical analysis software program (SPSS 7.0 for WINDOWS 95, SPSS Software, Chicago, IL). Results are expressed as medians (interquartiles) unless specified otherwise. The Mann-Whitney U-test was used for comparison of clinical and laboratory tests between survivors and non-survivors. For survivors the Wilcoxson signed rank test was used for comparison on different time points of different laboratory tests. Spearman's correlation coefficient (r) was used to evaluate the relationship between different parameters. Two-tailed p-values of 0.05 or less were considered statistically significant.

Results

Demographics

Twenty-six patients admitted to the PICU fulfilled the inclusion criteria and were included in the study: 16 males and 10 females (table 1). The median age was 23 months (range 4-185 months). Cultures of blood revealed *Neisseria meningitidis* in all 26 patients.

Age (months)	Sex	Duration of petechiae before admission (hours)	PRISM score	Survival
4	M	7	12	Yes
5	M	3.5	30	No
5.5	M	3	34	No
7	F	4	32	No
9	F	3.5	19	No
10	F	9	16	Yes
10	M	7	32	No
12	F	9	18	Yes
15	F	4	18	Yes
18	M	5.5	27	Yes
19	M	1	15	Yes
21	M	15	18	Yes
21	M	8	29	No
24	M	0.5	37	No
25	M	15	21	Yes
27	F	10	14	Yes
30	M	3	24	Yes
32	F	6	10	Yes
52	F	9	20	Yes
77	M	7	15	Yes
81	F	11	7	Yes
113	M	3.5	29	No
128	M	4	9	Yes
136	M	4	24	Yes
141	M	10	24	Yes
185	F	6	14	Yes

M=male, F=female, PRISM score = pediatric risk of mortality score

Table 1 Endocrine and metabolic parameters on admission

Clinical parameters

Eight children died after a median stay in the PICU for 10 hours (range 2-40), 18 children survived, they stayed in the PICU for a median of 86 hours (range 30-312). There was a significant difference in age between non-survivors and survivors (10 vs 29 months, $p<0.05$). The interval between appearance of petechiae and admission to the PICU was 5.8 hours (range 2.5-18). There was a significant difference between non-survivors and survivors for the interval between appearance of petechiae and admission to the PICU (3.5 vs 7.0 hours, $p<0.05$). Concomitant therapy during the study period included antibiotics, administration of plasma and inotropics for all patients (10 patients received dopamine, 22 noradrenaline and 25 dobutamine (24 patients received a combination of inotropics)). Non-survivors received significantly higher doses of inotropics (dobutamine and noradrenaline, $p<0.01$). The 18 survivors received inotropic therapy for a median of 49 hours

(range 9-170). Administration of corticosteroids is not a routine procedure in the Netherlands. For that reason none of the children received corticosteroid therapy on admission. Seventeen patients required mechanical ventilatory support and sedation with benzodiazepines. The parameters to monitor severity of disease were significantly different between non-survivors and survivors; PRISM-score (31 vs 17, p<0.01), arterial lactate levels (7.3 vs 3.2 mmol/l, p<0.01) and CRP levels (34 vs 78 mg/l, p<0.01) (table 2, figure 1). In survivors, compared with levels on admission, lactate levels decreased significantly after 24 and normalized after 48 hours and CRP levels were significantly increased after 24 and 48 hours.

		Non-Survivors		Survivors						
		T=0		T=0		T=24[ab]		T=48		Normal reference
PRISM		31	(29-34)	17	(14-17)**					
Cortisol	µmol/l	0.62	(0.49-0.79)	0.89	(0.77-1.15)^{a**}	0.54	(0.38-0.62)^{b**}			0.2-0.6
ACTH	ng/l	1234	(740-2915)	231	(48-665)^{a**}	18	(12-31)^{b**}			20-100
Cortisol/ACTH		0.58^{-3}	(0.17^{-3}-1.00^{-3})	6.1^{-3}	(1.8^{-3}-21.7^{-3})^{a**}	29^{-3}	(17^{-3}-34^{-3})^{a**}			
TSH	mE/l	0.97	(0.52-1.56)	0.29	(0.15-0.54)^{a**}	0.55	(0.17-1.80)	1.73	(0.36-2.41)^{a**}	< 4.5
T_4	nmol/l	38	(25-46)	44	(39-56)	49	(38-64)	45	(34-71)	64-132
T_3	nmol/l	0.53	(0.43-0.76)	0.38	(0.26-0.42)^{a*}	0.58	(0.27-0.85)^{b**}	0.63	(0.29-0.97)^{c*}	1.1-2.6
rT_3	nmol/l	0.75	(0.55-0.97)	1.44	(0.99-1.85)^{a**}	1.52	(1.29-2.21)	0.98	(0.75-1.35)^{b**c*}	0.15-0.43
T_3/rT_3		0.76	(0.64-0.85)	0.43	(0.17-0.35)^{a**}	0.43	(0.13-0.62)	0.87	(0.21-1.14)^{b**c*}	
fT_4	pmol/l	14	(13-21)	16	(15-19)	13	(12-19)	12	(9-19)^{c**}	11-25
Insulin	mU/l	5	(5-7)	13	(5-21)^{a**}	16	(10-29)	19	(12-29)^{c**}	
Glucose	mmol/l	3.9	(2.5-6.5)	6.3	(5.2-7.8)^{a**}	6.0	(5.7-8.0)	6.5	(5.5-7.2)	2.6-11
Insulin/glucose		1.8	(0.9-2.0)	1.8	(1.1-2.6)	2.7	(1.7-4.0)	3.1	(2.1-3.5)^{c*}	< 50
CRP	mg/l	34	(24-37)	78	(58-100)^{a**}	201	(172-244)^{a**}	165	(133-219)^{c**}	< 5
Lactate	mmol/l	7.3	(5.9-10.4)	3.2	(2.2-5.1)^{a**}	2.0	(1.5- 2.8)^{a**}	1.5	(1.2-2.0)^{c**}	< 2.0
NEFA	mmol/l	0.32	(0.25-0.50)	0.95	(0.7-1.4)^{a**}	0.73	(0.46-0.84)^{a**}	0.41	(0.28-0.77)^{c**}	0.3-1.1

Table 2 Endocrine and metabolic differences between survivors and non-survivors on admission and time course of survivors

all values are expressed as median and interquartiles. *p<0.05 **p<0.01

[a] significant difference between non-survivors and survivors

[ab] for cortisol and ACTH T=24 is 8.00 AM day 2

[c] significant difference between T=24 and T=0

[b] significant difference between T=48 and T=24

[c] significant difference between T=48 and T=0

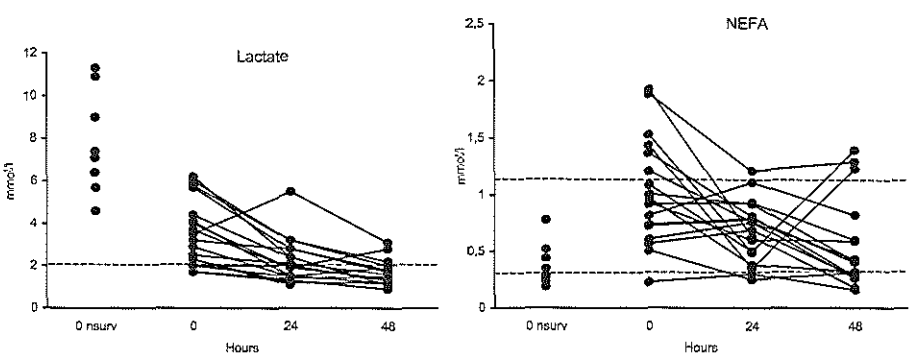

Figure 1 Levels of lactate and NEFA on admission for non-survivors (0 nsurv) and survivors (0) and the time course of these levels for survivors after 24 and 48 hours. Reference values below or between dotted lines.

Cortisol/ACTH

On admission non-survivors had significantly lower serum cortisol levels than survivors (0.62 vs 0.89 μmol/l, $p<0.05$), whereas the ACTH levels were extremely high in those who did not survive (1234 vs 231 ng/l, $p<0.01$)(table 2, figure 2). The ratio cortisol/ACTH was significantly different between non-survivors and survivors ($p<0.01$). ACTH and the ratio cortisol/ACTH correlated well with parameters to monitor the severity of disease (PRISM, lactate and CRP)(table 3). In those who survived twelve hours after admission levels of cortisol (0.73 μmol/l) and ACTH (31 ng/l) decreased significantly in comparison with levels on admission; on the second day after admission there was a further significant decrease of levels of cortisol (0,54 μmol/l) and ACTH (18 ng/l) in comparison with levels on admission and the levels of 12 hours after admission (table 2). In 14 survivors on day 2 a cortisol/ACTH profile was performed: in none of them a circadian rhythm could be detected in the 3 samples taken at 8.00, 14.00 and 20.00.

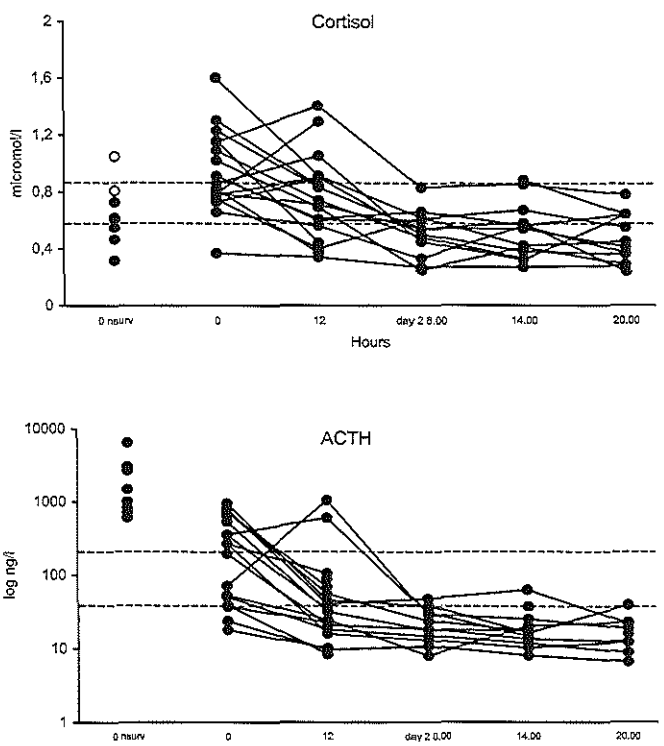

Figure 2 Levels of cortisol, ACTH and the ratio cortisol/ACTH on admission for non-survivors (0 nsurv) and for survivors (0) and the time course of cortisol and ACTH levels for survivors after 12 hours and on day 2 at 8.00 h, 14.00 h and 20.00 h. Reference values between dotted lines. Open circles in cortisol figure refers to the 2 non-survivors who lived > 24 hours

Thyroid hormones

On admission, data of non-survivors and survivors showed significant differences in levels of rT_3 (0.75 vs 1.44 nmol/l, $p<0.01$), the ratio T_3/rT_3 (0.76 vs 0.43, $p<0.01$), TSH (0.97 vs 0.29 nmol/l, $p<0.01$) and T_3 (0,53 vs 0,38 nmol/l, $p<0,05$), whereas the levels of T_4 and fT_4 were not significantly different between the two groups (table 2, fig 3). In comparison with normal reference values the levels of T_4 and T_3 were decreased in both non-survivors and survivors and those of rT_3 were increased. The median fT_4 and TSH levels were within the normal reference range. On admission there were, except for fT_4, significant correlations between levels of TSH, T_3, rT_3 and the ratio T_3/rT_3 with the parameters to monitor the severity of disease (table 3). After 48 hours survivors showed significantly increased levels of TSH, T_3 and the ratio T_3/rT_3 and significantly decreased levels of rT_3 and fT_4 in comparison with levels on admission whereas the level of T_4 did not change significantly. For survivors 48 hour after admission median levels of T_3, T_4 and rT_3 remained below normal reference values, whereas median fT_4 and TSH levels remained within the normal reference values (fig 2).

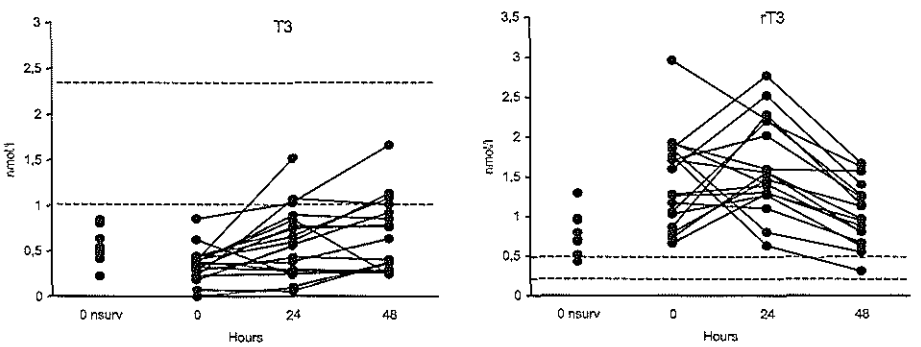

Figure 3 Levels of T_3, rT_3, TSH and ratio T_3/rT_3 on admission for non-survivors (0 nsurv) and for survivors (0) and the time course of these levels for survivors after 24 and 48 hours. Reference values below or between dotted lines.

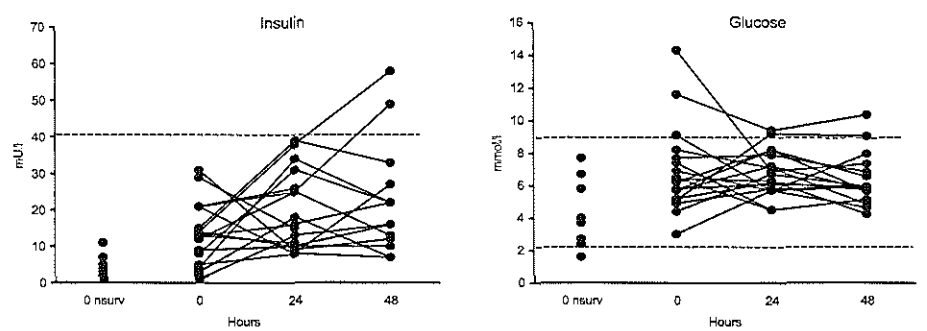

Figure 4 Levels of insulin and glucose on admission for non-survivors (0 nsurv) and survivors (0) and the time course of these levels for survivors after 24 and 48 hours. Reference values below or between dotted lines.

Insulin/glucose

On admission non-survivors showed compared to survivors significantly different levels of insulin (5 vs 13 mU/l, $p<0.05$) and glucose (3.9 vs 6.3 mmol/l, $p<0.05$) (table 2, figure 4). The ratio insulin/glucose did not show statistical significance between survivors and non-survivors (table 2). On admission two of the children had a hypoglycemia (2.4 and 1.6 mmol/l) and two children had a hyperglycemia (11.6 and 14.3 mmol/l). After 48 hours survivors showed significantly increased levels for insulin and the ratio insulin/glucose in comparison with levels on admission whereas the levels of glucose showed no changes (fig 4). There were no significant correlations between insulin, glucose and the ratio insulin/glucose versus the levels of cortisol and the parameters to monitor the severity of disease (table 3).

Non-esterified free fatty acids

On admission non-survivors had significantly lower NEFA levels than survivors (0.32 vs 0.95 mmol/l, $p<0.01$) (table 2, figure 1). Median NEFA levels for both non-survivors and survivors remained within normal reference values on admission. NEFA levels decreased significantly after 48 hours in comparison with the levels of admission and after 24 hours. Levels of NEFA correlated negatively with lactate and PRISM score ($p<0.01$) (table 3).

	Lactate	CRP	PRISM
Cortisol	-0.40*	0.10	-0.37
ACTH	0.57*	-0.64**	0,68**
Cort/ACTH	-0.63**	0.54*	-0.64**
TSH	0.53**	-0.43*	0.42*
T_4	0.07	0.06	-0.34
T_3	0.45*	-0.49*	0.29
rT_3	-0.34	0.48*	-0.41*
T_3/rT_3	0.42*	-0.51*	0.35
fT_4	0.40	-0.17	-0.41
Insulin	-0.09	0.18	-0.35
Glucose	-0.23	0.15	-0.36
Insulin/glucose	0.19	0.04	-0.06
NEFA	-0.47*	0.32	-0.48*

*$p<0.05$, **$p<0.01$

Table 3 Non linear significant correlation-coefficients (Spearman) between endocrine parameters, NEFA and parameters to monitor severity of disease (lactate, CRP and PRISM score) on admission

Nitrogen excretion

Nitrogen excretion was assessed in 16 survivors. The median nitrogen excretion was not significantly different between the first 24 hours and the second 24 hours after admission 271 mg/kg/day (range 64-940) vs 251 mg/kg/day (range 152-737)).

Caloric intake

For survivors the median difference between actual energy intake and calculated resting energy expenditure was −45% (range −83% to +24%) during the first 24 hours after admission and −42% (range -83% to +27%) during the second 24 hours after admission.

Time course in non-survivors

Six of the 8 non-survivors died within 14 hours because shock persisted in all; in 2 of these 6 children it was combined with pulmonary edema, in 1 child with convulsions, in 1 child with pulmonary hypertension and in 1 child with cerebral death. Two children died after 24 hours (25 and 40 hours). For these children the interval between appearance of petechiae and admission to the PICU was significant longer (7 and 8 hours) compared to the other 6 children (median 3.5 h (range 0.5-4 h) ($p<0.05$). These two children also showed higher cortisol levels on admission (0.73 and 1.05 μmol/l, fig 2 open circles) than the other 6 children. After 24 hours both children showed decreased levels of ACTH but these levels still remained above normal reference values (163 and 805 ng/l). In one of these 2 children a cortisol measurement was done after 24 hours showing only a slight improvement of the level of cortisol (from 0.73 to 0.80 μmol/l) in view of the ultimate state of stress. Furthermore, both children showed after 24 hours an increase in rT_3 levels (from 0.95 – 1.65 nmol/l) and CRP (from 34 to 111 mg/l). The level of lactate, however, increased in both children. In the child who lived for 40 hours shock persisted, recurrent convulsions developed and severe bradycardias occurred a few hours before death. In the child who lived for 25 hours shock persisted and a combination with severe pulmonary hypertension lead to death.

Discussion

Our study shows that children who do not survive meningococcal sepsis have an impaired adrenal response, altered thyroid hormones and decreased levels of NEFA, associated with a higher severity of disease score on admission. The observed endocrine and metabolic changes are of such clinical importance to reconsider therapeutic strategies.

One of the most striking alterations in our study concerned a significant difference in cortisol/ACTH response between non-survivors and survivors on admission. Non-survivors showed an inadequate cortisol response in combination with very high ACTH levels whereas survivors showed a normal stress response with significantly higher cortisol levels in combination with moderately increased ACTH levels. ACTH and the ratio cortisol/ACTH were strongly correlated with survival and parameters to monitor the severity of disease. Low cortisol levels in non-survivors and high cortisol levels in survivors have been reported in the past in children with meningococcal sepsis [21, 22]. More recently, only one other study in children with meningococcal sepsis reported the levels of ACTH in relation with the levels of cortisol. Significantly higher ACTH levels were found in those who died [23]. The decreased production of cortisol in relation with high levels of ACTH might be caused by various mechanisms. Firstly, the low production of cortisol might the result of bilateral adrenal hemorrhages (described previously as the Waterhouse-Friderichsen syndrome [21, 22]), due to severe coagulation disorders found in meningococcal sepsis [15]. Alternatively, an inadequate perfusion of the adrenal cortex due to hypotension might have lead to impaired adrenal function with diminished cortisol production. Our study seems to support this mechanism as we found a strong negative correlation between levels of cortisol and lactate. Thirdly, in the presence of higher levels of endotoxin and TNF-α (as seen in non-survivors) there might be a decreased adrenal ACTH receptor binding and a suppressed synthesis of cortisol [24, 25]. Salem reported a family of peptides (called corticostatins) that might impair the sensitivity of the adrenals to ACTH during sepsis [26]. It remains a question if treatment with supplemental steroids, which is not a routine treatment in the Netherlands, will be beneficial [27]. In our study there was a very rapid and aggressive course of the disease in 4 of the 5 non-survivors who died within 12 hours after admission. In non-survivors the interval between appearance of petechiae and admission to the PICU was significant shorter compared with survivors indicating the importance of the time course of disease. We believe that if corticosteroid treatment is started after referral and admission on the intensive care unit therapy might only benefit a very small group of children. In that case the time delay before initial corticosteroid treatment the infection process might have gone beyond any currently available therapeutic approach. Based on our data, however, we feel that a large randomized double-blind controlled study has to be designed to treat children with steroids as soon as possible after the first signs of disease. The results of this study might give an answer on the role of administration of steroids with in respect the time course of the disease.

During critical illness cortisol has an important role on the metabolism of fatty acids, glucose and protein [28]. There is evidence to suggest that NEFA may the preferred fuel for oxidation in critical illness [29]. In our study we found on admission a positive correlation between levels of cortisol and NEFA. Non-survivors had significant lower levels of NEFA than survivors, indicating a lack of an adequate metabolic stress response in those who died. Non-survivors also showed significant lower levels of glucose but there was no correlation with the levels of cortisol indicating other mechanisms causing hypoglycaemia [30].

In this study we examined the ACTH/cortisol axis as an important modulator of cardiovascular and metabolic homeostasis during critical illness. We did not examine the serum concentrations of the catecholamines and glucagon that might have influenced the anabolic effects of insulin and growth hormone. Preliminary data of a comparable group of children with meningococcal sepsis showed that non-survivors had significantly increased levels of growth-hormone and low levels of insulin-like growth factor-1 compared to survivors (manuscript in preparation). Research is in progress to assess the etiology and consequences of the alterations of the growth-hormone/ insulin-like growth factor-1 axis.

Alterations in the thyroid axis in critically ill children are called "the euthyroid sick syndrome" [8-12]. In our study both survivors and non-survivors showed the features of "the euthyroid sick syndrome": decreased levels of T_3 and T_4, increased levels of rT_3, normal levels of fT_4 and no compensatory increased levels for TSH. Non-survivors, however, showed significantly higher T_3 levels and lower rT_3 levels compared to survivors. Low levels of T_3 and increased levels of rT_3 are explained by an adaptive mechanism aimed at preventing protein catabolism and lowering energy requirements in severely ill patients. It might thus be postulated that non-survivors are not able to adapt in the same way as survivors [31-34]. The euthyroid sick syndrome may be mediated by cytokines and can be induced or aggravated by dopamine, glucocorticoids, while somatostatin may play a suppressive role [9, 28, 35-37]. In our study 10 of the survivors received dopamine. Our study was not designed to study the immediate relation of thyroid function after dopamine withdrawal as done previously in children after cardiac surgery [9, 28, 35-37].

In order to get a better insight in the resolution of the hormonal and metabolic changes in children who survived, we evaluated the time course. Cortisol and ACTH levels in those who survived were on day 2 already significantly lower compared with the levels on admission and after 12 hours. The cortisol and ACTH levels where within normal reference values, however, the circadian rhythm of cortisol was not seen. These findings suggest a persisting hyperactivity of the adrenal gland despite normalization of cortisol and ACTH levels. Furthermore 2 days after admission levels of total T_4 remained unaltered, whereas levels of fT_4 and TSH increased but remained within normal reference values. Levels of rT_3 decreased and levels of T_3 and the ratio T_3/rT_3 increased but all these levels still remained below normal reference values. These data indicate a slightly restoration but not a normalization of the thyroid function at day 2. Levels of NEFA had decreased significantly at 2 days after admission, indicating that lipolysis had

diminished. Levels of insulin and glucose showed a variable pattern within the normal range during the first 2 days after admission which is in contrast to studies in critically ill adults where hyperglycemia and glucose intolerance with elevated insulin levels are hallmarks of stressed metabolism [38]. Whether children with septic shock have a different insulin/glucose response compared to adults is not known. Although several hormonal axes and levels of NEFA and lactate indicated the restoration of anabolism at the second day after admission, the urea nitrogen excretion did not change significantly and remained high. This is in accordance with studies in critically ill adult patients in which protein breakdown remained increased after the return to baseline values of the stress hormones and cytokines [39]. In some studies of critically ill adult patients and critically ill children an improvement in protein synthetic rate or an improvement of nitrogen retention could be achieved by provision of exogenous nutrition [40-42]. In our study the provision of calories was far below the estimated need for calories during the first 2 days after admission (respectively 45% and 42% too low). The low caloric supply might therefore be responsible for the high and unaltered excretion of nitrogen during these days in our patients.

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Chapter 4

Low serum cortisol levels in combination with high ACTH levels are associated with poor outcome in children with severe meningococcal disease

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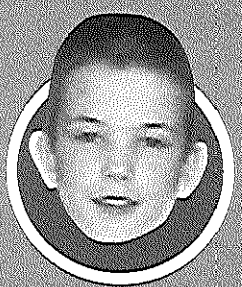

Abstract

Objectives: To study the correlation between serum levels of adrenocorticotrophic hormone (ACTH) and cortisol in relation to severity of disease in children with meningococcal sepsis.

Methods: Children with meningococcal sepsis, who were admitted to the pediatric intensive care unit were included in this study. Clinical data, laboratory parameters and blood samples were collected. Arterial cortisol, ACTH, IL-6 and TNFα levels were measured on admission and studied for their relation with severity of disease (sepsis, septic shock / survivors, septic shock/ non-survivors).

Results: 72 patients fulfilled the criteria for meningococcal sepsis. Sixty-two of these children with positive blood cultures of *Neisseria meningitidis*, who were not treated with corticosteroids before admission, were included. Fifty of these 62 patients had septic shock. Twelve of those children (24%) died. The median age of the children in this study was 2.6 years (0.3 –16.1 years). Cortisol levels were significantly lower in non-survivors (median 654 nmol/l) compared with survivors (median 2184 nmol/l) ($p<0.01$). ACTH levels were significantly higher in children who died (median 1271 ng/l) than in survivors (85 ng/l) ($p<0.01$). The median cortisol/ACTH ratio decreased significantly depending on the disease severity categories. Serum cortisol levels on admission were correlated with the PRISM score ($r = -0.37$, $p<0.01$), lactate levels ($r = -0.31$, $p=0.01$), IL-6 ($r=-0.41$, $p=0.04$), CRP ($r = 0.40$, $p<0.01$) and fibrinogen levels ($r = 0.64$, $p<0.01$). ACTH levels on admission were correlated with the PRISM score ($r = 0.62$, $p<0.01$), lactate levels ($r = 0.51$, $p<0.01$), Il-6 ($r=0.69$, $p<0.01$), TNFα ($r=0.77$, $p<0.01$), CRP ($r = -0.50$, $p<0.01$), fibrinogen ($r = -0.66$, $p<0.01$), glucose ($r=-0.33$, $p=0.01$) and also with age ($r = -0.31$, $p=0.02$).

Conclusions: Low serum cortisol levels in combination with high ACTH levels are associated with poor outcome in children with severe meningococcal disease.

Introduction

Severe meningococcal disease (SMD) is well known for its sudden onset and rapid progression. Despite early use of antibiotics, aggressive hemodynamic support and life sustaining care, the overall mortality of children with meningococcal disease remains high (10%) [1]. The systemic inflammatory response in severe meningococcal disease is partly mediated by activation of the hypothalamic-pituitary-adrenal axis (HPA) leading to increased serum concentrations of adrenocorticotrophic hormone (ACTH) and cortisol [2, 3]. This "stress response" is thought to contribute towards the restoration of homeostasis. In this respect, cortisol has a vital role in the maintenance of the vascular tone, endothelial integrity, vascular permeability and the distribution of total body water within the vascular compartment [2, 4, 5]. Cortisol also potentiates the vasoconstrictor actions of catecholamines [4, 5].

The mechanism of activation of the hypothalamic-pituitary-adrenal axis in severely ill patients is complex. During acute stress corticotropin-releasing hormone (CRH) and arginine vasopressin are released in the hypothalamus stimulating the secretion of ACTH in the pituitary gland. ACTH is the key stimulator of glucocorticoid secretion in the adrenal glands. Several circulating mediators (e.g. LPS and cytokines) play a major role in the activation of the HPA axis as part of the inflammatory response. Experimental and human studies have contributed to the understanding that cytokines (such as IL-6) predominantly stimulate [6-10], but may also inhibit (TNF-α) [11-13] the production of ACTH and cortisol. In children with meningococcal sepsis high serum levels of both inflammatory and anti-inflammatory cytokines are found on hospital admission [14, 15].

In adult patients with sepsis a significant correlation has been reported between severity of illness and mortality on the one hand, and serum cortisol levels on the other [16-18]. However, in children with meningococcal sepsis the lowest levels of cortisol have been found in children who died [19-25]. This raises the question, which levels of plasma cortisol are 'adequate' in critically ill patients. During the last decade, several controlled studies have indicated that the use of high doses of corticosteroids in adults with sepsis or septic shock does not improve outcome and may even increase morbidity and mortality [26-28]. However, adrenal insufficiency is considered to be an indication for treatment with corticosteroids. Recently, the discussion whether low dose corticosteroid treatment may improve outcome of septic shock was reinitiated. Studies in adult patients with septic shock reported hemodynamic improvements and beneficial effects on survival after treatment with low dose corticosteroids [29, 30]. Similarly, preliminary results of studies in children indicated the presence of relative adrenal insufficiency in non-survivors of severe meningococcal disease [24, 25]. The aim of the current study was to analyze the serum levels of circulating adrenocorticotrophic hormone (ACTH), cortisol and IL-6 and TNFα in children with severe meningococcal disease and study the association between the levels of these hormones and clinical outcome.

Materials and methods

Study design

Children between 1 month and 18 years of age, who were admitted between 1995 and 1999 with suspected meningococcal sepsis to the pediatric intensive care unit of the Sophia Children's Hospital were included in this study. Sepsis was defined as clinical diagnosis of infection with a systemic response: tachycardia and tachypnea (> 2 SD according to age) and fever or hypothermia (<36 °C or >38.5 °C). Septic shock was defined as persistent hypotension despite volume suppletion, described as <2SD systolic blood pressure according to age or evidence of poor end-organ perfusion, defined as at least two of the following: 1. Unexplained metabolic acidosis (pH<7.3 or base excess<-5 mmol/l or arterial plasma lactate levels > 2.0 mmol/l); 2. Arterial hypoxia (PO_2 <75 mmHg, a PO_2/FiO_2 ratio <250 or transcutaneous oxygen saturation <96%) in patients without overt cardiopulmonary disease; 3. Acute renal failure (diuresis <0.5 ml/kg/h for at least 1 hour despite volume loading or evidence of adequate intravascular volume without pre-existing renal disease): 4. Sudden deterioration of the baseline mental status.

After written informed consent of the parents, clinical data, laboratory parameters and blood samples were collected from all participants. The Paediatric Risk of Mortality score (PRISM II) was calculated based on the most abnormal values regarding 14 physiological variables during the first six hours of admission [31]. In patients who entered the study protocol after October 1997, serum cortisol and ACTH concentrations were measured within a few days and followed prospectively. Blood samples from participants before October 1997 were stored at −80°C until analysis was done. All patients received standard intensive care treatment. The administration of corticosteroids is no routine in our intensive care unit.

The study protocol was approved by the Medical Ethical Committee of the University Hospital of Rotterdam. Patients admitted to the ICU before May 1995 participated in a randomised, multicenter, placebo-controlled study with HA-1A a monoclonal anti-endotoxin antibody (Centocor). Patients admitted after July 1997 participated in a randomised double-blind trial of protein C (Baxter-Immuno, Vienna) versus placebo. Study medication was administered some hours after admission on the ICU and did therefore not influence the data on admission.

ACTH and cortisol assessments

Arterial blood samples were taken on admission (before study medication was given), and for some children also after 12 and 24 hours. In 14 patients, the diurnal rhythm for cortisol and ACTH was measured by taking blood samples on the second day of admission at 8.00 am, 2.00 pm and 8.00 pm. These data have previously been described [25]. Plasma concentrations of cortisol were measured by a competitive luminescence immunoassay (LIA). The detection limits of this assay are: 3-1380 nmol/l. The plasma concentrations of ACTH were measured by a immunoradiometric assay (IRMA, ELSA-ACTH CIS bio international), using two monoclonal antibodies. The within-

run coefficient of variation was 6.1% at 22 pg/ml, 2.9% at 59 pg/ml and 2.1% at 778 pg/ml. The between-run coefficient of variation was 5.3% at 40 pg/ml, 4.8% at 203 pg/ml and 1.3% at 1055 pg/ml. Reference values for cortisol were: 8.00 h: 200-600 nmol/l, 14.00h: 100-500 nmol/l, 20.00 h: 50-300 nmol/l. Reference values for ACTH were 20-100 ng/l. From the values of cortisol and ACTH the ratio cortisol/ACTH was calculated.

IL-6 and TNFα assessments

IL-6 serum levels and TNFα serum levels were measured by using commercial Immune reagents (CLB, Amsterdam, the Netherlands). The detection limit of IL-6 (lowest positive standard) was: 10 pg/ml. The detection limit of TNFα was: 5 pg/ml.

Statistics

Statistical analysis was performed using SPSS (version 9.0) computer package. Since the data were not normally distributed, results are described as median values (ranges) and non-parametric tests were used for analysis. Statistical differences between groups were assessed using the Mann-Whitney U test for independent groups. For comparison in groups vs time, the paired Wilcoxon signed rank test was used. Correlation's were calculated using the Pearson's correlation coefficient. A $p<0.05$ was considered as statistically significant.

Results

Population

In the period between October 1994 and September 1999, 72 patients were included in the study. Fifty-nine patients had a septic shock. Fourteen of these 59 did not survive. Thirteen patients had sepsis without features of shock. In 64 of the 72 patients, Neisseria meningitidis was cultured. Two patients were treated with corticosteroids before admission and therefore excluded from analyses. The clinical characteristics and laboratory parameters upon admission of the 62 children with a proven meningococcal disease are depicted in Table 1. The patients are classified into three disease severity categories, based on the clinical picture and outcome (sepsis, septic shock/survivor, septic shock/non-survivor). Forty-two percent of the patients were female. Only 17% of the non-survivors were female (P=0.06). The median age of the total group was 2.6 years (0.3 –16.1 years). Children with a more severe clinical picture had a significantly lower age. Hypoglycemia (≤ 2.6 mmol/l) was only present in 2 survivors of septic shock.

On admission

Serum ACTH and cortisol levels

Levels of serum cortisol on admission varied widely within all patients (median 954 nmol/l, range 296-2480 nmol/l). The cortisol levels of 84% of the patients were above the normal reference range for healthy, non-stressed children (200-600 nmol/l). Nine patients with meningococcal septic shock had a cortisol level within the normal reference range for healthy non-stressed children. Five of them died. None of the patients had a cortisol level below 138 nmol/l, as would be expected in case of adrenal insufficiency. Seven patients had cortisol levels between 138 nmol/l and 497 nmol/l, suggesting a partial adrenal insufficiency. Four of these children died. The median levels of serum cortisol were significantly different between the 3 clinical catagories (Table 1). There was a significant relationship between severity of disease and the serum cortisol levels (Table 1 and Figure 1).

In the total studygroup, serum ACTH levels had a median concentration of 223 ng/l (range 13.4 – 4120 ng/l). Twenty-seven percent of the patients had ACTH levels within the normal range (20 -100 ng/l), 8% had levels below and 65% above this range. Medians and ranges of ACTH concentrations in the three different clinical groups are shown in Tabel 1. The ACTH levels were significantly different ($p<0.01$) between the three groups with the highest levels in the group of non-survivors (Figure 2). All of the non-survivors had ACTH levels above the normal range.

The cortisol/ACTH ratio decreased with severity of disease (Table 1) and was significantly correlated with the PRISM score ($r=-0.60$, $p<0.01$). The cortisol and ACTH concentrations were also significantly correlated ($r = -0.46$, $p<0.01$) (figure 3). Serum cortisol levels were significantly correlated with the PRISM score ($r = -0.37$, $p<0.01$), lactate ($r = -0.31$, $p=0.01$), CRP ($r = 0.40$, $p<0.01$)

and fibrinogen (r = 0.64, p<0.01). ACTH levels on admission were significantly correlated with the PRISM score (r = 0.62, p<0.01), lactate (r = 0.51, p<0.01), CRP (r = -0.50, p<0.01), fibrinogen (r = -0.66, p<0.01), glucose (r=-0.33, p=0.01) and also with age (r = -0.31, p=0.02).

	Sepsis (all survivors) N=12	Septic shock Survivors N=38	Septic shock Non-survivors N=12	Normal range
Female	7 (58%)	18 (45%)	2 (17%)	
Respiratory support	1 (8.3%)	20 (53%)	12 (100%)	
Age (in years)				
Median	4.5 #	2.4	0.8 **	
Range	0.8-12.1	0.3-16.1	0.5-9.4	
CRP t=0 (mg/l)				
Median	114.5	91.0	34.0 **	0-10 mg/l
Range	13.0-250.0	21.6-284.0	6.0-84.0	
Fibrinogen t=0 (g/l)	N=8	N=24	N=10	
Median	4.8 ##	2.4	0.50 **	2-4 g/l
Range	3.2-6.8	0.3-4.9	0.4-1.6	
Lactate t=0 (mmol/l)				
Median	2.4 ##	4.2	6.8 **	<1.8 mmol/l
Range	1.3-5.7	1.7-11.8	3.2-13.8	
PRISM score				
Median	7.8 ##	18.5	30.5 **	
Range	2-19	3-36	15-37	
Glucose t=0 (mmol/l)	N=10			
Median	7.6 #	6.2	5.4	4.0-6.0 mmol/l
Range	4.1-44.9	1.3-12.7	2.8-10.0	
ACTH t=0 (ng/l)				
Median	49.2 ##	170	1272 **	20-100 ng/l
Range	13.4-360	14.5-4120	409-3010	
Cortisol t=0 (nmol/l)				8.00 h: 200-600 nmol/l
Median	1158 #	997	654 **	14.00h: 100-500nmol/l
Range	737-2150	296-2480	320-1050	20.00h: 50-300nmol/l
Cortisol/ACTH t=0				
Median	32 ##	7.4	0.53 **	2-33
Range	3.3-86	0.1-77	0.12-2.2	
Il-6 t=0 (pg/ml)		N=19	N=7	
Median		41041	1184641 **	<20 pg/ml
Range		650-313127	624496-2934110	
TNFα t=0 (pg/ml)		N=20	N=7	
Median		6.6	42 **	<20 pg/ml
Range		5-22	12-527	

Table 1 Clinical characteristics and laboratory parameters of 62 children with meningococcal sepsis

\# p<0.05 between sepsis patients and septic shock patients

p<0.01 between sepsis patients and septic shock patients

** p<0.01 between septic shock survivors and non-survivors

CRP= C-reactive protein; ACTH= Adrenocorticotrophic Hormone; IL-6= Interleukin 6; PRISM score= Pediatric Risk of Mortality Score; TNFα= tumor necrosis factor alpha

Cytokine levels

Il-6 levels were measured in 26 children with a septic shock of whom 7 patients died. The median Il-6 level was 76650 pg/ml (range 650 – 2934110 pg/ml). Non-survivors had significantly higher Il-6 levels than survivors from septic shock ($p<0.01$). The levels of Il-6 were significantly correlated with ACTH levels ($r= 0.69$, $p<0.01$) (figure 4). A negative correlation was found between cortisol and Il-6 levels ($r=-0.41$, $p=0.04$) and between IL-6 and the cortisol/ACTH ratio (r-0.83, $p<0.01$). There was also a significant correlation between Il-6 levels and the PRISM score (r=0.75

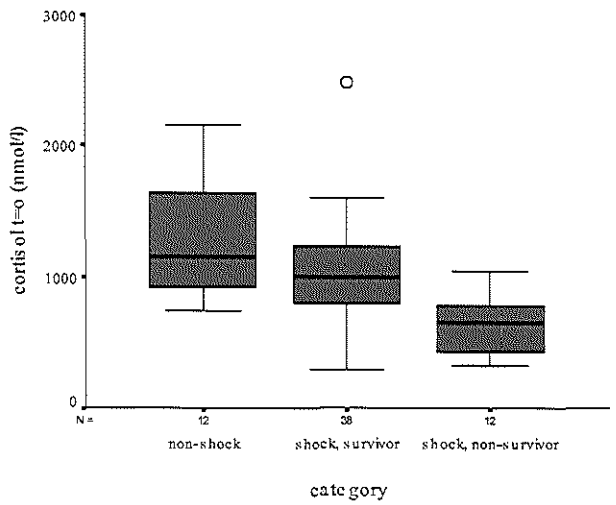

Figure 1 Serum cortisol levels on admission in different groups of children with meningococcal disease.

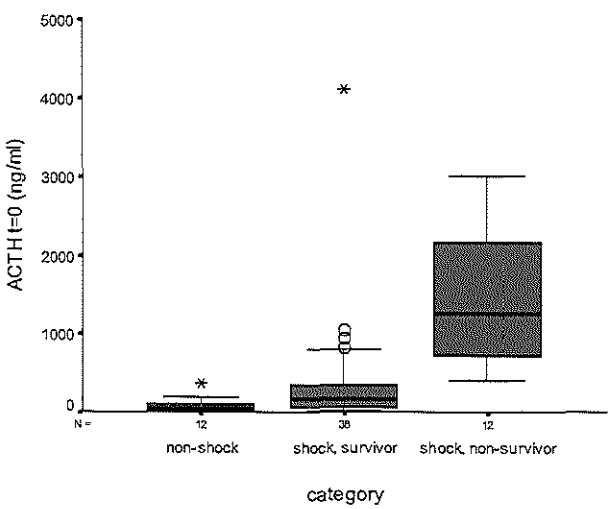

Figure 2 Serum ACTH levels on admission in different groups of children with meningococcal disease.

p<0.01), lactate (r=0.71 p<0.01), CRP (r=-0.55 p<0.01) and fibrinogen (r=-0.784 p<0.01). TNFα levels were measured in 27 children with a septic shock. The median level TNFα was 9.0 pg/ml (range 5-527). Non-survivors (n=7) had significantly higher median levels than survivors (resp. 42 (12-527) and 6.5 (5-22) pg/ml, p<0.01). The levels of TNFα correlated significantly with ACTH (r=0.77, p<0.01). There was no significant correlation with cortisol levels, but a negative correlation

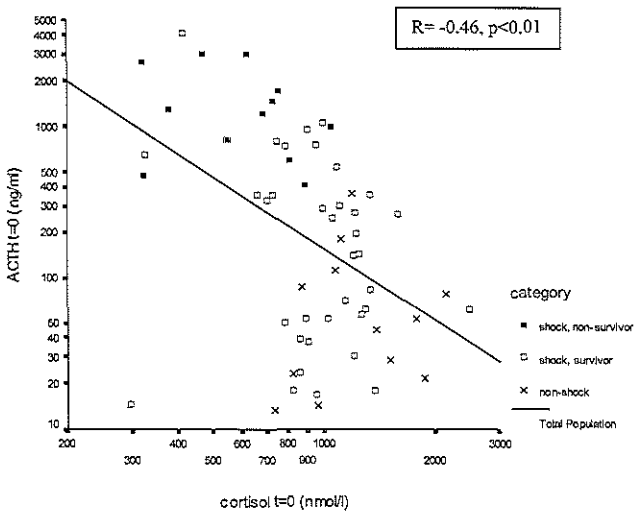

Figure 3 Correlation of cortisol and ACTH serum levels on admission. The children in the three clinical categories have different markers.

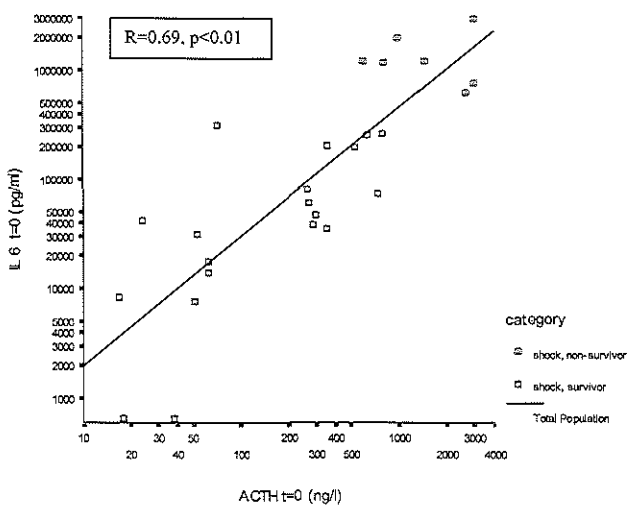

Figure 4 Correlation of ACTH and IL-6 serum levels on admission. Survivors and non-survivors of meningococcal septic shock are discriminated by different markers.

was present between TNFα and the cortisol/ACTH ratio (r=-0.67, p<0.01). TNFα levels correlated significantly with the PRISM score (r=0.72, p<0.01), CRP (r=-0.59, p<0.01), lactate (r=0.65, p<0.01) and fibrinogen (r=-0.87, p<0.01).

During stay in the ICU

Serum ACTH and cortisol levels

In 41 patients serum ACTH and cortisol concentrations were studied during the first 24 hours of admission in the ICU. The levels of cortisol and ACTH decreased within 24 hours in all three clinical categories. The three non-survivors who died after 12 or 24 hours showed also a decrease in cortisol and ACTH serum levels over time. In ten survivors (33%) with septic shock cortisol levels increased in the first day, whereas only 2 patients also showed an increase in ACTH. In 14 survivors with septic shock an ACTH/cortisol daycurve was performed on the second day after admission. In none of the patients a circadian rhythm could be detected.

Discussion

This study shows that median levels of ACTH and cortisol are significantly different in children with meningococcal sepsis, in survivors from meningococcal septic shock and in non-surviving children. Low serum cortisol levels and high serum ACTH levels are associated with poor outcome in children with severe meningococcal disease. The cortisol/ACTH ratio decreased with increasing severity of disease.

Our results are in striking contrast to those in adult patients, in which the highest cortisol levels are observed in non-survivors and in whom cortisol levels are positively correlated with severity of disease [17]. However, in a small number of adults with septic shock low levels of cortisol are also encountered [2, 32, 33]. Riordan et al. recently found low cortisol levels in a group of children with meningococcal disease, although they did not detect a correlation between serum ACTH and cortisol levels [24]. The difference in cortisol levels between patients with or without hypotension in this study was not statistically significant [24]. However, the definitions of sepsis and septic shock were not accurately described in their study. In our study, ACTH levels correlated significantly with cortisol levels, whereas ACTH and cortisol levels differed significantly between patients with a meningococcal sepsis and patients with a meningococcal septic shock.

The pathogenetic mechanisms leading to high ACTH in combination with low cortisol levels in children with a meningococcal sepsis are not well understood. The presence of extremely high levels of cytokines in children with meningococcal sepsis on admission, may play a crucial role. Il-6 is able to stimulate the stress-response by activation of the production of ACTH [9]. We found a positive correlation between Il-6 and ACTH on admission, and a negative correlation between serum Il-6 and cortisol levels. Alternatively, high TNFα levels in meningococcal sepsis may inhibit corticosteroid production [13]. The possibility that the adrenal cortisol production is inhibited by TNFα could not be confirmed, because no significant correlation was found between TNFα and cortisol levels. Riordan et al. did also not find a correlation between TNF-α and cortisol levels [24]. In this study we observed a positive correlation between TNFα and ACTH serum levels, which suggests that ACTH production is directly or indirectly activated by TNFα. Alternatively, low cortisol levels could be the result of adrenal haemorrhage or inadequate adrenal perfusion leading to a diminished function of the adrenal glands. In our study cortisol levels were significantly different between children with and without a septic shock. Unfortunately, post-mortem examinations enabling identification of adrenal infarction, were not performed. In meningococcal disease, the adrenal cortisol production seems not to follow a stress induced ACTH surge as reflected by the decreased cortisol/ACTH ratio with increasing severity of disease. This finding suggests a downregulation of the cortisol producing cells. The pathogenesis of this mechanism is not yet understood. Decreased responsiveness of the adrenal cortex to ACTH has also been described in rats and may be age-dependent [34]. One may speculate that the extreme high levels of cytokines in children with meningococcal sepsis may inhibit the receptor transduction in the adrenal cells.

In adult patients with sepsis, an altered glucocorticoid receptor function has recently found in the presence of an abundant production of glucocorticoids [35, 36]. No information is yet available on the glucocorticoid receptor function in children.

Hatherhill et al. found that the incidence of adrenal insufficiency as defined by a post-Synacthen cortisol increment of<200 nmol/l is 52%, in children with septic shock [23]. Mortality was not statistically different between children with adrenal insufficiency and in those without [23]. However, the validity and the interpretation of tests of responsiveness to synthetic adrenocorticotropin stimulation in stressed patients have been seriously questioned. The decreased cortisol/ACTH ratio is a strong indicator of relative adrenal insufficiency. Because of the life threatening situation of the patients a corticotropin stimulation test is deemed unnecessary. None of the patients had a definite adrenal insufficiency (cortisol < 138 nmol/l). A cortisol concentration of > 700 nmol/l may rule out the presence of adrenal insufficiency in stressed adults [37]. Interestingly 42% of the non-survivors had levels above 700 nmol/l, suggesting that adrenal insufficiency does not play a major role as cause of death. However, 16% of the survivors and 58% of the non-survivors showed cortisol levels below 700 nmol/l during maximum stress in the presence of high ACTH levels, indicating relative adrenal insufficiency. One may suggest on the basis of these relatively low levels of cortisol that this group of patients may benefit from early administration of corticosteroids. However, we are not yet able to predict whether the balance between pro and anti-inflammatory cytokines in children with meningococcal sepsis will be influenced in a positive or in a negative direction once corticosteroids are given. This implicate that it may be quite hazardous to prescribe corticosteroids to these patients. Further research is needed to provide more information of the pathophysiological mechanisms involved in the hypothalamic-pituitary-adrenal axis in children with meningococcal sepsis. The question whether corticosteroids deserve a position in the therapeutic regime of patients with meningococcal sepsis remains to be answered.

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Chapter 3

Validation of a prognostic scoring system for children with meningococcal septic shock: The Rotterdam Score.

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Abstract

Objective: To prospectively validate and evaluate the Rotterdam Score (RS), a previously developed prognostic risk of mortality score for children with meningococcal septic shock. The actual mortality rate will be compared with that predicted by the RS and with the mortality predicted by the PRISM score.

Design: Clinical and laboratory data obtained prospectively through questionnaires and retrospectively through hospital case notes analysis, were pooled to obtain a validation cohort.

Setting: Pediatric departments from 71 hospitals in the Netherlands and St. Mary's Hospital in London, UK.

Patients and Methods: During a twelve month period, selected clinical and laboratory parameters were prospectively evaluated for children (age range 3 months to 18 years) admitted with meningococcal septic shock to 71 pediatric departments in the Netherlands (group 1). The hospital records of children admitted with meningococcal septic shock to St. Mary's Hospital in London between January 1992 and July 1998, were reviewed and data recorded retrospectively (group 2). The performance of the RS, consisting of four laboratory parameters (C-reactive protein, potassium, arterial base excess, platelet count) was compared with the Pediatric Risk of Mortality (PRISM) score.

Results: Data of group 1 and 2 were pooled to obtain 148 cases for validation of the RS. Eighteen (12%) patients died. Median values for the risk of mortality calculated by the RS were statistically significant higher in non-survivors (96%) than in survivors (5%) ($P<0.01$). The correlation of the RS with the PRISM score was $r=0.532$ ($p<0.01$). Logistic regression analysis revealed that the RS had a higher predictive value for death and survival than the PRISM score.

Conclusion: The Rotterdam score is an institutionally independent, simple, objective and effective mortality prediction score for children with meningococcal septic shock. A 6-hour PRISM predicts mortality in this subgroup of children with reasonable accuracy.

Introduction

Patients with sepsis caused by *Neisseria meningitidis* rapidly develop the characteristic petechiae and clinical symptoms. Eighty to ninety percent of these patients remain clinically stable and do not deteriorate hemodynamically. However, in a small group a rapid progress of disease can occur [1]. 10-15% of cases of meningococcal disease (MD) present with isolated sepsis (with mortality rates as high as 50%) and 40% present with a mixed picture of meningitis and sepsis. The excessive response of the immune system on products of the meningococcus induces damage to the vascular endothelium resulting in capillary leakage and edema. The decrease in intravascular volume may result in a hypotensive, hypovolemic patient. The presence of hypotension and shock are prognostic indicators of poor outcome and are associated with a high morbidity and mortality [1-4]. A simplified objective mortality prediction score for patients with meningococcal sepsis has many applications. The scores may be applied as a tool for comparison of individual intensive care units. These scores are increasingly being used for laboratory research in MD. These scores generally facilitate in stratifying cases according to severity and allow accurate descriptions of the casemix in individual units. This is vital in assessing efficacy of conventional and experimental therapeutic interventions in controlled trials. For interventions to be effective in meningococcal septic shock (MSS), early detection and estimation of severity is desirable.

Several prognostic scores have been developed since Stiehm and Damrosch introduced their model for prediction of mortality risk of patients with acute meningococcal infection [3, 5-8]. Glasgow Meningococcal Sepsis Prognostic Score (GMSPS) and Pediatric Risk of Mortality Score (PRISM) have been used for mortality prediction in these patients. Some of these systems are only validated in a small number of patients or are not yet validated prospectively, others have obtained restricted validity across time by the loss of positive predictive value, or use only clinical variables with a large degree of inter-observer variability. The Rotterdam score (RS) has been developed with data from seventy-five patients, 18 years of age and younger who were admitted to the Pediatric Intensive Care Unit (PICU) of the Sophia Children's Hospital with meningococcal septic shock [9]. The medical records of these children were prospectively evaluated between October 1988 and June 1995. Logistic regression analysis identified four independent variables predicting the likelihood of survival; serum CRP level, serum potassium level, base excess, and platelet count. Kornelisse et al. compared the RS with several other scoring systems [9-14]. The RS is simple, objective and highly specific for patients with meningococcal septic shock.

Aims

The aim of the current study was to prospectively validate and evaluate the Rotterdam Score in a new set of patients with meningococcal septic shock. The actual mortality rate will be compared with that predicted by the RS and with the mortality predicted by the PRISM score.

Materials and methods

Patients

All children between 3 months and 18 years of age, admitted to the hospital with a presumed meningococcal septic shock (MSS), were included for data collection. Information needed for validation of the scoring system was obtained through two questionnaires. The first list included questions about laboratory results and clinical features of the patient assessed within the first six hours after admission to the hospital. Information regarding disease outcome was obtained with a follow-up questionnaire some weeks later. Informed consent was not required, since information needed for this validation study was limited to a review of medical records and was analysed anonymously. Patients were included in the study if they met the following criteria:

1. Presence of petechiae and /or purpura
2. Presence of shock defined as hypotension or at least two signs of poor end-organ perfusion. Hypotension was defined according to age: systolic blood pressure < 75 mm Hg in children below the age of 1 year; < 80 mm Hg in children aged 1-5 years; < 85 mm Hg in children 6-12 years and < 100 mm Hg in children older than 12 years. The following signs of poor end-organ perfusion were defined: unexplained metabolic acidosis defined as pH ≤ 7.3 or base deficit ≥ 5 or plasma lactate levels > 2.0 mmol/l; arterial hypoxia defined as $PO_2 \leq 75$ mm Hg, a PO_2/FiO_2 ratio < 250 or transcutaneous $SaO_2 \leq 96\%$ in patients without pre-existing cardiopulmonary problems; acute renal failure (urine output < 0.5 ml/kg/h for at least one hour despite acute volume loading or evidence of adequate intravascular volume) without pre-existing renal disease; sudden deterioration of patients baseline mental status which does not appear to be solely due to meningitis;
3. Confirmation of an invasive infection with *Neisseria meningitidis* by isolation of meningococci from blood, detection of meningococcal antigen, detection of meningococcal genome in the blood by polymerase chain reaction (PCR), or by isolation of meningococci in skin scraping from purpuric lesions.

Patients were excluded from the study if their questionnaire was incomplete, if laboratory studies failed to confirm meningococcal infection or if patients did not have features of shock. Patients with meningococcal meningitis without shock on admission were excluded from the study

Data collection

In phase 1 of the study, pediatricians from hospitals in the Netherlands were invited to participate in this prospective study over a period of 12 months (November 1997-November1998). When a child with suspected MSS was admitted to their department they were requested to complete the first questionnaire and return it to the investigators. The follow-up questionnaire was

sent to the attending specialist at these hospitals. Clinical data in the first questionnaire included institution, date and time of entry, age, gender, weight, date and time of onset of the first petechial rash, history suggestive of immunodeficiency, systolic and diastolic blood pressure (BP), heart rate and respiratory rate, medication given and highest dose in the first 6 hours after admission, mechanical ventilation or requirement for respiratory support, Pediatric Glasgow Coma Score [15] and pupillary response to light.

Laboratory data in the first questionnaire included $PaCO_2$; PaO_2; pH; Base Excess; bicarbonate and lactate concentration; serum potassium level; platelet count and serum CRP, white blood cell count; levels of serum total calcium, glucose and bilirubin; prothrombin time; partial thromboplastin time; creatinin level and urine output.

The second evaluation list, a few weeks later, included questions concerning outcome of the patient: whether the patient died; date, time and cause of death and complications. Additional data in this questionnaire included blood and CSF cultures, antigen detection or skin scrapings, systolic and diastolic blood pressure, heart rate, respiratory rate, Pediatric Glasgow Coma Score and current medical status and supportive therapies (including ventilation).

The second list contained no laboratory variables. The most abnormal value of a variable within the first six hours after admission was recorded. Only data needed for validation of the Rotterdam score have been used and described. The remaining information concerning this group of patients will be reported in future publications.

In phase 2 of this study, medical records of patients who were admitted to St. Mary's Hospital with a final diagnosis of meningococcal septic shock (satisfying the above inclusion criteria), between January 1992 and July 1998 were analyzed retrospectively by one of the authors (AME B).

Calculation of the two scoring systems

The mathematical expression for translation of the Rotterdam score into patient probabilities of mortality is $e^{RS}/(1+e^{RS})$, where the Rotterdam score (RS) = 1.01 + (1.21 × serum potassium level [mmol/l]) − (0.29 × base excess [mmol/l]) − (0.024 × platelet count [x 10^9 /l]) − (3.75 × log serum CRP level [mg/l]) [9]. The value of the Rotterdam score predicts outcome of death: a very low Rotterdam score results in a mortality probability near 0%, a high Rotterdam score in a mortality probability near 100%. All Rotterdam scores obtained from patients of this validation set were transformed into predicted probabilities of mortality. Subsequently, patients were ranked, based on their predicted mortality, and divided into mortality risk categories. The variables of the PRISM score included systolic and diastolic blood pressures, heart rate, respiratory rate, coma status, response of pupils to light, levels of serum potassium, bicarbonate, total bilirubin, calcium and glucose, prothrombin/ partial thromboplastin times, PCO_2, PaO_2/ FiO_2. The systolic blood pressure, heart rate, and respiratory rate are stratified by age. The PRISM score is obtained by summing for each variable the weight corresponding to the most abnormal value observed in the first six hours after admission [13].

Statistical Methods

Logistic regression was used to test differences between fatality rate, age, sex and variables included in the Rotterdam score of groups 1 and 2. Descriptive statistics were used to describe groups (mean, median, SD, range). The Rotterdam score as well as the PRISM score were determined for patients included in the validation set, and the individual predicted mortality risks were averaged to obtain a predicted average mortality risk for the whole group for both scoring systems. The Mann-Whitney test was used for comparison of medians between survivors and non-survivors. A significance level of 5% (two-tailed $p<.05$) was used in the interpretation of the results. Pearson's correlation coefficient (r) was used to evaluate the relation between the Rotterdam score and the PRISM score. Linear logistic regression analysis was also used to compare the scores for survivors and non-survivors. Observed mortality was compared to predicted mortality using Pearson's chi-square test for fit.

Results

Over 95% of all pediatric institutions in the Netherlands agreed to participate in this study.

Two-hundred-eighteen questionnaires of patients with possible meningococcal disease were received. Information about the causative organism was acquired in 194 cases (*Neisseria meningitidis* in 193 and *Haemophilus influenzae* in 1 patient). *Neisseria meningitidis* was cultured from blood (n= 80), cerebrospinal fluid (CSF) (n=36) and both CSF and blood (n=73) and from skin scrapings of purpuric lesions (n=4). Of the 157 patients with an isolate of *N. meningitidis* from blood (with or without an isolate of meningococci from CSF) or in skin scrapings, 80 patients had features of shock. Eighteen cases were excluded from this group because of incomplete data (of the laboratory assessments included in the Rotterdam score). Arterial base excess is included in the scoring system. 28 patients who either had venous or capillary base excess were excluded from validation set. Of 34 cases available for analysis 4 (12%) patients died.

Medical records of 121 patients with MSS admitted to St Mary's Hospital in London between January 1992 and July 1998 were retrospectively reviewed (group 2). Eight cases were excluded because of missing data (of the laboratory assessments included in the Rotterdam score). In the remaining 113 children with MSS, a case-fatality rate was 12%. None of the patients from either group 1 or 2 that enrolled in the study had an underlying disease. No significant differences in age, gender, mortality rate, PRISM scores for survivors and non-survivors were found between the two patient groups. Furthermore, the same selection criteria for MSS were used and hence data of both groups were pooled and used as validation set.

Characteristics	the Netherlands group 1 (n=34)	London, St. Mary's PICU group 2 (n=113)	Validation set (n=147)
Age (months): median (range)	27 (4.8-193.8)	37 (3.5-193.0)	*Total group* 34 (3.5-193.8) *Survivors* 36 (3.5-193.8) *Non-survivors* 26 (4.75-193)
Female sex: n (%)	18 (53%)	48 (43%)	*Total group* 66 (45%) *Survivors* 58 (45%) *Non-survivors* 8 (44%)
Deaths: n (%)	4 (12%)	14 (12%)	18 (12%)
Mean PRISM score ± SD	17 ± 9 (n=30)	17 ± 8 (n=113)	17 ± 8 (n=143)
Survivors ± SD	16 ± 9 (n=26)	15 ± 6 (n=99)	16 ± 7 (n=125)
Deaths ± SD	22 ± 5 (n=4)	28 ± 9 (n=14)	26 ± 9 (n=18)

Table 1 Characteristics of patients with a meningococcal septic shock

Validation set

After pooling data of patients of both groups, there were 147 cases available for validation of the Rotterdam score. No statistical significant differences were found in age and sex between survivors and non-survivors of the validation set. Table 1 shows patient characteristics for the validation set. The Rotterdam score was obtained for every patient of the validation set. Figure 1 illustrates the relationship of mortality predicted by the model classified into 10 mortality risk categories to observed mortality within each category. In the predicted mortality category of 45% only two patients were included: both survived. In the following three categories a slight decrease of the observed fatality rate is seen, and none of the patients studied had a predicted probability whitin the 75% mortality risk category. All average predicted probabilities are within the 95% confidence interval of the estimated probability of mortality based on the relative frequency of mortality within each subgroup. For patients with a predicted mortality rate less than 10% an observed mortality of 1% was seen. The observed mortality rate of the 14 patients in the last category with a predicted mortality > 90%, was 86%. Median values for the mortality predicted by the Rotterdam score were significantly higher in non-survivors (96%, ranging from 3 to 100%) than in survivors (5%, ranging from 0 to 99 %). The median value of the PRISM score was also higher for patients who died (24, ranging from 8 to 41) than for survivors (15, ranging from 1 to 39) ($p<.001$ for both scoring systems). The correlation of the Rotterdam score with the PRISM score was statistically significant at the $p<.01$ level ($r=.53$). Figure 2 shows the relation between PRISM

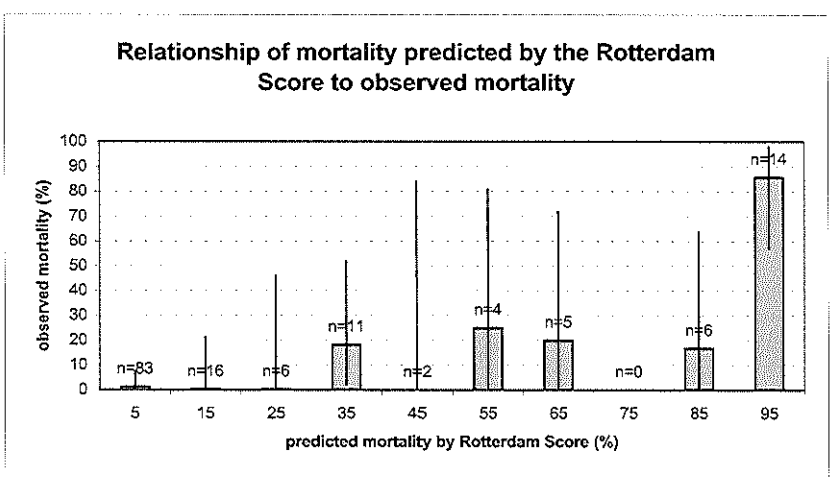

Figure 1 Relationship of mortality predicted by the Rotterdam Score to observed mortality.
The bars show the observed mortality in the 10% range of predicted mortality by the Rotterdam Score. The 95% confidence interval based on the relative frequency of mortality is depicted by lines in each subgroup. Number of participants is given for each subgroup.

score and Rotterdam score for patients of the validation set with different marks for survivors and non-survivors. With logistic regression analysis, the Rotterdam score showed a higher predictive value for death and survival than the PRISM score (Rotterdam score: $p<0.01$, PRISM score: $p=0.15$). The predictive ability of the two scores is shown in figure 3. The area under the ROC curve did

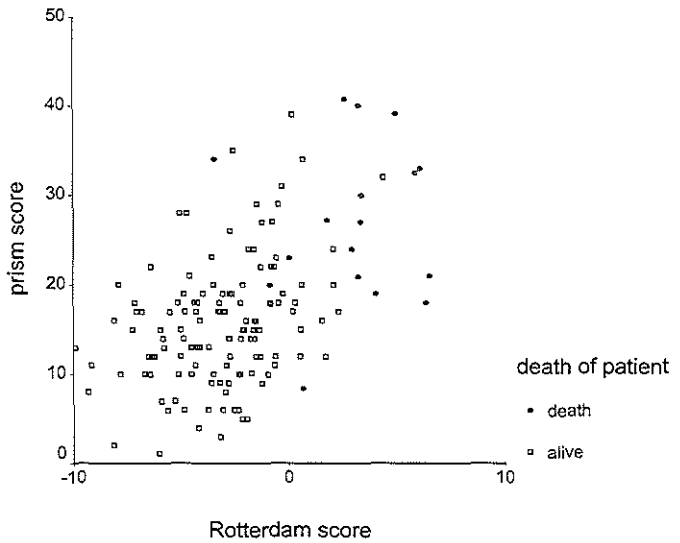

Figure 2 Relation between the PRISM Score and the Rotterdam Score with different marks for survivors and non-survivors

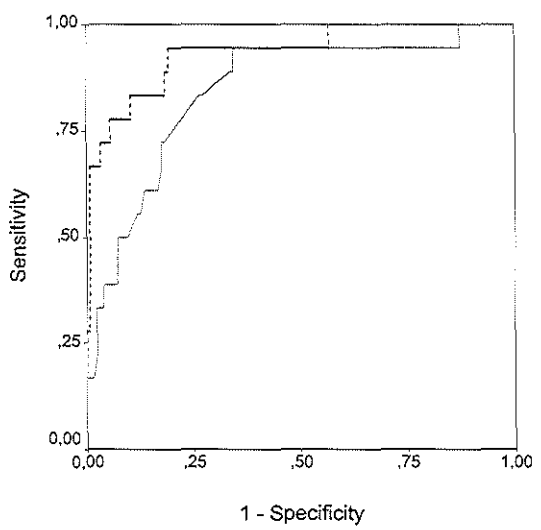

Figure 3 ROC-curve of the Rotterdam score (dotted line) and the PRISM score (solid line).

not significantly differ (p=0.14) between the Rotterdam score (auc=0.93) and the PRISM score (auc=0.84). The expected mortality for the total group based on the Rotterdam score was 23%. The observed mortality in the total group however was significantly lower (18/147=12%). The same difference was found for PRISM based expected mortality, which was 25%.

Discussion

Despite significant advances in our understanding of the disease process and improvement in rapid diagnostic and therapeutic measures, including intensive care, mortality due to meningococcal sepsis has changed little over the last few decades. To facilitate both clinical and laboratory research in this field, specific simple and effective prognostic score would stratify patient groups according to disease severity and risk of mortality.

Validation of the Rotterdam score with the present study population shows that this prognostic model predicts a significantly higher risk of mortality for non-survivors than survivors and provides a good discrimination between these two groups on admission to a pediatric ward. Figure 1 shows that most of the patients (57%) have a predicted mortality risk less than 10% (observed mortality 1%) and over 10% of the patients have a probability of mortality greater than 90% (observed mortality 86%). One third of the total patients are classified in the categories with a predicted mortality between 10% and 90%. The small number of patients included in these groups might explain the decreased fatality rate seen in the predicted mortality ranges from 55 to 85%, where it was difficult to discriminate between survivors and non-survivors by the Rotterdam score. In these middle ranges the Rotterdam score is not efficient. A larger validation set will be required for accurate assessment of individual risk categories for RS.

Scoring systems have been based on objective/laboratory and subjective/clinical variables. The RS includes four objective laboratory values and is therefore easy to reproduce and minimises inter-physician variation. This distinguishes the RS from the Glasgow Meningococcal Septicaemia Prognostic Score (GMSPS) which is based on clinical observations, which can be repeated during the hospital stay. The admission GMSPS score has a lower sensitivity for predicting mortality [8]. Furthermore, the calculation of coma scale (part of the GMSPS) is of questionable accuracy in post-ictal states and for patients requiring paralysis and/or sedation for ventilatory support. The score incorporates hypotension which is a late, pre-terminal sign of shock in children and may be absent in the acute stage. The laboratory parameters used in RS reflect disease pathology in meningococcal septic shock (MSS) and are not prone to observer bias. Dutch data was collected by means of questionnaires completed and returned by many specialists. The questionnaires were simple and easy to interpret in an attempt to minimize reporting bias and only laboratory data was required for calculation of the RS.

In this study, the Rotterdam score provided a better distinction in prediction between patients with fatal and non-fatal outcome than the PRISM score as illustrated in Figure 2 and Figure 3. The RS was specifically developed to determine risk of mortality in children with MSS, while the PRISM score was developed in predicting mortality in a general population of critically ill patients. PRISM score has shown to predict accurately the mortality rates for groups of patients with meningococcal disease [16, 17]. However, there are many methodological difficulties with the use of PRISM score in MD. The variables for PRISM score are difficult to collect and may not

be routinely recorded in every hospital. Moreover, there have been doubts about PRISM score overestimating risk of mortality in ICU patients [18]. One of the most significant difficulties in using PRISM for mortality predictions in MSS is that it incorporates the worst parameter in the first 24 hours after admission, with the minimum observation period being 8 hours [13]. Since, most experimental treatment strategies in MD are likely to be administered early in the disease, PRISM is less useful as a selection tool for clinical trials. RS is calculated from the worst parameters in the first 6 hours after admission. This makes it a valuable tool for stratification of groups early in the disease process. For the purposes of our study, we recorded the PRISM score using the worst parameter in the first 6 hours after admission to referring hospital. Our data showed a statistically significant difference in scores between survivors and non-survivors, using this time period for recording PRISM.

After selection based on inclusion criteria of MSS, 28 patients of group 1 were excluded from the validation set because they had either a venous or capillary base excess (BE). An arterially obtained BE is required for calculation of the Rotterdam score. In the presence of severe circulatory failure, comparison of non-arterial (capillary/venous) blood samples with arterial samples is not appropriate because of a wide difference in acid-base profiles [19, 20]. Although no significant difference was found between these two groups when a comparison was made between mean Rotterdam scores, the group of patients with a non-arterial BE was excluded. Calculation of the Rotterdam score in these cases will be possible when a correcting factor is found.

Case fatality rate for children with MSS in this study population is lower than reported in some other studies [7, 21, 22]. The observed mortality was also lower than the expected mortality for the whole group based on the PRISM score or the Rotterdam score. The UK patients formed the biggest part of the validationset. Serogroup C is much more frequent in the UK and the cause of disease in older children, corresponding with a lower mortality. Enrolment in the present validation study was based on bacteriological confirmed cases with Neisseria meningitidis. Pre-admission administration of antibiotics to patients with suspected meningococcal infection and the recognition of risks associated with obtained CSF in typical cases has decreased the likelihood of obtaining an isolate and culture confirmation. Diagnosis of meningococcal disease will be established by culture of the organism from the blood in less than two-third of cases, and meningococcal antigens may be detected in the CSF or blood in approximately 60% of culture-negative patients, depending on the serogroup of the causative organism [4]. This may result in under-reporting of culture negative cases of MD (especially in severely ill patients who die on their way to the hospital or just after admission) [23]. This may have resulted in failure to recruit some non-survivors with atypical clinical features and without bacteriological confirmation of diagnosis. However, PCR based DNA detection, recently available in reference laboratories and used in Group 2 patients in our study, has shown to be a very sensitive test for meningococcal detection [24]. This would have enhanced recruitment for cases in our study. The lower observed

mortality rate in the validation group as compared to the predicted mortality according to the Rotterdam score might possibly be explained by an improved awareness of the disease amongst both parents and physicians, resulting in early diagnosis, initial resuscitation and prompt referral and administration of intensive care where appropriate. The advances in intensive care and improved understanding of the pathogenesis of the disease may have lead to improvement of the management of children with MSS over this time period. The predicted mortality becomes more accurate by changing the leading constant. The updated formula of the Rotterdam score now reads: (RS) = −1.00 + (1.21 × serum potassium level) − (0.29 × base excess) − (0.024 × platelet count) − (3.75 × log serum CRP level).

In conclusion, this study has validated the Rotterdam score in international populations as well as in different centers as a prognostic score for children with MSS. The Rotterdam score is currently the only available scoring system using objective laboratory values, which is specific for children with meningococcal septic shock. The Rotterdam score shows a higher predictive value for death and survival than the PRISM score in children with MSS. A 6-hours PRISM can predict mortality in MSS patients with reasonable accuracy.

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Chapter 6

Administration of Protein C concentrate in children with severe meningococcal disease results in dose-related increases in serum protein C and activated protein C levels

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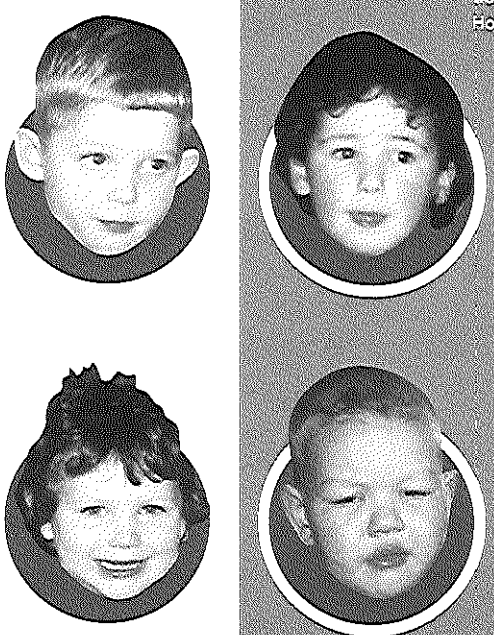

Summary

Background: Meningococcal septic shock has a high mortality and morbidity and is frequently complicated by the occurrence of severe disseminated intravascular coagulation. Decreased protein C levels in patients with meningococcal septic shock are associated with poor outcome and an increasing severity of thrombotic lesions. The aim of this study was to investigate the optimal dosing regime and the safety of protein C concentrate in children with meningococcal septic shock.

Methods: Forty children with meningococcal septic shock participated in this randomised, placebo-controlled, dose finding trial. All patients received standard therapy for septic shock including antibiotics, inotropic drugs, vasopressors and blood products. Within the first hours after admission in the ICU patients were treated with one of three dosages of protein C concentrate (50 U/kg, 100 U/kg, 150 U/kg) or with placebo (albumin 1%) for a maximum of 7 days. Clinical and laboratory data including serum levels of Protein C and activated protein C, were collected at various time points.

Findings: No adverse reactions related to the study medication were observed. All adverse events were consistent with complications of meningococcal septic shock. Nine of the 40 (23%) patients died. Five survivors needed amputation. There was no significant difference in the occurrence of amputations between treated and non-treated patients. AUCs of protein C and APC levels following the first gift of study medication correlated with the dosage of protein C concentrate administered. Activation of coagulation normalised significantly faster with increasing dosage of protein C concentrate, as reflected by levels of D-dimers after 12 hours of treatment.

Interpretation: Treatment with protein C is safe in children with meningococcal septic shock and leads to dose related increases of APC levels. There were no significant differences in mortality and amputations between the various treatment groups.

Introduction

Meningococcal infection is a world-wide occurring infectious disease with high morbidity and mortality. Meningococcal sepsis is associated with clinical and laboratory evidence of disseminated intravascular coagulation (DIC). In DIC, generation of large amounts of thrombin, combined with dysfunction of control mechanisms, may result in fibrin deposition and consequently tissue ischemia. The strong activation of coagulation results in depletion of hemostatic proteins. In meningococcal sepsis levels of one of these proteins, protein C, are particularly decreased [1]. A strong correlation between the decrease in protein C levels and the severity of thrombotic lesions and poor outcome has been demonstrated [1-8]. This correlation likely is causal because hereditary homozygous protein C deficiency [9, 10] leads to lethal neonatal thrombosis that can be corrected by protein C replacement [11-16].

Activated protein C is a natural anti-coagulant contributing to the homeostasis of the coagulation system. The protein C pathway is activated in the microcirculation where activated protein C (APC) is generated from protein C following the binding of thrombin to the endothelial receptor thrombomodulin [17]. This activation process is enhanced when protein C binds to the recently described endothelial cell protein C receptor (EPCR) [18]. APC and its cofactor protein S inactivate coagulation factors Va and VIIIa. Furthermore, APC promotes fibrinolysis by the inhibition of plasminogen activator inhibitor [19], and by the reduction of activation of the thrombin activatable fibrinolysis inhibitor (TAFI) [20]. In addition to its anticoagulant properties, APC has strong anti-inflammatory properties. Infusion of APC protects baboons from a lethal response to LD_{100} *Escherichia coli* and inhibition of protein C activation exacerbates the response to a sublethal challenge with numbers of *E. coli* [21, 22]. The role of the protein C pathway in the inflammatory response has not yet been fully elucidated. The mechanism for this modulation of the inflammatory response is probably related to the ability of APC to inhibit the production of proinflammatory cytokines by mononuclear phagocytes, presumably via its effect on recently identified receptors for protein C on monocytes or endothelial cells [23].

Administration of activated protein C improves outcome in meningococcal endotoxin shock in rabbits [24]. Initial clinical results in limited numbers of patients have suggested that protein C infusion may be beneficial in severe meningococcal disease and other forms of septic shock [25-30]. The aim of this study was to investigate the optimal dosing regime and the safety of protein C concentrate in children in an early stage of meningococcal septic shock. To this purpose we performed a randomised, placebo-controlled, dose finding study in forty children.

Patients and methods

Patients

Patients were recruited from two hospitals (Sophia Children's Hospital, Rotterdam, the Netherlands and from the Guy's Hospital, London, UK). The medical ethical committees of both hospitals approved the protocol. Patients were eligible to enter the study according to the criteria shown in Table 1. After obtaining written informed consent, patients were randomised to receive one of three different intravenous doses of protein C concentrate (Human Vapor Heated, Immuno; 50 U/kg, 100 U/kg or 150 U/kg) or placebo (Albumin Human 1%, Immuno). All patients received standard therapy for septic shock, including antibiotics, inotropic drugs, vasopressors and blood products including fresh frozen plasma (virus inactivated). Study medication was given as an intravenous bolus every 6 hours for the first three days followed by every 12 hours with a maximum of 7 days. In patients who recovered in less than 7 days, the study medication was discontinued earlier.

Inclusion criteria

- Age between 1 month and 18 years
- Presumed meningococcal disease
- Petechial rash and/or purpura fulminans
- *Either* persistent hypotension (on two consecutive measurements plus volume load in between) based on the systolic blood pressure according to age (1-12 months: <75; 1-5 years: <80; 6-12 years: <85; >12 years:<100)
or two or more features of poor end-organ perfusion:
  - unexplained metabolic acidosis defined as pH≤7.3, base deficit ≥8, or plasma lactate levels >2.0 mmol/l
  - arterial hypoxia defined as PO2 ≤75 mm Hg, a PO2/FiO2 ratio <250 or TcO2 saturation ≤ 96% in patients without overt pulmonary disease as the cause
  - acute renal failure defined as oliguria with urine output less than 0.5 ml/kg/hr for at least 1 hour despite acute fluid volume loading or despite evidence of adequate intravascular volume and without renal disease
  - sudden detoriation of baseline mental status not resulting from meningitis.

Exclusion criteria
- The diagnosis of septic shock was made ≥ 6 hours before entry in this study - High dose heparin therapy (>5 units/kg/hr) - Participation in another clinical trial - Females >13 years of age with a positive pregnancy test.

Table 1 Inclusion and exclusion criteria in 40 children with meningococcal septic shock

Measurements

Clinical and laboratory data were collected every 24 hours until the end of day 3, on day 7,and after 1 and 3 months. Serum samples were collected before infusion of study medication, ½, 1, 4 and 6 hours after the first infusion and every 24 hours until the end of the treatment period and analysed for protein C levels, activated protein C, cytokines and coagulation parameters. To measure severity of disease different scoring systems were calculated: the Pediatric Risk of Mortality (PRISM) score [31], Sepsis-related Organ Failure assessment (SOFA) score adapted for pediatric patients [32] and a DIC-score [1].

Protein C activity was measured using a commercially available amidolytic test (Immunochrom Protein C) on the coagulation Analyser STA (Hoffmann La Roche, Diagnostic, Switzerland). Activated protein C was assayed according to a modification of the enzyme capture assay (ECA) from Gruber et al., 1992 [33], where a specific chromogenic substrate was used to determine APC (Chromogenix, Sweden). The detection limit was 0.3 ng/ml. IL-6, IL-8, IL-10 and TNFα antigen serum levels were measured with commercial assays (CLB, Amsterdam, the Netherlands). The detection limit (lowest positive standard) was for IL-6: 10 pg/ml, for IL-8: 20 pg/ml, for IL-10: 50 pg/ml and for TNFα: 5 pg/ml. PAI-1 antigen levels were measured by an ELISA which was modified from the radio-immuno-assay described by de Boer et al. [34]. The intra- and interassay coefficient variation is <15%. D-dimers were measured by an Enzyme Immunoassay (Dade Behring). Thrombin-antithrombin III (TAT) complexes were performed by an ELISA as described by Boermeester et al. [35]. Plasmin-antiplasmin complexes (PAP) were performed by a radioimmunoassay as described by Levi et al. [36]

Outcomes

The major clinical outcome parameters in this study were death and amputations. The minor outcome parameters were decline in inflammatory responses (Il-6 and IL-8 levels), decline in parameters for activation of coagulation (D-dimers, TAT) and an increase in fibrinolysis (PAP, PAI-1 levels).

Statistics

The multigroup log-rank test was used for linear dose trend of mortality, amputations and skin-transplantations. Cox model was used to discriminate the variables to predict mortality. Spearman's rho correlations (r_s) were used to measure the correlation between the area under the curves of protein C and APC levels and dosage protein C given.

Spearman's rho correlations were also used to measure the correlations between APC levels and laboratory values on admission. Half-life was calculated according to the compartmental model of Lee et al (1990) using the protein C levels in the first 6 hours. Mixed model ANOVA (random coefficients model) was used to estimate the mean intercept and slope with time of all individuals. This model was used to measure and test differences in the course of several continuos outcome variables. The outcome variables considered (Protein C, APC, D-dimers, TAT

complexes, PAP complexes and cytokines) were analysed after ln-transformation because of their positive skewness. For D-dimers, TAT and PAP complexes, a precourse log-linear ("broken stick") model was assumed, allowing the slope with time in the first twelve hours to be different from the slope thereafter.

Results

Patient characteristics

Between July 1997 and February 2000, 38 children with meningococcal septic shock were entered by the Sophia Children's Hospital, Rotterdam, the Netherlands and 2 by the Guy's Hospital, London, UK. All parents gave written informed consent. The children (23 male/17 female) had a median age of 2.3 years (0.2-16.1). The forty patients were almost equally distributed in the four treatment groups. Treatment with study medication started with a median of 2.87 hours (1.25-10.25) after admission on the ICU. Baseline clinical and laboratory characteristics are summarised in Table 2. All patients received antibiotics, inotropic drugs and blood product transfusions. In 31 patients the diagnosis of meningococcal infection was confirmed by bacterial cultures. Plasma kinetics of protein C and APC following protein C infusion were only performed in patients in the Sophia Children's Hospital. The baseline of special coagulation parameters and of serum levels of cytokines in the 38 Dutch children are depicted in Table 3. In 35 patients on admission, levels of TAT complexes were increased (median 35 ng/ml, range 4 -1573) reflecting the presence of DIC. Protein C levels were decreased in all patients with a median level of 0.22 U/ml (0.03-0.63). Levels of activated protein C were increased (median 2.76 ng/ml, range 0.07 – 41.81) in relation to the levels in the control samples on month 3 (0.20 – 0.75 ng/ml) of the surviving patients.

Safety

During infusion of study medication no acute adverse reactions were observed. No apparent adverse event could be related to the protein C study medication. All adverse events were consistent with complications of meningococcal septic shock. Serious adverse events included death, amputation, skin transplantation and gastrointestinal haemorrhage.

	Treatment groups				
General	***Placebo*** ***N=10***	***50 U/kg*** ***N=11***	***100 U/kg*** ***N=9***	***150 U/kg*** ***N=10***	
Age (yr)	1.3 (0.5-11.8)	4.4 (0.8-10.6)	2.7 (0.7-15.5)	1.9 (0.3-16.1)	
Weight (kg)	12.0 (7.5-40)	18.0 (5.5-60)	18.0 (9.0-60)	12.2 (6.0-70)	
Sex	4 female/ 6 male	4 female/ 7 male	6 female/ 3 male	3 female/ 7 male	
PRISM score	26.5 (12-33)	20 (9-30)	18 (10-34)	25 (4-43)	
SOFA score	12 (6-16)	10 (4-14)	10 (4-17)	13 (6-19)	
Positive culture with *Neisseria meningitidis*	10	8	8	7	
DIC present/absent	6/4	4/7	7/2	6/4	
Laboratory meassurements					*normal ranges*
C-reactive protein (mg/l)	74.5 (24-118)	80.0 (7-144)	74.0 (29-132)	60.0 (6-259)	0-10
WBC (x 10^9/l)	7.0 (1.5-33)	6.7 (1.7-19.2)	12.8 (1.3-21.7)	6,9 (2,2-26)	0-12 months 5-21 >12 months 4-10
Platelets (x 10^9/l)	96 (16-163)	102 (38-163)	84 (21-151)	54 (13-272)	1-12 months 200-473 >12 months 150-450
Fibrinogen (g/l)	2.3 (0.4-2.9)	3.0 (0.4-4.5)	2.1 (0.4-6.0)	1.7(0.4-4.4)	2-4
PT (%)	35.5 (13.6-75)	24 (4.8-47)	29.2 (17.8-60)	22.9 (13.5-44)	70-130
aPTT (sec)	88.0 (44-200)	58.0(1-200)	76.0(41-118)	68.5(31-200)	28-40

Table 2 Baseline clinical and laboratory characteristics in 40 children with meningococcal septic shock

Abbreviations: PRISM= the Pediatric Risk of Mortality score; SOFA= Sepsis-related Organ Failure assessment score; DIC score = disseminated intravascular coagulation score; WBC= white blood cell counts; PT= prothrombin time; aPTT= activated partial thromboplastin time

All data are presented as median (range)

	Treatment groups				
Laboratory measurements Median (range)	***Placebo N=10***	***50 U/kg N=9***	***100 U/kg N=9***	***150 U/kg N=10***	*Normal range*
Protein C (U/ml)	0.15 (0.03-0.63)	0.13 (0.06-0.34)	0.21(0.13-0.44)	0.18 (0.05-0.35)	0.7-1.2
APC (ng/ml)	2.3 (0.89-17.37)	2.6 (0.53-14.52)	3.77 (0.35-21.48)	7.87 (0.7-41.81)	0.20-0.75
TATc (ng/ml)	26 (4-320)	15 (4-538)	22 (8.1-138)	815 (4-1573)	< 4
PAPc (nmol/l)	20 (4.5-104)	22 (7.4 -215)	43 (8.1-58.0)	25 (6.7-64)	<7
D-dimer (*n*g/l)	1530 (216-33101)	1951 (237-44897)	2880 (317-23892)	3943 (421-13084)	<78 µg/ml
IL-6 (pg/ml)	$12.7\ 10^4$ ($580\text{-}118\ 10^4$)	$4.2\ 10^4$ ($650\text{-}196\ 10^4$)	$6.1\ 10^4$ ($205\text{-}123\ 10^4$)	$50.4\ 10^4$ ($0.75\ 10^4$- $293\ 10^4$)	<10
IL-8 (pg/ml)	$2.20\ 10^4$ ($311\text{-}29.8\ 10^4$)	$0.90\ 10^4$ ($450\text{-}46.0\ 10^4$)	$2.04\ 10^4$ ($140\text{-}48.1\ 10^4$)	$13.5\ 10^4$ ($0.25\ 10^4$- $151\ 10^4$)	<20
TNF-*α* (pg/ml)	8.1 (5-26)	5.0 (5.0-97)	7.8 (5-22)	12 (5-527)	<5
IL-10 (pg/ml)	7034 (679-18094)	8562 (581-20406)	5850 (180-409292)	8635 (98-67627)	<50
PAI-1 (ng/ml)	4693 (528-13539)	4286 (572-14841)	4224 (1804-12263)	7003 (1505-30892)	4-40

Table 3 Baseline coagulation and inflammatory parameters in 38 patients with meningococcal septic shock

APC= activated protein C; TATc= thrombin-antithrombin III complexes; PAPc= plasmin-antiplasmin complexes ; IL= interleukin; TNF-α= tumor necrosis factor α; PAI= plasminogen activator inhibitor.

Protein C and APC kinetics

Kinetics of protein C and APC levels in the first 6 hours after the first gift of study medication are summarised in Figure 1 and Figure 2. Peak levels of protein C were reached after 30 minutes. The median (range) areas under the curve (AUC) for protein C levels were 1.25 (0.84-4.12) in the placebo group, 3.20 (1.74-4.63) in the 50 U/kg protein C treatment group, 6.32 (1.74-4.63) in the 100 U/kg protein C treatment group and 7.10 (4.35-13.68) in the 150 U/kg protein C treatment group. The AUCs correlated significantly with the dosage of protein C given (r_s=0.85, p<0.01). The median half live of clearance from plasma of protein C ranged from 6.8 hours after administration of 50 U/kg to 8.0 hours after 100 U/kg and 150 U/kg.

Peak levels of APC were reached after 30 minutes. The median (range) AUCs of APC were 7.7 (3.3-106.2) in the placebo group, 16.7 (3.2-120.1) in the 50 U/kg treatment group, 22.9 (5.9-133.0) in the 100 U/kg treatment group and 54.0 (6.6-454.8) in the 150 U/kg treatment group. The AUCs of APC levels correlated significantly with the dosage of protein C (r_s=0.44, p<0.01).

After 24 hours steady state levels of both protein C as well as APC were obtained (Figure 3 and 4). Administration of 50 U/kg protein C concentrate four times daily resulted in a normalisation of protein C levels. The dosages of 100 U/kg and 150 U/kg of protein C concentrate resulted in serum levels which were 2 times and 2.5 times higher than normal. According to mixed model ANOVA analysis, the course of protein C levels was significantly different between the treatment groups (p<0.01) until the end of the treatment period. APC levels decreased in time over the treatment period. The decreases were significantly less in patients receiving higher dosage of protein C (p<0.01). The course of APC levels was not significantly different between the 100 U/kg treatment group and the 150 U/kg treatment group.

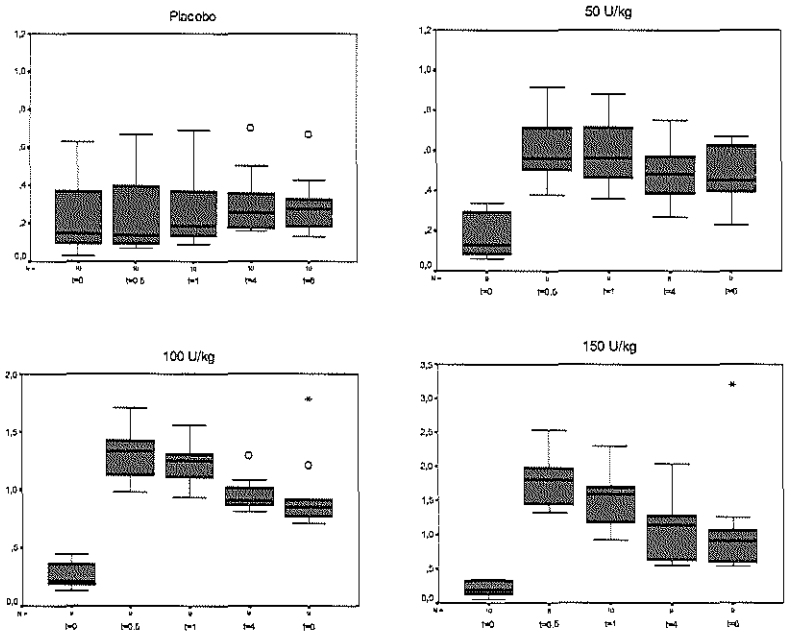

Figure 1 Protein C levels in the first 6 hours after studymedication

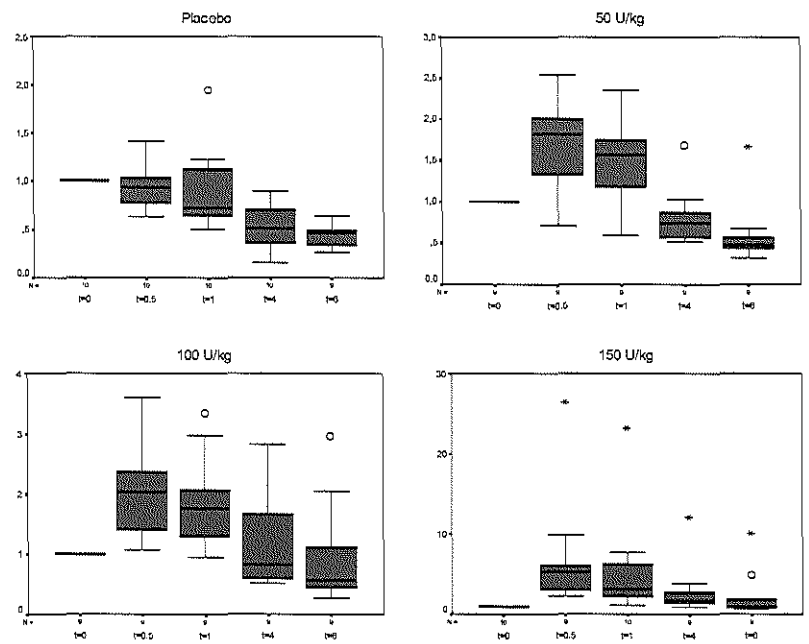

Figure 2 relative APC levels in the first 6 hours after studymedication

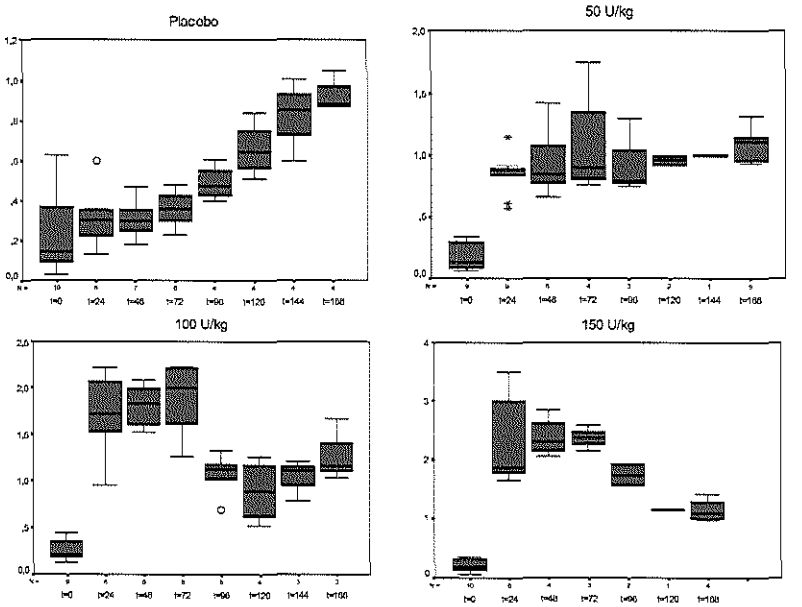

Figure 3 Protein C levels during the first 7 days after admission in the ICU

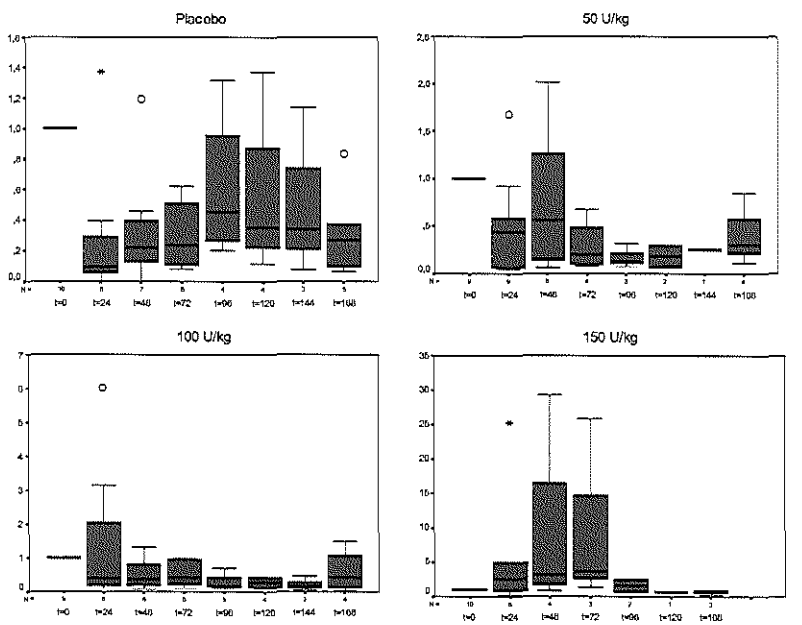

Figure 4 Relative APC levels during the first 7 days after admission in the ICU

Clinical outcome

Clinical outcome of the forty children is summarised in Table 4. Nine of the forty patients died (23%). The median (range) survival time on the ICU in these nine patients was 12.3 hours (7.5-42.2). Five patients died in the high dose group (150U protein C concentrate per kg). The mortality rates were not significantly different between the four treatment groups. The median (range) stay of the survivors on the ICU was 5 days (2-46). Five patients survived with amputations varying from a spontaneously-amputated toe to an arm amputation above the elbow. The incidence of amputations was not significantly different between the treatment groups. On admission, mortality was predicted significantly by APC levels (hazard ratio 1.36, p=0.01), PRISM score (HR 0.30, p=0.01) and age (HR 1.34, p=0.01). Levels of APC on admission correlated significantly with serum levels of D-dimers (r_s=0.53, p<0.01), IL-6 (r_s =0.87, p<0.01), IL-8 (r_s =0.86, p<0.01), PAI-1 (r_s =0.84, p<0.01), TAT complexes (r_s =0.75, p<0.01), PAP complexes (r_s =0.57, p<0.01), TAT/PAP ratio (r_s =0.59, p<0.01), SOFA score (r_s =0.57, p<0.01) and PRISM score (r_s =0.54, p<0.01).

	Treatment groups			
General	***Placebo*** ***N=10***	***50 U/kg*** ***N=11***	***100 U/kg*** ***N=9***	***150 U/kg*** ***N=10***
Death	2	1	1	5
Treatment hours	78 (6-180)	43 (18-168)	120 (6-180)	33 (6-108)
Ventilatory support	8 (80%)	5 (45%)	5 (56%)	8 (80%)
ICU days (survivors only)	6.5 (3-46)	5.0 (2-19)	8.0 (3-16)	5.0 (2-7)
Amputations (total)	2	2	1	0
Mild *	1	1	0	
Moderate	1	0	1	
Severe	0	1	0	
Skin transplantations	2	1	2	0
Blood products received				
Fresh frozen plasma ml/kg	6.08	8.62	2.32	3.00
ESDEP ml/kg	122.82	93.26	62.60	171.21

Table 4 Clinical outcome parameters in 40 children with meningococcal septic shock

* Mild=loss of digit distal to distal interphalangeal joint (DIP); moderate=amputation proximal to DIP but excluding below knee or elbow; severe=above knee or elbow or more extreme amputations

Normalisation of coagulation and cytokines

According to the mixed model ANOVA analysis, levels of D-dimers showed a faster normalisation in time with increasing dosage of protein C concentrate (p<0.01). No significant differences were seen in the time course of TAT complexes, PAP complexes or cytokine levels including PAI-1.

Discussion

This first randomised, placebo controlled, dose finding trial with protein C treatment shows that administration of protein C concentrate is safe in children with meningococcal septic shock. Treatment with protein C in these ill patients leads to increased plasma levels of protein C and to an enhanced formation of APC. However, we did not observe significant differences in mortality or amputation rates between the various treatment groups. The highest mortality but also the lowest number of amputations were present in the treatment group with the highest dosage of protein C. Levels of D-dimers dose-dependently decreased upon protein C treatment.

Disseminated intravascular coagulation is part of the abundant systemic inflammatory response ensuing in meningococcal sepsis and already present in most patients on admission of the ICU. Organ dysfunction due to microthrombosis is supposed to be one of the major causes of morbidity and mortality [2]. In patients with meningococcal sepsis levels of protein C indeed are reduced, and to a greater extent than the other anticoagulants [37]. Hence, these patients may benefit from administration of protein C concentrate to correct the dysbalance between coagulation and fibrinolysis.

Expression of thrombomodulin and the endothelial cell protein C receptor is reduced in vessels in skin biopsies of patients with meningococcal sepsis, which suggest an impairment in protein C activation [38] and hence a less potent anticoagulation. Our study indicates the presence of a positive correlation between the APC levels and severity of disease reflected by the PRISM score and a dose-dependent increase of APC during the first hours after protein C infusion. Thus, in spite of the potential deficiency of thrombomodulin and protein C receptor, low APC levels are most likely due to decreased protein C levels rather than to impaired activation of this anticoagulant. APC levels on admission were increased in most (33 of the 38) patients and hence decreased protein C levels in these patients resulted from increased activation. Moreover, APC levels had a hazard ratio of 1.36 in the prediction of mortality. These levels also correlated significantly with severity of disease (PRISM score, SOFA score, cytokines), with activation of coagulation (TAT) and with the dysbalance between coagulation and fibrinolysis (TAT/PAP ratio). Whether this increase of APC is sufficient to restore the impaired balance between the coagulant and the anticoagulant system, is not known. Probably, increased APC levels are not detrimental for patients as was reputed after treatment of adult septic patients with recombinant APC (rAPC) is reported to be safe.

Protein C treatment had no positive effects on mortality in this study. The mortality rate was not significantly different between the treatment groups, although the highest mortality occurred in the group which was treated with the highest dose of protein C. However, in spite of randomisation, the children in the highest dose group had more severe meningococcal disease than the other groups (higher PRISM score, higher cytokine levels, younger age). Since this study intended to be a dose finding, the groups of patients studied are rather small. No significant

difference was found in the amputation rate between the different treatment groups.

A significantly more rapid decrease of D-dimers was found after the first 12 hours with increasing doses of protein C. This may reflect the inhibition of the activated coagulation by APC, which may in this turn result in a decreased rate of complications such as amputations and skin transplantations. APC did not significantly influence the fibrinolysis system by means of inhibition of plasminogen activator inhibitor-1 (PAI-1). Also, we did not observe an inhibition of the inflammatory process, since no significant changes were found in the decrease of the cytokine levels in response to treatment with protein C. This may be explained by the extremely high levels of Il-6, Il-8 and also of PAI-1 in patients with meningococcal septic shock and their rapid decrease. In this study only a late effect (after 12 hours) of protein C treatment was demonstrated, when the cytokine levels already were decreasing and 6 out of 9 patients were already death.

During the last decade several innovative immunomodulatory approaches have been used to treat patients with meningococcal disease. Interventions to neutralise endotoxin may be effective given in a very early phase of the disease. The results of the multicenter trial with a human monoclonal antibody to endotoxin (HA-1A) were disappointing [39]. A phase III trial with recombinant bactericidal/permeability–increasing protein (rBPI) in children with meningococcal sepsis showed no reduction in mortality but a trend towards improved outcome [40]. Recent studies seeking to modulate the host responses to endotoxin have focused on the role of natural anticoagulants with the greatest attention to antithrombin III, tissue factor pathway inhibitor (TFPI) and protein C. Administration of recombinant TFPI In human volunteers resulted in anticoagulant effects without a concurrent effect on fibrinolysis or cytokine release [41]. Administration of antithrombin III may reduce the duration of DIC in adult sepsis patients, without a reduction in mortality. [42]. In meningococcal sepsis levels of protein C are particularly decreased [1]. Substitution of this hemostatic protein may be therefore a treatment of choice in patients with meningococcal sepsis. The question remains, whether recombinant APC is more effective than concentrated protein C. Recombinant APC when given intravenously, is directly present in the circulation at high levels of activated form. On the other hand high circulating levels of protein C can be activated when needed till high levels as shown in this study. Additional large randomised trials are needed to study whether protein C is really effective in the reduction of morbidity or mortality of children with severe meningococcal diseases.

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Chapter 7

Vaccination against infections by serogroup B meningococci

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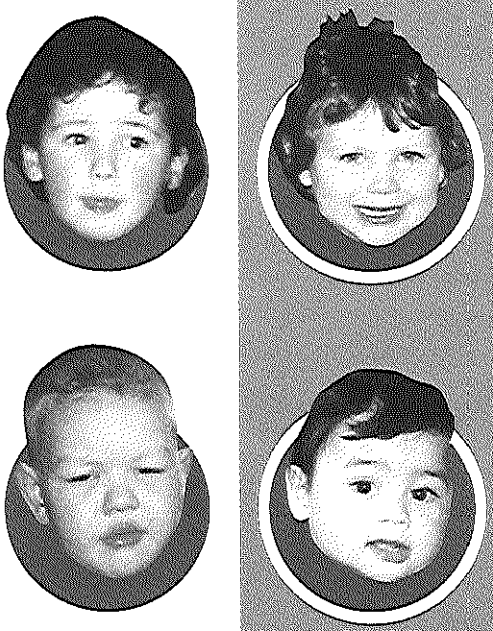

Abstract

Meningococcal disease is one of the leading infectious cause of death in early childhood in industrialised countries. *Neisseria meningitidis* of serogroup B remains the causing organism in these areas. Till now, polysaccharide vaccines and protein-polysaccharide conjugated vaccines are available against serogroup A, C, W125 and Y. Against serogroup B, protein based vaccines have been studied with intermediate efficacy, but no protection was present in young children. Based on the capsular structure of the meningococcal, promising vaccine candidates are under investigation. This review provides an overview of possible future vaccine candidates and discusses recent advances in the development of an effective vaccine against serogroup B meningococci.

Introduction

Neisseria meningitidis is far the most common cause of bacterial meningitis world-wide. Infection may develop within days after colonisation of *Neisseria meningitidis* of the nasopharynx. The predominant clinical expression of disease is either meningitis or sepsis. A subset of patients with sepsis develops septic shock leading to death within a few hours after the initiation of disease symptoms. The overall mortality of meningococcal disease is still 10% even when antibiotics are rapidly administrated and when tertiary care is available [1,2]. The disease affects mostly healthy young children [3-5]. The incidence of meningococcal disease varies between 1-3/100,000 inhabitants/year in industrialised countries (Table1) and 10-25/100,000 in third world countries [6-10]. During epidemic peaks in the 'meningitis belt' of Sub-Saharan countries, the disease incidence may reach 1000/100,000 inhabitants/year [11,12]. Most epidemic and endemic cases of meningococcal disease are caused by strains of a limited number of genetically well defined clonal groups [13-16]. Serogroups A, B and C meningococci (classified on the basis of different capsular polysaccharides) are responsible for 90% of the cases world-wide. Children and adults frequently carry and transmit meningococci in their nasopharynx. However, most of the meningococci (e.g. *Neisseria lactamica* or other non encapsulated Neisseriae) isolated from the nasopharynx of carriers have a limited pathogenic potential [17]. During non-epidemic periods, when disease is rare, 3 to 39% of the population may be colonised by the meningococcus [18-23]. The prevalence of meningococcal carriage is strongly associated with age. Peak rates are observed among people aged 15-30 year [24,25]. Meningococcal carriage may result in the induction of systemic protective antibody responses [26]. Natural immunisation probably occurs also through unrelated but serologically cross-reactive bacteria (e.g. *Escherichia coli*). From the

High Incidence (>3.08)		Medium Incidence (1.08-3.08)		Low Incidence (<1.08)	
Iceland	7.79	Denmark	2.79	Greece	1.04
Scotland	5.86	Belgium	2.35	Latvia*	0.91
England & Wales	3.82	Norway	2.28	Israel	0.81
Netherlands	3.18	Slovak Republic	1.94	France	0.60
		Lithuania**	1.81	Germany	0.44
		Spain	1.63	Estonia	0.38
		Russia, Moscow*	1.39	Slovenia*	0.20
		Austria	1.21		
		Czech Republic	1.10		

Table 1 Countries and incidence rates of meningococcal disease for the 1997/1998 epidemiological year (Data from the European Meningitis Surveillance Group: N.Noah, Department of Public Health and Epidemiology, London)

Incidence rates per 100.000 inhabitants

* data only available for three quarters ** data only available for two quarters

age of 2 years onwards antibody concentrations increase. Each year 5% of all children develop serum bactericidal activity against *N. meningitidis* of serogroups A, B and C. Specific antibodies and the complement system play a key role in the host defence against *Neisseria meningitidis*. Protection has been associated with the presence of bactericidal antibodies. Various lines of evidence highlight the importance of humoral bactericidal activity in the host defence against *Neisseria meningitidis*. The presence of bactericidal antibodies correlates inversely with the incidence of meningococcal disease [27]. Assays quantifying serum bactericidal activity have become the best available method to determine the protective potential of vaccine candidates. In addition, several reports indicate that opsonic antibodies may be important for protection [28-30]. The meningococcal serogroups differ in their susceptibility to serum bactericidal activity or phagocytosis. Serogroup B meningococci are relatively resistant to serum bactericidal activity and more susceptible to killing by serum opsonins and polymorphonuclear leukocytes [29]. In this respect, the whole blood assay, which assesses the bactericidal activity of whole blood, seems to be a sensitive test of immune response after infection and vaccination [31,32].

Attempts to prevent meningococcal disease started as early as in 1907-1912 with whole cell and autolysate vaccines [33]. After the good results with pneumococcal polysaccharides in 1945 [34], the same technology was used to prepare meningococcal capsular polysaccharide vaccines. Currently, polysaccharide vaccines are available against meningococci of serogroup A, C and/or W135 and Y. Vaccines consist of purified capsular polysaccharides of one to four of these serogroups. These polysaccharide vaccines can induce protective immunity against the respective serogroups. However, these antigens elicit antibody responses with no memory function [35,36], with the possible exception of serogroup A [37]. They are therefore called T-cell independent antigens. Unfortunately, these vaccines offer little protection in children under the age of 2 years [36,38,39]. To improve the immunological memory and protection in young infants, conjugate polysaccharide vaccines have recently been developed in which polysaccharides are covalently linked to a protein [40-43]. This strategy has not been successful to prevent infections by group B meningococci. The capsular polysaccharide (CPS) of serogroup B is poorly immunogenic [44,45], because the homopolymer of $\alpha(2\rightarrow8)$ N-acetylneuraminic acid is also present in glycoproteins of most human brain tissues during development and in lower amounts in adult tissues [46-48]. The immune response against this polysaccharide is weak. Since the early 1980s, non-capsular antigens, especially the outer membrane proteins (OMPs) have been studied as alternative vaccine-candidates. This review provides an overview of possible future vaccine candidates and discusses recent advances in the development of an effective vaccine against serogroup B meningococci.

Structures of the cell wall and relevant components as vaccine candidates

Neisseria meningitidis is a diplococcus with a cell envelope characteristic for gram-negative bacteria. The meningococcal cell surface is exposed to the human host. Four different groups of antigenic structures can be distinguished on meningococci: surface appendages known as pili, capsular polysaccharides (CPS), outer membrane proteins (OMPs), and lipopolysaccharides (LPS; located in the outer membrane). A characteristic of meningococci is strong 'blebbing', the shedding of outer membrane vesicles. Such blebs contain lipids, LPS, OMPs and CPS. The major components of the meningococcal cell surface are depicted in figure 1 [49]. Anti-CPS antibodies and anti-OMP antibodies are in general bactericidal and facilitate phagocytosis [27,50-52]. The role of anti-meningococcal LPS antibodies remains unclear, although epitopes are present on meningococcal LPS eliciting bactericidal antibodies [53]. Vaccine candidates are summarised in Table 2.

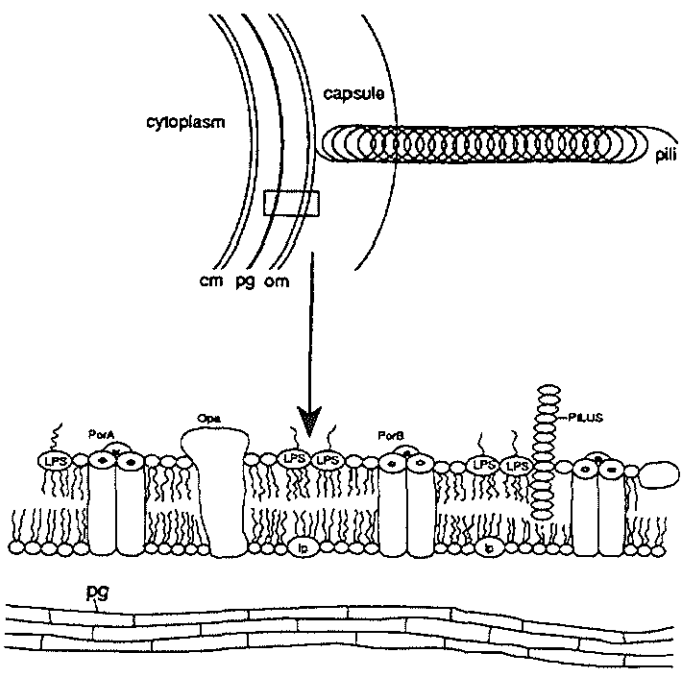

Figure 1 Structure of the meningococcal cell wall
Pili connect in the outer membrane. Capsule with the capsular polysaccharides. The outer membrane in detail with LPS and the major outer membrane proteins (PorA, PorB and Opa).
Cm = cytoplasm membrane, pg = peptidoglycan layer; om = outer membrane, LPS = lipopolysaccharides.
(From: Poolman [49])

Structure	references
Pili	[54]
Capsular polysaccharides	[55 - 57]
Outer membrane proteins	
Major OMPs	
Class 1 (PorA)	[58]
Class 2/3 (PorB)	[59]
Class 4 (Rmp)	[60, 61]
Class 5 (Opa, Opc)	[62, 63]
Iron regulated proteins	
Transferrin binding proteins (TbpA, TbpB)	[64, 65]
Lactoferrin binding proteins (Lbps)	[66, 67]
Iron binding protein (Fbp)	[68]
Haemoglobin-haptoglobin utilising protein (Hpu)	[69]
RTX cytotoxin-related proteins (FrpA, FrpB, FrpC)	[70, 71]
Neisseria surface protein A (NspA)	[72, 73]
Lipopolysaccharides	[76, 77]
Chiron Genome Analysis	[151 - 153]
Newly identified, highly conserved, surface proteins	
GNA33	
GNA1162	
GNA1220	
GNA1946	
GNA2001	

Table 2 Meningococcal vaccine components

Pili

Pili are filamentous projections from the meningococcal cell surface. Meningococci express two different classes of pili (class I and class II). Pili are involved in adhesion and play a role in acquisition of heterologous DNA from the environment. They show a high degree of antigenic variability and are thus not useful vaccine candidates [54].

Serogroup B capsular polysaccharides

Attempts are ongoing to produce a capsular polysaccharide based serogroup B vaccine, despite the complicating issue of possible induction of human autoimmune responses. It has been proposed that conformational determinants on B CPS, as presented on intact meningococci, provoke the development of antibodies that do not cross-react with the linear $\alpha(2\rightarrow8)$-linked determinants. The conformational structure is therefore seen to be more important in the generation of protective immunity than the primary polysaccharide structure [55]. Various ways of stabilising the molecule in order to present an appropriate conformation were therefore investigated. Another approach to generate T-cell dependent protective IgG responses has involved attempts to modify the structure of B CPS itself prior to conjugation, by replacing the N-acetyl groups of the sialic acid with N-proprionyl groups [56,57].

Outer membrane proteins

The best characterised outer membrane proteins are the so called major outer membrane proteins (mOMPs). These OMPs are expressed at high levels and are divided in five different classes (1 to 5) proteins. The class 1 protein (the serosubtype antigen [58]) and the class 2 and 3 proteins (two alleles of the serotype antigen [59]) are porins and have been studied widely. Meningococcal strains are sub-divided into more than 20 serotypes and more than 30 serosubtypes on the basis of the immunological hypervariability. Despite this antigenic variation, these antigens are still considered attractive candidate vaccine antigens. Class 4 OMP (or RmpM) can bind with specific antibodies which inhibit the bactericidal activity of class 1, class 2 and 3 OMP specific antibodies [60]. It has therefore been suggested that class 4 OMP may adversely affect the bactericidal activity of meningococcal vaccines containing outer membrane proteins [60]. However, the study of Rosenqvist et al. gave no evidence that vaccination with a meningococcal outer membrane vesicle vaccine containing the class 4 OMP induces blocking antibodies [61]. Class 5 OMPs, Opa and Opc are important in bacterial adhesion to and invasion of host cells [62]. Opa and Opc have genetic features resulting in high variability of Opa and Opc expression by meningococci [63].
Among the environmentally regulated proteins, iron-regulated proteins (FeRPs) are also considered as possible vaccine candidates. Meningococci have a number of proteins, which are expressed only under iron-depleted conditions. These FeRPs include transferrin-binding proteins (Tbps) [64,65], lactoferrin binding protein (Lbps) [66,67], iron binding protein (Fbp) [68], a haemaglobin-haptoglobin utilising protein (Hpu) [69], two RTX cytotoxin-related proteins (FrpA and FrpC) and a 70 kDa (FrpB) protein [70,71]. The transferrin binding proteins, TbpA and TbpB have been studied as vaccine candidates. TbpA and TbpB are major components of meningococcal transferrin receptors. Antibodies against Tbps are bactericidal and may interfere with the chelation and uptake of iron from transferrin and thereby inhibit the survival and growth of the organism in vivo. TbpA and TbpB are surface-exposed proteins and seem to have characteristics of broadly cross-reactive vaccine candidates. To date, no one has reported the occurrence of natural meningococcal mutants lacking Tbps.

Neisseria surface protein A (NspA) is a highly conserved membrane protein that is reported to elicit protective antibody responses against Neisseria meningitidis serogroups A, B and C in mice [72,73]. Although, not all meningococcal strains contain the NspA surface epitope [74]. Given these strain differences in NspA surface accessibility, an rNspA-based meningococcal B vaccine may have to be supplemented by additional antigens.

Lipopolysaccharides

Meningococcal LPS (or endotoxin) plays an important role in the pathophysiology of meningococcal disease. LPS remains a complex structure with only minor differences in primary oligosaccharide structures, but with distinctly different immunological and immunochemical behaviours (Figure 2). The immunotypes in the phenotypic classification system are based on

differences in the oligosaccharide structure of the meningococcal LPS. Most meningococcal strains express more than one immunotype-specific epitope on their LPS.

Meningococcal LPS immunotypes have revealed a conserved inner core structure. A short chain LPS may increase exposure of the LPS inner core [75]. The toxicity of the lipid A part of meningococcal LPS prevents the use in combination with OMV vaccines, since high levels of LPS per vaccine dose may lead to undesirable side effects. The similarity of the oligosaccharide chain to host antigens makes the use of native LPS in vaccine formulations undesirable [76,77]. Detoxified LPS can be conjugated to an OMP based vaccine to induce better and broader protection against meningococcal infections. This kind of vaccine is potentially capable of inducing neutralising antibodies against endotoxin.

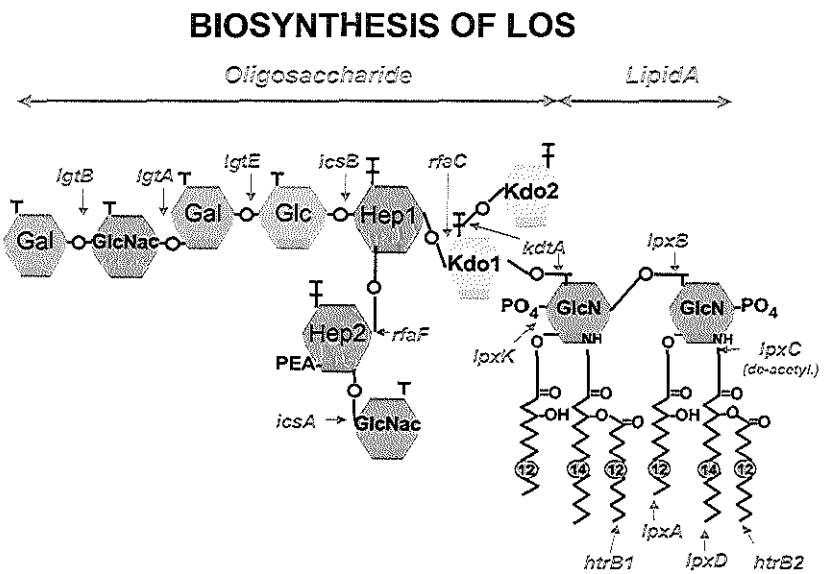

Figure 2 Structure of lipo-oligosaccharide.
(courtesy to Peter van der Ley and Wilma Witkamp, RIVM, the Netherlands)

Serogroup B capsular polysaccharide vaccines

Because the group B meningococcal polysaccharide is the best conserved antigenic structure on serogroup B meningococci, a polysaccharide-based vaccine would be the vaccine of choice, provided one could overcome its poor immunogenicity and prevent possible unwanted autoimmune effects. Conjugation of serogroup B CPS to protein carriers to form T-dependent antigens did lead to enhanced polysaccharide-specific antibody levels in animals and human volunteers. These antibody levels were generally low. Also there was no serum bactericidal activity, although prevention of disease was demonstrated when mice were challenged with serogroup B meningococci [78-83]. Under appropriate conditions, serogroup B CPS antibodies are bactericidal in vitro in the presence of homologous complement and to a higher extent in the presence of rabbit complement [84-87]. The bactericidal activity of antibodies elicited by various serogroup B PS conjugate vaccines appeared to be highly diverse in rhesus monkeys [88]. Covalent coupling of the serogroup B PS to meningococcal OMV enhanced the immunogenicity of both components in those nonhuman primates [88].

To modify immune tolerance and to increase immunogenicity, the serogroup B PS was chemically modified at the C-5 position wherein *N*-acetyl groups are replaced with *N*-propionyl groups. *N*-propionylated group B polysaccharide conjugated to the tetanus toxoid (NPrGBMP-TT) induced bactericidal antibodies in mice and non-human primates [56,87]. The antibodies were bactericidal *in vitro* against homologous group B strains of several different serotypes [57,87]. In vivo, these antibodies protected mice against bacteremia with group B meningococci [89]. Devi et al. showed that serogroup B CPS or *N*-propionylated serogroup B CPS or *E.coli* K92 capsular polysaccharide conjugated to various carrier proteins are immunogenic in nonhuman primates. Geometric mean titres against serogroup B PS increased minimally ninefold, and showed T-cell dependent properties [90]. The combination of the chemically modified serogroup B polysaccharide (NPr-GBMP) and a recombinant class 3 outer membrane protein (rPorB) as carrier protein elicited high PS-specific bactericidal titres in mice, adult and infant rhesus monkeys [87,91]. NPrGBMP-TT produced two distinct populations of antibodies, only one of which was associated with the high molecular weight serogroup B meningococcal polysaccharide but is not bactericidal, while the other was associated with a unique bactericidal epitope on the surface of serogroup B meningococci containing all bactericidal activity [57]. These observations suggest the presence of distinct epitopes in the capsular polysaccharide, some of which could be used to construct a specific meningococcal B vaccine.

It is possible to overcome the poor immunogenicity of serogroup B CPS as can be illustrated by the results of different studies. However, the presence of similar epitopes in humans still raises safety questions. The two studies with human volunteers have demonstrated no serious side effects or clinically significant haematological or biochemical abnormalities due to PS specific antibodies after immunisation with serogroup B PS antibodies complexed to OMV vaccines

[83,92]. Also, no adverse effects have been observed in studies with mice and nonhuman primates [80,87,90]. Injection of α2-8 polysialic acid antibodies into pregnant rats did not result in binding to foetal brain [93]. In addition, naturally occurring anti-B PS antibodies are present in 80 to 90% of healthy adults [92] and in mothers who have given birth to healthy full-term babies [82]. Full-term newborns also have polysialic acid IgG levels equal to or higher than those of their mothers, suggesting the absence of binding of these antibodies to foetal tissue in utero [82]. On the other hand, a careful evaluation of cross-reactivity is advised prior to actual vaccination trials. Not only is the structure of serogroup B CPS similar to glycoproteins of human brain tissues [46-48], but also auto-immune antibodies reacting in vitro with human embryonic neural cell adhesion molecules have been detected in sera of meningococcal B meningitis patients [94]. Some antibodies to the *N*-propionylated meningococcal B polysaccharide conjugate also bind to human embryonal brain polysialosyl glycopeptides, raising further concerns about the safety of this vaccine in humans [95].

Vaccines based on outer membrane proteins

Major outer membrane proteins

The idea of using OMPs in a meningococcal vaccine arose from observations that the presence of bactericidal antibodies correlates with protection. After meningococcal B infection, bactericidal antibodies are directed against non-capsular surface antigens. Convalescent sera of meningococcal patients showed a high rate of seroconversion to PorB protein (class 2/3 OMP), PorA protein (class 1 OMP) and class 5 OMP [51,96]. Antibodies to class 1 OMP and LPS are the most effective, whereas antibodies to class 2/3 OMPs are only occasionally protective and bactericidal [53]. Opa proteins (class 5 OMP) have been shown to be very immunogenic, though they do have a high variability and their synthesis is switched on/off at high frequency and are therefore of lesser interest for vaccine development. [97].

Meningococcal vaccines based on outer membrane proteins have been studied in large-scale field trials with two-dose schedules used (Table 3). The efficacy of the OMP vaccines varied between 57% and 83% [98-103]. However, infants were not protected [99,100] and duration of immunity was unsatisfactory [104]. The outcomes of the efficacy trials varied, which is probably a result of the differences in the OMV vaccines used in combination with the specific protection of the vaccine against the meningococcal vaccine strain. Tappero et al. compared the Cuban vaccine and the Norwegian vaccine in different age groups (infants, children and adults) [105]. High titres of serum bactericidal activity have been shown in all groups, especially in infants, but bactericidal responses seem to be limited to the vaccine strain [105,106].

Country	Vaccine component	N	Efficacy	SBA ≥ 1:4	Reference
Cuba (1987)	purified total OMP: 4: P1.15, and C polysaccharides *2 doses*	N=106000 (10-14 years) N=133600 (5 months-24 years)	83% efficacy confirmed: 83%-94%		[103]
Brazil (1989)	Same Cuban vaccine *2 doses*	N=2.4 million (3months-6 years)	<24 months -37% (-100 to 73) 24-47 months 47% (-72 to 84) >47 months 74% (16-92)	22% 45% 52% overall 40%	[100, 101] [102]
Norway (1988)	OMV vaccine: B:15:P1.7,16:L3,7 With class 5 OMP and Iron regulated protein *2 doses*	N=171800 (students 14-16 years)	57.2% (lower CI 27%)		[98]
Chile (1987)	Purified class 1, 3, 4 OMP15 P1.3, L3,7 LPS 0.1% with C polysaccharides *2 doses*	N=40811 (1-21yrs)	1-4 years -39% 5-21 years 70%	41% 83%	[99]

Table 3 efficacy trials

One way to improve OMP vaccines lies in the area of vaccine formulation, in which the presentation form of PorA was of great importance for the generation of bactericidal antibodies [107-109].

Outer membrane vesicle vaccines are prepared by isolating the OMP in the form of vesicles (OMV) depleted of LPS by treatment with a detergent. To improve solubility and immunogenicity, the OMV can be complexed with one or more meningococcal CPS [110]. The OMV vaccines containing OMP and limited amounts of LPS, have a three-dimensional structure in which the OMP are stabilised in their native conformation (Figure3) [111]. The OMV vaccines are immunogenic in adults and young children, and the antibodies are bactericidal and capable of enhancing phagocytosis [28,51,52]. The protection is considered to be OMP specific. OMV vaccines induced serosubtype (class 1OMP)-specific protection, and therefore a large number of serosubtypes must be included to achieve broad protection. Within a geographic area, the vaccine should contain 5 to 10 different (sub)serotype OMPs to achieve broad protection. This problem can hardly be solved by combining the various (sub)serotype OMV preparations, because of the levels of LPS

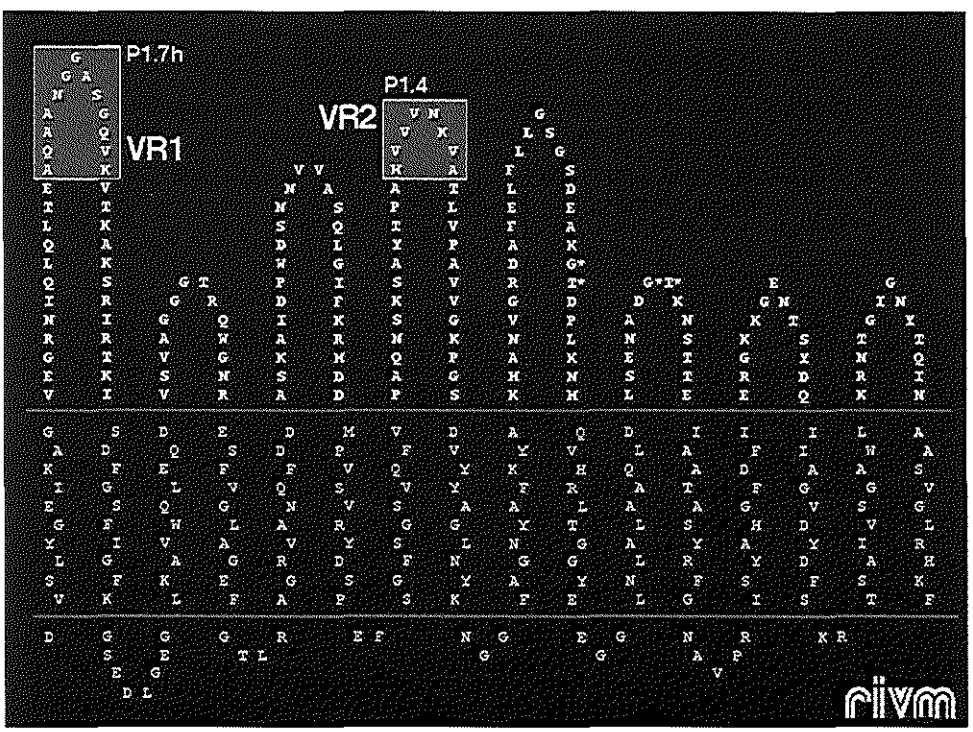

Figure 3 Model of the class 1 outer membrane protein (serosubtype P1.7h,4) showing the two variable regions VR1 and VR2.
(courtesy to Betsy Kuipers, RIVM, the Netherlands)

with the risk of unacceptable side effects. New meningococcal OMV vaccines are constructed by DNA techniques in which more serosubtype OMP (class 1 OMP) are expressed [112]. In this way a hexavalent meningococcal OMV vaccine based on class 1 OMP was produced [113]. This hexavalent meningococcal OMV vaccine covered approximately 80% of the meningococcal strains cultured in the Netherlands and in the UK. This vaccine was safe and induced SBA in human volunteers [114], children [115] and infants [116]. After four vaccinations, 100% of the infants showed at least a SBA titre of 1:4 against the six isogenic meningococcal strains present in the hexavalent vaccine [116]. The SBA against wild type isolates were comparable to the isogenic strains [117].

Vaccines administrated directly onto mucosal surfaces may induce local mucosal as well as systemic responses [118]. Nasal vaccines against meningococci are also investigated. This meningococcal OMV vaccine initiated a systemic as well as local mucosal immune response when presented in a nasal formulation [119,120]. The mucosal immunisation however requires a much higher dose.

Iron-regulated proteins

Meningococci contain a few thousand transferrin-binding sites, when fully expressed. The transferrin receptor is formed by the transferrin binding proteins TbpA and TbpB. Both Tbps are surface-exposed [121] and show considerable molecular and antigenic heterogeneity amongst different strains of *Neisseria meningitidis* [122-125]. TbpA proteins are believed to be less antigenically variable than TbpB [126]. Antibodies in convalescent sera have identified the expression of these proteins in vivo [127]. Purified transferrin binding proteins protected mice passively and actively against a lethal challenge with the homologue meningococcal strain [128]. Only mice and rabbit antisera specific for TbpB displayed bactericidal activity against the parent strain [129]. Bactericidal assays showed that the protection against various isolates in animal models was incomplete. Transferrin binding proteins vary among strains [128,130]. This variation is independent of the typing systems used. In view of the lack of cross-reaction in the antibody responses against various meningococcal isolates [123,128,130,131], patients and carriers responded with fully cross-reactive antibodies to TbpB and reacted weakly with antibodies to conformational epitopes of TbpA [132,133]. In another study, human antibodies recognised more frequently TbpA epitopes than the epitopes of TbpB, which are thought to be more exposed. Stevenson et al. showed that common antigenic domains exist in the TbpB molecules of all meningococcal isolates examined as well as in the analogous TbpB protein of *Haemophilus influenzae* type b [134]. These domains offer considerable interest as vaccine components. A growing amount of information on the genes encoding Tbps has become available [126,135-137].

Finally, with regard to possible cross-reaction between meningococcal and human transferrin receptors, it is now increasingly clear that, despite their common function, these receptors have

no common structural or immunological properties. Animal sera raised against meningococcal transferrin receptors failed to recognise human lymphocytes or human placental transferrin receptors [132,134]. A phase I study with recombinant TbpB B16B6 in 11 human volunteers has been reported [138]. Preliminary results showed that the vaccine was safe and immunogenic.

Lipopolysaccharides as vaccine candidate

Of the 12 different immunotypes known, only 3 are prevalent in the western world [139]. Combined vaccines with immunotype-specific epitopes will probably be able to induce better and broader protection against infections with *Neisseria meningitidis*. However, conflicting results have been reported about the bactericidal capacity of meningococcal anti-LPS antibodies in human sera [53,140,141]. Epitopes as target for bactericidal antibodies are present on meningococcal LPS. Monoclonal antibodies specific for these epitopes have been developed [53]. Oligosaccharide-OMP conjugate vaccines will probably elicit serosubtype-specific and immunotype specific antibodies. This kind of vaccine is potentially capable of inducing neutralising antibodies against endotoxin. A disadvantage using complete meningococcal oligosaccharides is the presence of the terminal lacto-N-neotetraose unit or the terminal α-D-Galp (1-4)-β-D-Galp moiety. These units are also detected on human precursors of blood group antigens and human epithelial cells [77,142]. Deletion of these determinants will avoid the risk of autoantibody induction. Another problem is that coupling of LPS derived oligosaccharide to a protein conjugate will probably determine the immunological and immunochemical characteristics of the resulting conjugate. Furthermore, it is not known what meningococcal LPS is composed of after colonisation or infection. In the log phase meningococci contain shorter LPS molecules than in the stationary phase [143].

LPS-derived oligosaccharide protein conjugates are tested in animal models. Rabbits were immunised with dephosphorylated immunotype L2, L3,9, L5 and L10 oligosaccharides coupled to tetanus toxoid [144]. The immunotypes L2, L5 and L10 conjugates were highly immunogenic, inducing antibodies against the homologous and heterologous immunotypes. Bactericidal antibodies were only present against the homologous immunotype [144]. Using an other coupling method, immunotype L2 and L3,7,9 oligosaccharide tetanus toxoid conjugates were highly immunogenic in rabbits [145]. Antibodies reacted with both homologous and heterologous immunotype LPS, but were not bactericidal in rabbits and mice [142]. These results show that it is very difficult to induce bactericidal antibodies against different immunotypes by using LPS derived oligosaccharide conjugates.

Meningococcal LPS plays a role in preserving conformational epitopes of some OMPs and may function as an adjuvant to enhance the immune response to antigens [146]. Free LPS is highly toxic, but the toxicity is reduced when LPS is integrated in OMVs [147,148]. Outer membrane complexes derived from an LPS-deficient meningococcal mutant were poorly immunogenic [149]. The immunogenicity could be restored with adjuvants less toxic than wild type LPS [149,150]. The presence of LPS, with or without a certain carbohydrate chain length, is required to induce bactericidal antibodies against PorA protein epitopes [75,149]. LPS mutants with a good ratio of activity and toxicity are good candidates to reduce the side effects of OMV vaccines without affecting adjuvant activity.

Discussion

Successful meningococcal vaccines must be safe and immunogenic in young children, whose immune responses are immature. The fact that infection at a very young age may confer protection against reinfection confirms the possibility of the development of a good vaccine against meningococci. Infection with one strain gives life-long immunity to infection with other heterologous serogroups. To date, attempts to produce such a successful meningococcal B vaccine have failed. However, several promising vaccine candidates are under investigation.

Most information is available about the OMV vaccines based on class 1 OMP or total outer membrane proteins. These vaccines are safe and immunological responses have been widely studied. Vaccines based on the most prevalent serotype or serosubtype will need a continuous detailed epidemiological surveillance of disease-associated organisms in order to predict the optimal vaccine. From time to time, the vaccine has to be reformulated as the prevalent serotypes and/or subtypes change. Serologically non-serotypable and non-serosubtypable strains have been isolated. Nasopharyngeal replacement or switching of capsule or outer membrane components may affect the impact of a meningococcal vaccine. A meningococcal vaccine needs to be as broadly protective as possible. Therefore new combinations of vaccine candidates should be integrated in the OMV formula. Recently, the complete DNA sequences of a serogroup A and B strain of *Neisseria meningitidis* have been unravelled [151-153]. Insights into the commensal and virulence behaviour of *N. meningitidis* can be deducted in the future from the genome, in which sequences for structural proteins are clustered and several coding regions unique to the serogroup capsular polysaccharide synthesis have been identified. The entire genome sequence of a virulent serogroup B strain is used to identify vaccine candidates. An alternative approach is the development of vaccines against serogroup B meningococcal infections based on the application of recombinant DNA techniques to attenuate group B strains and us them as live, intranasal vaccines. The major concern with a live, attenuated strain is off course the safety.

Serum bactericidal activity has been evaluated as a serological correlate of protection. Fourfold rise in SBA after vaccination is the best correlate for protection up to now, but the converse may not be true [106]. In addition, protection may be caused by opsonophagocytosis by PMNs. Therefore, both assays should be included in future vaccine efficacy trials to obtain a more reliable correlate of the protective activity conferred by the vaccine. An animal model can be a more direct way to study the protective efficacy of existing candidate vaccines, although the question remains how well the protection in an animal model corresponds with protection from human disease.

The search for a good, broad protective vaccine against serogroup B meningococci has not yet ended. Thusfar, the meningococcal OMP vaccines are most promising and have been

tested in efficacy trials showing intermediate effects. Certainly the elucidation of the genomic structure of *Neisseria meningitidis* will accelerate the understanding of the pathogenesis of meningococcal infections and will be a further contribution to the characterisation of promising vaccine candidates.

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Chapter 6

Immunogenicity and safety of a hexavalent meningococcal outer-membrane-vesicle vaccine in children of 2–3 and 7–8 years of age

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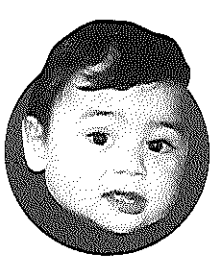
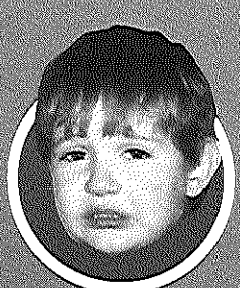




Abstract

To study the reactogenicity and immunogenicity of a hexavalent meningococcal outer-membrane-vesicle vaccine (OMV), two different dosages of this vaccine (7.5 µg and 15 µg of individual PorA proteins) consisting of vesicles expressing class 1 outer membrane proteins (OMPs) of subtypes P1.7,16; P1.5,2; P1.19,15 and P1.5^{c},10; P1.12,13; P1.7^{h},4 were administered to a group of 7-8 year (n=165) and a group of 2-3 year old children (n=172). Control groups of children with similar ages were vaccinated against hepatitis B. All participants received three injections. Pre- and post- immunisation sera were tested for bactericidal antibodies against six isogenic meningococcal vaccine strains expressing different PorA proteins. Antibody titres against OMP of the two different vesicles (PL16215 and PL10124) were measured by ELISA. The meningococcal hexavalent OMV vaccine was well tolerated. No statistically significant differences were seen between the high and low dose of hexavalent meningococcal OMV vaccine. The percentage of children showing a 4 fold increase of bactericidal antibody titres against the specific serosubtype varied in toddlers from 28% to 98% and in older children from 16% to 100%. Both ELISA antibody titres and bactericidal activity showed the highest level in the youngest agegroup.

Introduction

Meningococcal disease forms a major health problem in many countries with still an overall mortality of 7-10%. The clinical picture varies from self-limiting bacteremia, to meningitis or septic shock. In the Netherlands, the annual number of patients with meningococcal disease has increased steadily from the beginning of the eighties. In 1996, these infections were mostly caused by *Neisseria meningitidis* serogroup B (80%) which predominates also in other European countries with a temperate climate [1, 2]. Therefore, production of a meningococcal vaccine capable of generating long-lasting immunity in all age groups has a high priority in the Netherlands health care programme. Vaccines against *Neisseria meningitidis* serogroup B have not been widely available yet. Development of such a vaccine has been complicated by the inability of group B capsule polysaccharides to induce significant increases in antibodies [3]. The poor immunogenicity of the antigen is probably associated with immunological tolerance since antibodies against group B polysaccharide cross-react with host antigens [4, 5]. However, conjugate vaccines, in which capsular polysaccharides and proteins are covalently linked, elicit some immune response against the normally non-immunogenic group B polysaccharide [6-9]. Fusco et al. showed bactericidal activity in monkeys after three injections of modified N-propionylated group B meningococcal polysaccharide conjugated to a recombinant class 3 porin [10]. Other immunodominant antigens at the cell surface of *N. meningitidis* are also able to induce strong bactericidal activity and are widely used as candidate for vaccine development. Patients with meningococcal disease have shown immune responses to various meningococcal outer-membrane proteins (OMP), suggesting that these proteins could be an important component of meningococcal vaccines [11-17]. Vaccines consisting of membrane vesicles containing a mixture of OMPs were safe and immunogenic in volunteers [18-21]. Efficacy studies with these vaccines demonstrated that antibodies to these antigens do provide a significant protection against serogroup B meningococcal disease [22-26]. However, these studies showed that the protective efficacy was between 50 and 80%, age dependent and of limited duration [24-26]. Class 1 proteins (PorA) appeared to be better inducers of bactericidal antibodies than class 2 or class 3 proteins. These bactericidal antibodies were shown to be the best correlates for protection against group B meningococcal disease [20, 21, 27-35]. In the National Institute of Public Health and the Environment (RIVM) in the Netherlands, two trivalent vaccine strains have been developed containing and expressing genes encoding the prevalent PorA 6 serosubtypes [36, 37]. The PorA serosubtypes represent together 80% of current isolates from blood and cerebrospinal fluid of patients in the Netherlands [1]. Using the two trivalent vaccine strains a hexavalent meningococcal outer membrane vesicle (OMV) vaccine was produced including the serosubtype antigens: P1.7,16; P1.5,2; P1.19,15 and P1.5^{c},10; P1.12,13; P1.7^{h},4. The vaccine consisted for 90% of PorA OMPs and had very low expression of class 4 and 5 OMPs. Classes 2/3 OMPs as well as the B-capsular polysaccharide are not expressed due to gene deletions. Phase I clinical trials with this vaccine

showed that the vaccine is safe and that after one vaccination with the highest dose (15 μg of the individual PorA proteins) 50% of the volunteers showed a 4 fold increase in bactericidal antibody activity against six test-strains expressing the specific serosubtypes [21]. In infants in the UK, this hexavalent OMV vaccine was safe and evoked encouraging immune responses [38]. The current phase II study was designed to analyse the safety and the immunogenicity of two different dose of this hexavalent meningococcal OMV vaccine in healthy Netherlands toddlers and school children.

Materials and Methods

Study population

Healthy children living in Rotterdam (The Netherlands) 7 to 8 years old (born in 1988) or 2 to 3 years old (born in 1993) were recruited by letter to participate in this trial. Children were included in the study after a positive reply, a health check and written informed consent. The study subjects did not receive a hepatitis B or meningococcal vaccine prior to entry in the study, were known not to be allergic to vaccine-components, had no immunodeficiency and were never diagnosed with meningococcal disease. No other vaccinations were given during the trial. After approval of the protocol by the Medical Ethical Committee of the University Hospital of Rotterdam, the study started in January 1996 and was ended in September 1996.

Study design

An open randomised, placebo controlled trial including 3 vaccines (meningococcal OMV low dose, meningococcal OMV high dose, Hepatitis B) was designed. The choice of the vaccine was allocated by a computer-generated list of random numbers. One Group received three times a low dosage hexavalent meningococcal vaccine containing 7.5 µgram class 1 protein per dose (low dose meningococcal vaccine). The second group received three times a high dosage hexavalent meningococcal vaccine containing 15 µgram class 1 protein per dose (high dose meningococcal vaccine). The control group received three times a hepatitis B vaccine (HepB). Vaccines (0.5 ml) were given by injection with a 0.8 x 40 mm needle in the deltoid muscle. Children born in 1988 (school children) were vaccinated in a local community health centre. Children born in 1993 (toddlers) received vaccine from the research-physician involved in this project (EdK). In all groups, the time schedule for vaccinations was similar. The first vaccination was administered at the age of 7 year (children born in 1988) or at the age of 2-3 year (children born in 1993). The second vaccination should be given between 7 and 13 weeks after the first one. The third (booster) vaccination should be given 5 to 9 months after the second vaccination. Blood samples were obtained before the first vaccination, 4 to 6 weeks after the second vaccination, at the most 2 weeks before the third vaccination and 4 to 6 weeks after the third vaccination. Blood samples were collected by venipuncture and sent to the RIVM, Bilthoven, the Netherlands by regular mail. Serum was stored at -20 °C until distribution of aliquots for blinded specific antibody measurements. All sera were heat inactivated for 30 min at 56°C.

Vaccines

Production and characterisation of the hexavalent meningococcal group B OMV vaccine was described by Claassen et al. [39]. The final vaccine contained equal amounts of OMV of the two recombinant engineered trivalent meningococcal strains PL 16215 (- : - :P1.7,16; P1.5,2; P1.19,15) and PL 10124 (- : - :P1.5^{c},10; P1.12,13; P1.7^{h},4). PorA mounted to approximately 90% of the vaccine

protein. The remaining proteins were class 4 and class 5 OMP. The vaccine consists of 2.5 to 10% LPS relative to the protein content. Two meningococcal OMV vaccine lots were tested, i.e. lot E 9282 (50μg total protein = low dose meningococcal vaccine) and lot E 9281 (100μg total protein = high dose meningococcal vaccine) which implies respectively 7.5 μg or 15 μg of each of the individual PorA's in a vaccine dose. The proteins were absorbed to $AlPO_4$ and the vaccine contains sucrose as a stabiliser. The vaccines contained: 1.5 mg $AlPO_4$; 10 mg sucrose; 0.05 mg thiomersal per dose, in a volume of 0.5 ml. A licensed hepatitis B vaccine (HB-vax DNA, Pasteur-Merieux MSD, lot HA 41120) served as a control. A 0.5 ml dose consisted of 10 μg/ml HBsAg (hepatitis B surface antigen, recombinant). As the labelling of study vaccines and control vaccine was different, the study could not be blinded. However, antibody titrations were done blinded.

Monitoring for adverse reactions

Participants were monitored for local and systemic adverse reactions by home visits between 18 and 30 hours after each vaccination. Parents were asked to keep a diary until 7 days after vaccination to record local and systemic adverse reactions. During the next visit adverse reactions were registered by a structured interview. Local adverse reactions registered were the presence of redness, redness more than 2.5 cm, swelling larger than 2.5 cm, itching, pain and inability to use the arm. Systemic adverse reactions registered were the presence of fever, defined as a rectal temperature above 38,5 °C, headache, drooping, decreased appetite, nausea, joint complaints, cutaneous symptoms, school non-attendance, sleepiness, unusual crying, use of medication, contacts with the health care system and illness in the family.

Antibodies to OMV

Antibody titers to OMV from the vaccine strains PL16215 and PL10124 were determined by enzyme linked immunosorbent assay (ELISA) as described by Peeters et al.[21]. In short, after overnight coating of the microtiter plates at room temperature with the two different OMVs the plates were incubated with threefold serial dilutions of serum samples for 90 min at 37°C. After subsequent incubation with peroxidase conjugated rabbit anti-human IgG (90 minutes at 37°C) and peroxidase substrate the developed colour was read at 450 nm. IgG antibody titres were expressed as the dilution which gave 50% of ODmax-ODmin.

Serum bactericidal activity assay

Bactericidal activity of antibodies against isogenic variants of strain H44/76 was determined as described by Peeters et al. & Rouppe van der Voort et al. [21, 40]. In short, 2-fold dilutions of heat inactivated sera (30 min at 56°C) were incubated with complement (final concentration 10% (v/v)) and 2.5 x 10^2 c.f.u. bacteria. Six isogenic strains expressing the serosubtype specific class 1 proteins present in the two trivalent vaccine strains (H44/76, TR52, TR15 and TR10, TR1213, TR4) were used to determine subtype specific bactericidal activity of the antibodies. After plating samples on GC

agar and 18 -20h incubation at 37°C in 5% CO_2 in the presence of 10% human complement, the colonies from time zero and 60-min-incubation were counted. The average number of c.f.u. at time zero was set at 100%. The serum bactericidal titre was reported as the lowest 2log reciprocal of the serum dilution yielding $\geq$ 90% killing.

Statistics

Chi-squared tests or Fisher's exact tests were used to evaluate the significance of the differences of local and general reactions between the hexavalent meningococcal OMV vaccine group and the hepatitis B group. The significance of differences in Elisa IgG titres against OMV and bactericidal GMT titres between the different groups were determined by the Mann-Whitney U test.

Results

Study populations

After written informed consent by their parents or their legal representatives, a total of 165 healthy children of 7 year old (78 female, 87 male = group A) and 172 healthy toddlers of 2 to 3 year old (76 female, 96 male =group B) were willing to participate. In group A, three participants dropped out after the first vaccination, without any serious adverse event (2 of the high dose meningococcal vaccine and 1 of the HepB vaccinated children). Two additional children were excluded because they received a wrong dosage meningococcal vaccine at the third vaccination. In group B, 2 children dropped out before the third vaccination (1 of the low dose meningococcal vaccine and 1 of the high dose meningococcal vaccine). One child was lost for follow up and the other dropped out without a serious adverse event. The time-interval between the first and the second vaccination was 9.0 (7 - 12.0) weeks. The third (booster) vaccination was given 27.4 (25 – 31.4) weeks after the second vaccination.

Safety

No serious adverse events occurred. In general, mild adverse reactions were reported more frequently for the hexavalent meningococcal OMV vaccine groups than the hepatitis B control group in both age groups. There were no statistically significant differences in side effects between the low and high doses of the hexavalent meningococcal OMV vaccine. Adverse reactions peaked within 24 hours after vaccination and declined rapidly within 1 week. Local and systemic reactions monitored during home visits, 18-30 hours after vaccination are documented in Table 1. The monitoring at home before 18 hours or after 30 hours after vaccination, and for children receiving another vaccine during the study period were excluded. Local reactions generally consisted of mild inflammation at the injection site. Local reactions were more frequently seen than systemic reactions, with a higher frequency in older children. In the meningococcal vaccine group 74.4% versus 18.4% in the hepatitis B vaccine group showed any local reaction, mostly pain and redness $\leq$ 2.5 cm 24 hours after at least one of the three vaccinations ($p<0.01$). The rates of swelling larger than 2.5 cm increased with each vaccination in the group of school children. For toddlers the rate of local pain increased after each vaccination. Any systemic reaction 24 hours after vaccination was documented by 29.9% of the school children and by 23.1% of the toddlers after at least one of the three meningococcal OMV vaccinations. This was significantly more ($p<0.01$) than in the hepatitis B vaccine group (14.7% and 11.6%). Pain medication (paracetamol) in the week after vaccination was used by 4.2% of the children after meningococcal vaccination and by 2.5% of the children after hepatitis B vaccination ($p=0.03$). There were no differences between the meningococcal vaccine group and the control group in visits to the family doctor during the observation period after vaccination.

Schoolchildren	Vaccination 1					Vaccination 2					Vaccination 3				
Reaction	MenB (N=123)		HB (N=39)			MenB (N=122)		HB (N=39)			MenB (N=120)		HB (N=39)		
	N	%	N	%		N	%	N	%		N	%	N	%	
Any local reaction	110	89.4	12	30.8	*	110	90.2	13	33.3	*	97	80.8	9	23.1	*
Pain	107	87.0	7	17.9	*	105	86.1	12	30.8	*	88	73.3	7	17.9	*
Redness	47	38.2	2	5.1	*	59	48.4	0		*	46	38.3	3	7.7	*
Redness >2.5cm	13	10.6	0		**	25	20.5	0		**	14	11.7	0		**
Swelling >2.5cm	15	12.2	3	7.7		25	20.5	0		**	36	30.0	0		*
Not using arm	9	7.4	1	2.6		12	9.8	2	5.1		11	9.2	0		
Itching	9	7.3	0			6	4.9	1	2.6		5	4.2	1	2.6	
Any systemic reaction	43	35.0	9	23.1		44	36.1	5	12.8	**	27	22.5	5	12.8	
Fever (> 38.5 °C)	0		0			3	2.5	0			2	1.7	0		
Headache	20	16.3	2	5.1		20	16.4	2	5.1		17	14.2	4	10.3	
Drooping	21	17.1	2	5.1		22	18.0	1	2.6	**	13	10.8	3	7.7	
Less appetite	9	7.3	1	2.6		12	9.8	1	2.6		9	7.5	0		
Nausea	7	5.7	2	5.1		6	4.9	1	2.6		7	5.8	0		
Joint complication	6	4.9	0			2	1.6	0			0		1	2.6	
Cutaneous symptoms	2	1.6	3	7.7		5	4.1	1	2.6		0		1	2.6	
Absence school	2	1.6	1	2.6		14	11.5	1	2.6		8	6.7	3	7.7	

Toddlers	Vaccination 1					Vaccination 2					Vaccination 3				
Reaction	MenB (N=111)		HB (N=53)			MenB (N=114)		HB (N=56)			MenB (N=112)		HB (N=55)		
	N	%	N	%		N	%	N	%		N	%	N	%	
Any local reaction	63	56.8	5	9.4	*	70	61.4	7	12.5	*	72	64.3	5	9.1	*
Pain	49	44.1	5	9.4	*	56	49.1	7	12.5	*	67	59.8	4	7.3	*
Redness	31	27.9	0		*	24	21.1	0		*	24	21.4	1	1.8	*
Redness >2.5cm	1	0.9	0			2	1.8	0			2	1.8	0		
Swelling >2.5cm	2	1.8	0			2	1.8	0			2	1.8	0		
Not using arm	10	9.0	0		**	14	12.3	1	1.8	**	6	5.4	2	3.6	
Itching	0		0			0		0			0		0		
Any systemic reaction	28	25.2	7	13.2		26	22.8	8	14.3		26	23.2	4	7.3	**
Fever (> 38.5 °C)	5	4.5	2	3.8		5	4.4	1	1.8		2	1.8	1	1.8	
Headache	1	0.9	1	1.9		2	1.8	0			0		0		
Drooping	16	14.4	5	9.4		20	17.5	2	3.6	**	20	17.9	4	7.3	
Sleepy	1	0.9	1	1.9		0		0			4	3.6	0		
Unusual crying	13	11.7	2	3.8		13	11.4	5	8.9		8	7.1	0		**
Less appetite	10	9.0	2	3.8		7	6.1	1	1.8		9	8.0	2	3.6	
Nausea	3	2.7	0			2	1.8	0			3	2.7	0		
Joint complication	0		0			0		0			0		0		
Cutaneous symptoms	0		1	1.9		1	0.9	0			0		0		

Table 1 Side effects

Data obtained with low dosage and high dosage of meningococcal vaccine are combined. Children may have more than one symptom.

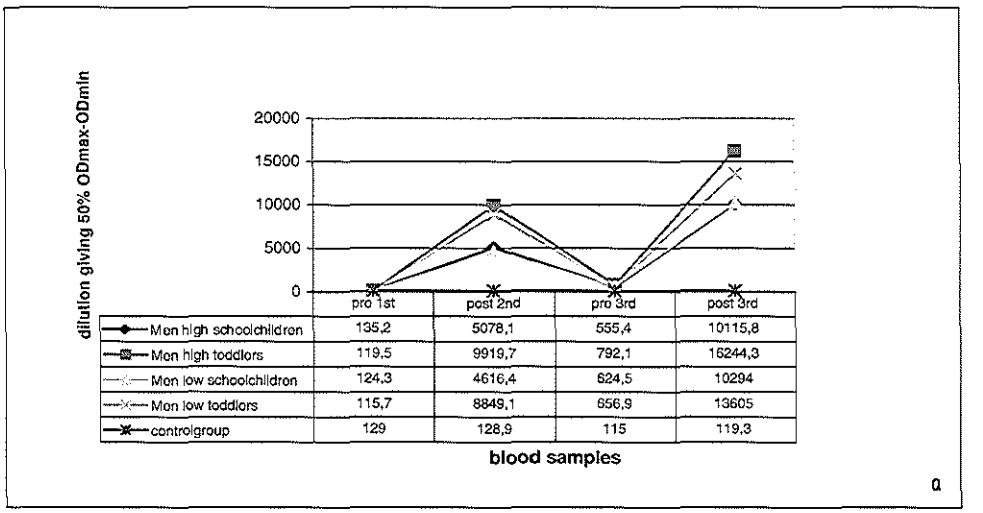
MenB: RIVM meningococcal OMV vaccine, low and high dosage * p<0.01 (Chi Squared test)

HB: hepatitis B vaccine ** p=0.01- 0.05

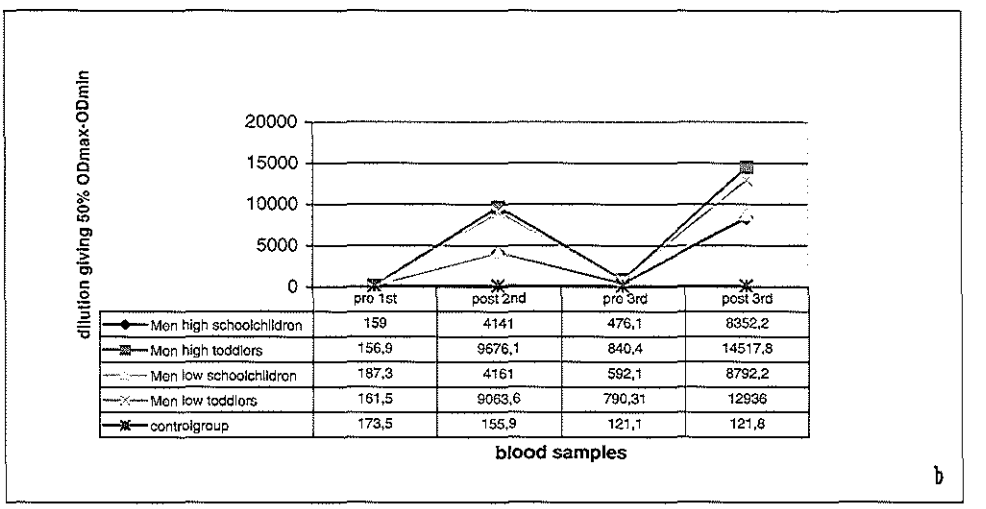
Antibodies to OMV

Sera of all volunteers were analysed, although in a small number of children the volume of blood samples was too small to perform all serological tests.

The geometric mean titre of IgG antibody against both trivalent vaccine vesicles (PL10124 and PL16215) are shown in Figure 1. In general, the toddlers had a greater increase in IgG geometric mean titres than the school children. After the second and the booster vaccination mean titres of



	pre 1st	post 2nd	pre 3rd	post 3rd
Men high schoolchildren	135,2	5078,1	555,4	10115,8
Men high toddlers	119,5	9919,7	792,1	16244,3
Men low schoolchildren	124,3	4616,4	624,5	10294
Men low toddlers	115,7	8849,1	656,9	13605
controlgroup	129	128,9	115	119,3



	pre 1st	post 2nd	pre 3rd	post 3rd
Men high schoolchildren	159	4141	476,1	8352,2
Men high toddlers	156,9	9676,1	840,4	14517,8
Men low schoolchildren	187,3	4161	592,1	8792,2
Men low toddlers	161,5	9063,6	790,31	12936
controlgroup	173,5	155,9	121,1	121,8

Figure 1a and 1b

Total IgG geometric mean titre to outer membrane proteins of strain PL16215 (figure 1a) and strain PL10124 (figure 1b)

Men high: RIVM meningococcal OMV vaccine, high dosage

Men low: RIVM meningococcal OMV vaccine, low dosage

Control group: group of children receiving hepatitis B vaccine

the IgG antibodies in toddlers were all significantly higher than in school children against both vaccine strains. There were no significant differences in the response to meningococcal OMV between the high and the low dose of the hexavalent meningococcal OMV vaccine within the two age groups, with one exception. After the booster vaccination, toddlers receiving a high dose of meningococcal OMV vaccine showed a statistically significant higher IgG titre against strain

Schoolchildren													
Blood sample / Vaccine / Strain		*Prevaccination 1* Men Low	Men high	HepB	*Postprimary 2* Men Low	Men high	HepB	*Prebooster 3* Men Low	Men high	HepB	*Postbooster 4* Men Low	Men high	HepB
P1.12,13	N	61	64	40	60	62	39	60	62	39	58	62	37
(TR1213)	GMT	0.11	0.06	0.03	1.50	1.94	0.10	0.30	0.29	0.00	2.14	1.89	0.05
	s.d	0.69	0.39	0.16	2.01	1.96	0.64	1.20	0.82	0.00	2.29	2.00	0.33
	%>=1:4	3.3	1.6	0.0	40.0	50.0	2.6	6.7	11.3	0.0	58.6	50.0	2.7
P1.5c,10	N	61	64	40	60	61	39	60	62	39	58	62	37
(TR10)	GMT	0.18	0.09	0.05	5.02	5.33	0.21	2.45	2.18	0.21	6.79	6.82	0.41
	s.d	0.81	0.53	0.32	2.16	2.38	0.73	2.28	2.26	0.73	1.63	2.24	1.24
	%>=1:4	4.9	3.1	2.5	93.3	93.4	7.7	65.0	53.2	7.7	100.0	93.5	10.8
P1.7h,4	N	61	64	40	60	62	39	60	62	39	58	62	37
(TR4)	GMT	0.05	0.03	0.08	0.57	0.31	0.13	0.28	0.15	0.10	1.09	0.69	0.22
	s.d	0.38	0.25	0.35	1.69	0.90	0.57	1.01	0.72	0.50	1.93	1.65	0.95
	%>=1:4	1.6	1.6	2.5	10.0	9.7	5.2	8.3	4.8	2.6	25.9	16.1	5.4
P1.5,2	N	61	64	40	60	61	39	60	62	39	58	62	37
(TR52)	GMT	0.20	0.08	0.05	2.53	2.33	0.13	0.95	0.65	0.21	4.38	4.76	0.11
	s.d	0.95	0.41	0.32	2.40	2.13	0.52	1.85	1.45	0.73	2.43	2.02	0.52
	%>=1:4	4.9	1.6	2.5	63.3	62.3	2.6	23.3	17.7	7.7	84.5	95.2	2.7
P1.7,16	N	61	64	40	60	62	39	60	62	39	58	62	37
(H44/76)	GMT	0.00	0.03	0.08	0.63	0.60	0.10	0.37	0.27	0.03	1.60	1.84	0.08
	s.d	0.00	0.25	0.35	1.60	1.64	0.45	1.41	1.16	0.16	1.96	2.28	0.49
	%>=1:4	0.0	1.6	2.5	16.7	12.9	5.1	6.7	6.5	0.0	43.1	38.7	2.7
P1.19,15	N	61	64	40	60	62	39	60	62	39	58	62	37
(TR15)	GMT	0.00	0.17	0.15	0.45	0.87	0.15	0.27	0.32	0.00	0.72	1.08	0.08
	s.d	0.00	0.97	0.58	1.42	2.00	0.67	1.45	1.16	0.00	1.60	2.07	0.49
	%>=1:4	0.0	3.1	5.0	10.0	16.1	5.1	3.3	9.7	0.0	19.0	24.2	2.7

Toddlers													
Blood sample / Vaccine / Strain		*Prevaccination 1* Men Low	Men high	HepB	*Postprimary 2* Men Low	Men high	HepB	*Prebooster 3* Men Low	Men high	HepB	*Postbooster 4* Men Low	Men high	HepB
P1.12,13	N	62	54	56	60	54	54	60	52	55	61	51	55
(TR1213)	GMT	0.06	0.02	0.04	2.43	2.54	0.02	0.27	0.23	0.02	3.16	2.71	0.05
	s.d	0.51	0.14	0.19	2.23	1.86	0.14	1.16	0.90	0.13	2.07	2.09	0.30
	%>=1:4	1.6	0.0	0.0	60.0	70.4	0.0	5.0	3.8	0.0	78.7	68.6	1.8
P1.5c,10	N	62	54	56	60	53	54	60	52	55	61	51	55
(TR10)	GMT	0.13	0.02	0.27	7.00	7.09	0.30	3.25	3.56	0.29	7.67	7.58	0.29
	s.d	1.02	0.14	1.00	1.68	1.44	1.24	2.38	2.21	1.20	0.83	1.30	1.20
	%>=1:4	1.6	0.0	7.1	98.3	100.0	7.4	73.3	80.8	7.4	100.0	98.0	7.5
P1.7h,4	N	61	54	56	59	54	54	60	52	55	61	51	54
(TR4)	GMT	0.00	0.00	0.11	0.49	0.57	0.09	0.18	0.06	0.15	1.43	1.57	0.09
	s.d	0.00	0.00	0.59	1.21	1.22	0.49	1.10	0.31	0.76	2.00	2.00	0.49
	%>=1:4	0.0	0.0	3.6	15.3	11.1	3.7	3.3	1.9	3.6	34.4	41.2	3.7
P1.5,2	N	62	54	56	60	53	54	60	52	55	61	51	54
(TR52)	GMT	0.02	0.06	0.21	3.37	3.66	0.15	0.67	0.52	0.13	5.15	5.65	0.11
	s.d	0.13	0.41	0.99	2.02	2.03	0.76	1.63	1.50	0.55	1.96	1.96	0.60
	%>=1:4	0.0	1.9	5.4	83.3	84.9	3.7	15.0	11.5	5.6	95.1	94.1	3.8
P1.7,16	N	61	54	56	59	54	54	60	52	55	61	51	54
(H44/76)	GMT	0.00	0.00	0.04	0.64	0.89	0.00	0.17	0.12	0.02	1.70	2.55	0.00
	s.d	0.00	0.00	0.27	1.24	1.63	0.00	1.06	0.62	0.13	2.43	2.34	0.00
	%>=1:4	0.0	0.0	1.8	20.3	24.1	0.0	3.3	3.8	0.0	37.7	58.8	0.0
P1.19,15	N	62	54	56	60	54	54	60	52	55	61	51	54
(TR15)	GMT	0.00	0.11	0.05	0.53	0.72	0.00	0.22	0.31	0.00	1.10	0.96	0.00
	s.d	0.00	0.82	0.40	1.03	1.82	0.00	1.12	1.29	0.00	1.80	1.72	0.00
	%>=1:4	0.0	1.9	1.8	18.3	14.8	0.0	5.0	5.8	0.0	29.5	27.5	0.0

Table 2 Meningococcal bactericidal activity of schoolchildren and toddlers against six serosubtype specific test strains.

Men low : RIVM meningococcal OMV vaccine, low dosage

Men high: RIVM meningococcal OMV vaccine, high dosage

Z >= 1:4 percentage of children with a bactericidal titre higher or equal to 1:4

Hep B: hepatitis B vaccine

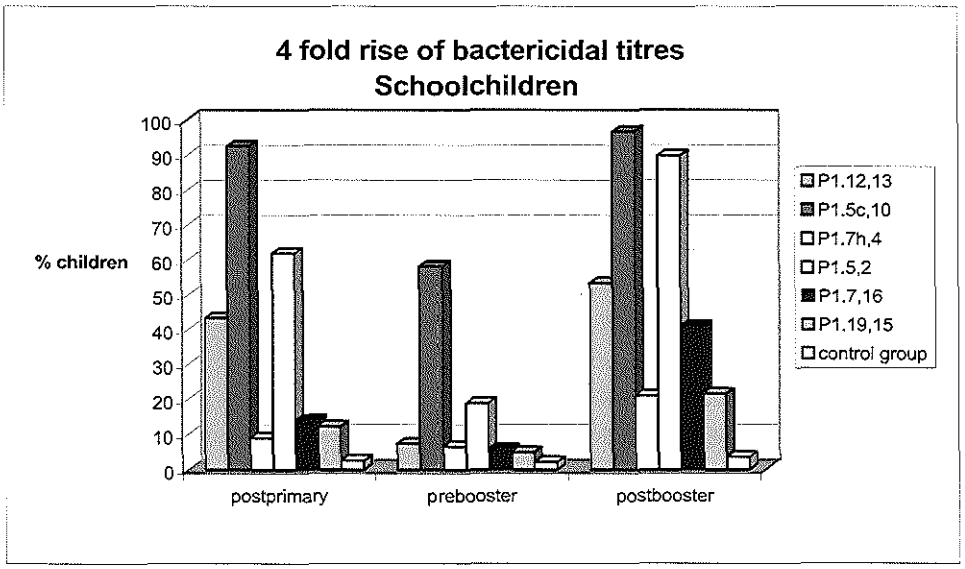
N : number of participants

GMT: mean 2log titre

PL16215 (p=0.04) than toddlers receiving a low dose of meningococcal OMV vaccine. For all meningococcal vaccine groups, the geometric mean titres of IgG antibody were increased against both strains after two meningococcal vaccinations and fell off within 6 months after the two primary vaccinations. The mean titre 6 months after the primary meningococcal vaccinations remained above the pre-immunisation levels and was significantly higher than in the control group ($p<0.01$). In the meningococcal vaccine group the mean antibody titre was 2.6 times higher than the mean antibody response after the primary vaccinations, indicating a booster response. The antibody titres after the third vaccination were statistically significant higher than the antibody titres after the second vaccination ($p<0.01$).

Bactericidal antibody response

Sera from the volunteers were tested for bactericidal activity (SBA) against six isogenic strains expressing the serosubtype specific class 1 proteins present in the vaccine strains (H44/76, TR52, TR15 and TR10, TR1213, TR4)(Table2). Before vaccination 10.1% of the children had SBA activity against at least one of the meningococcal test strains. After the two primary meningococcal OMV vaccinations SBA activity increased. SBA activity against strain TR10 (P1.5c,10) and TR52 (P1.5,2) showed the highest increase. After 2 meningococcal OMV vaccinations, the percentage of children with a fourfold increase of SBA activity varied from 11.1% (P1.7h,4) to 95.3% (P1.5c,10) depending on the serosubtype specific meningococcal test strain (Figure 2). Six months later, before the booster vaccination, SBA activity for all test strains was reduced. SBA activity against TR4 (P1.7h,4) and H44/76 (P1.7,16) in the meningococcal vaccine group was not significantly different from SBA activity in the control group. Activity against TR10 (P1.5c,10) and TR52 (P1.5,2) remained relatively high, 66.7% and 16.2% of the children receiving meningococcal OMV vaccine maintained a fourfold increase of SBA activity against TR10 (P1.5c,10) and TR52 (1.5,2). The third meningococcal vaccination induced a rapid SBA response. The booster response resulted in higher SBA activity against all strains than the two primary meningococcal vaccinations. The percentage of children with at least a fourfold increase of SBA activity compared with prevaccination levels varied for the tested serosubtype specific meningococcal strain between 16.1% (P1.7h,4) and 100% (P1.5c,10). Both age groups showed the same response pattern, although the toddlers had the strongest increase in geometric mean titres. After 3 meningococcal OMV vaccinations, toddlers had a statistically significant higher titre than the school children against 4 of the 6 strains (TR1213, TR10, TR4 and TR52). There were no significant differences in the SBA activity response between the high and the low dose of the hexavalent meningococcal OMV vaccine, with one exception. After the booster vaccination toddlers receiving a high dose of meningococcal OMV vaccine showed a statistical significantly ($p=0.02$) higher SBA activity against strain H44/76 (P1.7,16) than toddlers receiving a low dose of meningococcal OMV vaccine.



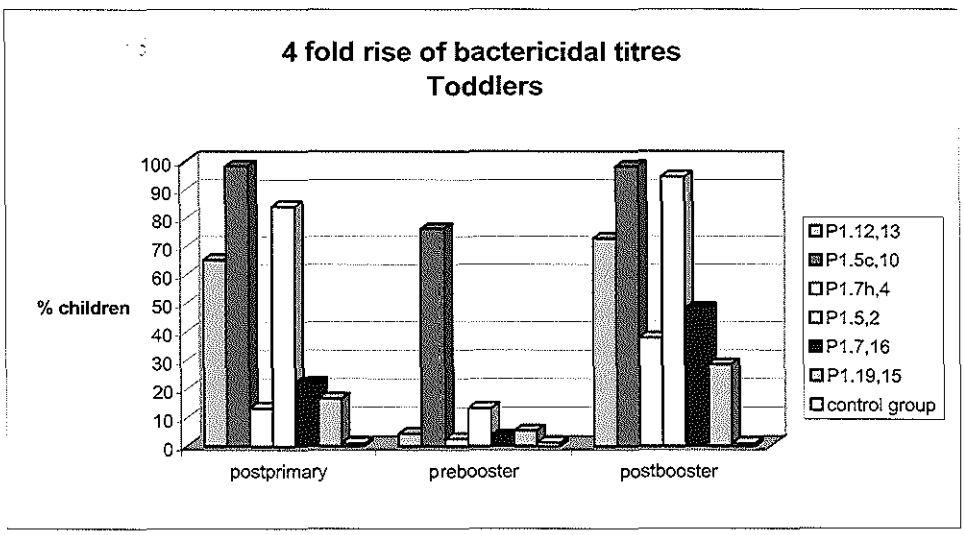


Figure 2a and 2b

Schoolchildren (figure 2a) and toddlers (figure 2b) with a fourfold increase of prevaccination serum bactericidal activity.

- Data obtained with high and low dose of meningococcal OMV vaccine are combined
- Controlgroup = percentage of children vaccinated with a hepatitis B vaccine, mean response against the six meningococcal test strains

Discussion

The hexavalent RIVM meningococcal OMV vaccine used in this study was shown to be safe and able to induce bactericidal antibodies in 2 to 3 and 7 to 8 years old children. Adverse reactions, mostly mild local inflammation were seen more frequently after meningococcal vaccination than after hepatitis B vaccination. Side-effects after both high and low dose of meningococcal OMV vaccine were minimal and comparable with meningococcal OMP vaccines in children [38, 41, 42]. In this study, the IgG antibody responses were increased against OMV of both meningococcal test strains after two meningococcal OMV vaccinations. Antibody levels decreased relatively rapidly after the first two injections, similar as found in studies with the Norwegian OMV vaccine [20, 35, 43, 44]. After administration of a third meningococcal OMV vaccine booster responses were noted, suggesting that the first two doses had induced immunological memory. In our study clear differences were seen between the two different age groups in both ELISA specific IgG titres and SBA activity. Children 2 to 3 years old had statistically significant higher responses in both assays in comparison with children aged 7 or 8 year. Boslego et al. also measured higher IgG ELISA titres against purified outer membrane proteins in younger children, although these children showed the lowest bactericidal activity [42]. Vaccination with meningococcal outer membrane vesicles gives rise to IgG1 and IgG3 antibodies, and some IgG2 antibodies [45-47]. IgG3 and also IgG1 have a strong ability to activate complement, which may explain the higher serum bactericidal activity in younger children. We tested the hypothesis that the children responded after vaccination with a class 1 OMP containing meningococcal OMV vaccine mostly with IgG3, which show relatively higher levels in younger children. Post vaccination sera from 44 toddlers and 46 school children were analysed for isotype distribution in ELISA using isotype specific (IgG1,2,3,4) monoclonal conjugates [48]. IgG1 antibodies dominated the response followed by IgG3 and low levels of IgG2 and IgG4. All IgG isotype levels were higher in toddlers compared to school children. No correlation was observed between the bactericidal titres and the levels of IgG antibodies or any of the isotype specific IgG subclass titres.

After 3 meningococcal OMV vaccinations, the percentage of children with at least a fourfold increase of SBA activity varied between 16% (P1.7h,4) and 100% (P1.5c,10) depending on the meningococcal strain used for representation of different serosubtypes in the bactericidal assay. Also the SBA activity showed the kinetics of priming with a booster response after the third vaccination. The youngest children showed the highest increase in SBA activity, which is in contrasts with the results of SBA activity and protection in young children in efficacy trials [24, 25]. The RIVM meningococcal OMV vaccine in UK infants showed even higher SBA titres than in Dutch children [38]. However the UK SBA test results are not fully comparable with the tests done in the Netherlands, since SBA assays in the UK and the Netherlands were slightly different giving higher results in the UK. Further harmonisation of the SBA test with regard to standard and methodology is therefore necessary. In the study of Tappero et al. infants vaccinated with an FI-produced or

NIPH-produced meningococcal OMV vaccine were showing the highest SBA activity against the homologous target strain in comparison with children and adults [41]. However, these infants did not respond to heterologous strains. Positive sera of our study against the H44/76b isogenic strain expressing P1.7h,4 were analysed for the ability to kill wild type isolates from the Netherlands and New Zealand (H91/40: B:4:P1.4, H92/53: B:4:P1.4, H94/5: B:4:P1.4, H97/47: -:14:P1.4, H97/81: B:14:P1.4, H97/122: B:1:P1.4, H96/211: C:2a:P1.4). The SBA activity against the wild type isolates were comparable to the isogenic strain [49].

After each vaccination the SBAs were preferentially induced against one of the three PorA proteins of each vesicle (P1.5c,10 and P1.5,2). This may be due to immunological interference or variation in immunogenicity of the different por A subtypes. The percentages of children with a fourfold increase of SBA against the homologous strains of serosubtype P1.5c,10 and P1.5,2 are comparable with results shown by Tappero and Perkins [35, 41]. In the Netherlands, P1.4 is the most frequently isolated serosubtype. Of all meningococcal isolates collected in 1996 from patients, 48% is subtype P1.4 [1]. In the hexavalent meningococcal OMV vaccine this serosubtype (P1.7h,4) induced the lowest SBA response. The question remains if the vaccine has to be improved or that the antibody response is sufficient for protection. Phase 3 efficacy studies need to be done to show if the amounts of these antibodies found after 3 vaccinations are sufficient for protection. Goldschneider et al. have reported a protecting SBA titre as 1:4 or greater for antipolysaccharide antibodies [27, 28, 50]. There is also some evidence that SBA titres correlate with protection against serogroup B meningococcal disease [24, 25, 31, 42]. However, SBA tests are not yet standardised and the lowest titre required for protection against meningococcal disease is not known.

In conclusion: Hexavalent meningococcal OMV vaccine is considered to be safe. After 3 hexavalent meningococcal vaccinations increased levels of IgG against OMV and increased bactericidal activity were measured with the highest levels in the youngest children. Fourfold rise in bactericidal antibody titre in vaccinated children assumed to be indicative for protection varied for the different meningococcal serosubtypes. SBA activity against the wild type isolates was comparable to the isogenic strains.

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Chapter 3

Serum bactericidal activity and isotype distribution of antibodies in toddlers and schoolchildren after vaccination with RIVM hexavalent PorA vesicle vaccine

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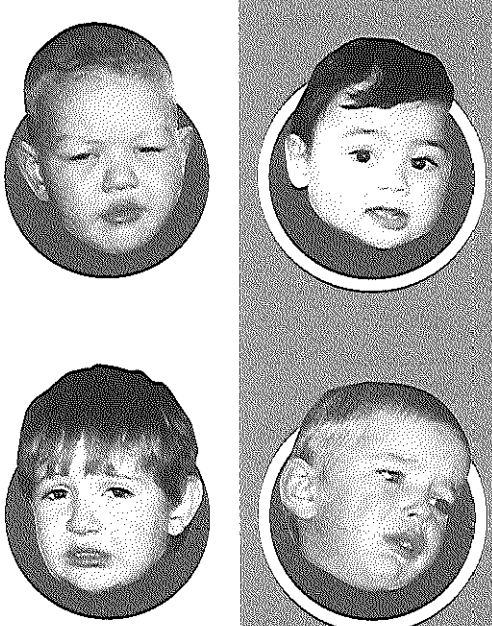

Abstract

A clinical phase II trial with the RIVM hexavalent OMV vaccine containing 6 different PorAs was carried out in toddlers (2-3 years) and schoolchildren (7-8 years) in the Netherlands. Children were vaccinated 3 times (0-2-8 months). Sera after 2 and 3 vaccinations were analyzed for bactericidal activity (SBA) and isotype distribution in whole cell ELISA. The SBA after vaccination against the 6 PorAs was significantly different. We investigated whether the age specific and PorA specific differences in SBA titers correlated with differences in PorA specific IgG isotype distribution The SBA titers were higher in toddlers compared with schoolchildren. After vaccination, IgG1 antibodies dominated the response followed by IgG3 antibodies. IgG2 levels were low, whereas IgG4 was not detected. Irrespective of PorA, IgG total and isotype specific titers after 2 and 3 vaccinations were significantly higher in toddlers than in schoolchildren. A weak correlation was found between IgG total or IgG1 and SBA. Although the immunogenicity of the 6 PorAs is very different, the isotype distribution was similar for all 6 tested PorAs.

We conclude that the RIVM hexavalent PorA vesicle vaccine induces bactericidal antibodies mainly of the IgG1 and IgG3 isotypes which are considered to be most important for protection against disease. The isotype distribution of the response is not age dependent.

Introduction

Meningococcal disease remains a major health problem in many countries. Clinical manifestations vary from self-limiting bacteremia to meningitis or fulminant sepsis. IgG antibodies are important for protection against meningococcal infections. IgG antibodies exert two major effector functions: activation of complement and opsonisation. Not all IgG isotypes are equally effective in protective effector functions such as complement mediated bactericidal activity and opsonophagocytosis. IgG1 and IgG3 are most effective for complement binding and activation, whereas IgG2 is only effective at high epitope density. [1-4]. IgG4 does not activate complement. All IgG isotypes have the ability to induce phagocytosis mediated by FcγR and complement receptors, but only IgG1 and IgG3 are highly effective [1, 5]. The IgG isotype distribution in specific antibody responses has been found to vary with the structure of the antigen, its dose and route of entry as well as with the genetic constitution and age of the host. IgG1 and IgG3 are mainly directed against protein antigens [6, 7] whereas IgG2 antibodies are induced by vaccination with polysaccharide antigens [8]. IgG4 antibodies appear only after prolonged antigenic stimulation [9].

Vaccines based on non-capsular surface antigens of serogroup B have been developed and used in several trials [10-12]. A hexavalent vesicle vaccine has been developed at the RIVM in which six PorA-proteins (P1.7,16; P1.5a,2c; P1.19,15a; P1.5c,10; P1.12a,13; P1.7b,4) are embedded in outer membrane vesicles [13, 14]. Immunogenicity studies with different immunisation schedules were carried out in English infants [15] and Dutch toddlers and schoolchildren [16]. This vaccine was safe en well tolerated. The hexavalent vaccine is immunogenic in infants [15] and in toddlers and schoolchildren [16], although multiple doses of vaccine are required to induce a significant serum bactericidal activity (SBA) and differences are found in the magnitudes of SBA responses to different PorA's. This variability may be caused by differences in immunogenic properties of the various PorA antigens. Interestingly, the percentage of responders with a fourfold increase in SBA was highest in infants followed by toddlers and the least in the schoolchildren. For SBA against meningococci of serogroup B, is the generation of adequate levels of IgG1 and IgG3 important and this is possibly age-dependent [17]. The aim of this study was to test whether the differences in SBA are due to differences in IgG isotype distribution in these two age groups after the second (post-primary) and third (post-boost) vaccination. Furthermore, we studied whether the differences in SBA titres against the various PorAs are related to differences in IgG isotype distribution to the various PorAs.

Materials and methods

Vaccine

The hexavalent meningococcal vesicle vaccine was produced using two different trivalent Neisseria meningitidis strains, PL16215 (Capsular polysaccharide- (CPS-), P1.7,16,5a,2c,19,15a) and PL10124 (CPS-, P1.5c,10,12a,13,7b,4) [13]. Each strain produces three different class I (PorA) proteins. About 90% of the vaccine protein consisted of PorA. Low amounts of class 4 and class 5 outer membrane proteins (OMP) are also present. The vaccine consists of 2.6-10% GalE LPS relative to the protein content. In this study, only the sera from the vaccinees who received 100 µg total protein (lot E 9281) were tested. In this vaccine, 15 µg of every single PorA is present. Production, characterization and control of the hexavalent PorA OMV vaccine have been described by Claassen et al.[18].

Vaccinees, immunization schedule and sampling

An open randomised, placebo controlled phase II study, approved of by the Medical Ethical Committee of the Academical Hospital Rotterdam, was carried out in 172 healthy toddlers (2-3 years old) and 165 healthy schoolchildren (7-8 years old) in Rotterdam, the Netherlands. The study design has previously been described [16]. Vaccinations were given at 0, 2 and 8 months. In this study serum samples of 60 toddlers and 61 schoolchildren that received the highest dose (100 µg protein) were studied. Serum samples from 4-6 weeks after the second (post primary) and 4-6 weeks after the third vaccination (post boost) were analysed.

Bactericidal assay

The bactericidal activity against strain H44/76 (B:15:P1.7,16) and the 5 isogenic PorA strains (TR52, TR15, TR1213, TR4 and TR10) was determined in post primary and post booster vaccination serum samples from 60 toddlers and 61 schoolchildren. Human complement (10%) served as an exogenous complement source [19]. The serum bactericidal titer was calculated as the 2 log reciprocal of the serum dilution yielding $\geq$ 90% killing.

Whole cell ELISA

IgG total and isotype antibody titres to whole cells from H44/76 (P1.7,16) and 5 isogenic strains: TR52 (P1.5a,2c); TR10 (P1.5c,10); TR1213 (P1.12a,13); TR15 (P1.19,15a); TR4 (P1.7b,4) and H1.--5 (PorA deficient strain) were determined by enzyme linked immunosorbent assay (ELISA). We used class 3 and 4 deficient variants of these strains to minimize measurement of responses to other proteins then PorA. Bacteria were grown overnight at 37° C on GC agar plates. The bacteria were resuspended in sterile phosphate-buffered saline (PBS), heat inactivated at 56° C for 30 minutes and then diluted to an optical density (OD) of 0.1 at 620 nm. Flat-bottomed 96-well microtitre plates (Nunc, Immulon 2) were coated overnight at 37° C with inactivated bacteria in

PBS. After overnight incubation the plates were washed three times with water-Tween-80 (0.03%). The plates were then incubated for 90 minutes at 37° C with threefold serial dilutions of the serum samples in PBS containing 0.1% Tween-80. After incubation, the plates were washed again three times. The following conjugates (CLB, The Netherlands) were used: mouse anti-human IgG1 conjugated to horseradish peroxidase(HRP) (clone MH; 1:4000) mouse anti-human IgG2-HRP(clone MH; 1:200), mouse anti-human IgG3-HRP(clone MH; 1:100) and mouse anti-human IgG4-HRP (clone MH 64.4; 1:200). Total IgG was measured using anti-human IgG-HRP (DAKO;1:5000). All conjugates were diluted in PBS containing 0.1% Tween-80 and 0.5% skimmed milk powder (Protifar, Nutricia, The Netherlands) and added to the plates. Plates were sealed and incubated at 37° C for 90 minutes. Plates were washed again and 100 µl of the peroxidase substrate (3,3',5,5'-tetramethylbenzidine with 0.01% H_2O_2 in 0.11 M sodium acetate buffer pH 5.5) was added to each well, which was incubated for 10 minutes at room temperature. The reaction was stopped by adding 100 µl of 2M H_2SO_4 (SVM, The Netherlands) to each well. IgG antibody titres were expressed as the 10 log of the serum dilution giving 50% of the ODmax.

Statistical methods

Wilcoxon signed ranks test was used to evaluate the significance of differences between the titers after 2 and 3 immunizations. Mann-Whitney test was used to evaluate the significance of differences between the responses between toddlers and schoolchildren. The Spearman correlation coefficients were determined in the correlation analyses between isotype and SBA. All statistical analyses were performed with SPSS (version 9.0) for windows software.

Results

SBA

The serum bactericidal antibody response was assessed against 6 isogenic PorA strains (Fig.1). Overall toddlers had higher bactericidal antibody titers than schoolchildren after the primary series and after the boost vaccination. The SBA titers against P1.5c.10 P1.5a,2c and P1.12a,13 were significantly higher in toddlers than in schoolchildren. The SBA titers against all isogenic strains increased significantly after the booster immunization in toddlers and schoolchildren (Fig 1).

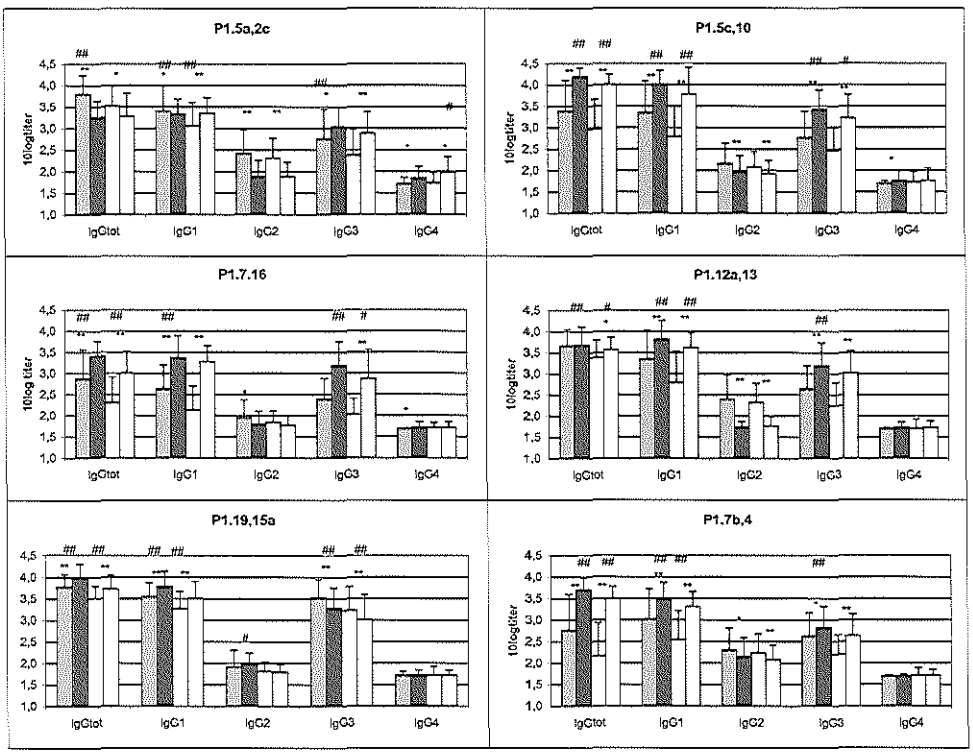

Figure 1 Serum bactericidal antibodies against six different PorA variants of *Neisseria meningitidis* in sera of toddlers and schoolchildren after 2 and 3 immunizations with a hexavalent OMV vaccine.
* significant differences between SBA titers post primary and post boost
# significant differences between SBA titers of toddlers and schoolchildren

toddlers 2: post 2 vaccination
toddlers 4: post third vaccination
schoolchildren 2: post 2 vaccination
schoolchildren 4: post third vaccination
Legend: see figure 2

The highest bactericidal titers were found against P1.5c,10, followed by P1.5a,2c. Intermediate bactericidal responses were seen against P1.12a,13 and P1.7,16. The lowest response was found to P1.7b,4 and P1.19,15a in both toddlers and schoolchildren.

IgG total and isotype distribution

The total IgG and isotype profiles of anti-PorA antibodies were investigated in toddlers and schoolchildren vaccinated with the hexavalent RIVM OMV vaccine. Serum samples from 4-6 weeks after the second (post primary series) and 4-6 weeks after the third vaccination (post boost) were analyzed. Irrespective of the PorA, IgG total and isotype titers found after 2 or 3 immunizations were significantly higher in toddlers compared with schoolchildren (Fig 2). After 2 immunizations IgG1 antibodies dominated the response followed by IgG3 and low levels of IgG2. No IgG4 was detected. The IgG1 and IgG3 antibody titers increased significantly after the third vaccination compared with the second vaccination in toddlers and in schoolchildren. In contrast, the IgG2 responses decreased significantly. No differences were found in the isotype distribution pattern between toddlers and schoolchildren. In both age groups the distribution was IgG1>IgG3>IgG2>IgG4.

The levels of antibody responses to the 6 different PorA varied. After two vaccinations the IgG titers against P1.5a,2c were the highest, followed by P1.5c,10; P1.12a,13; P1.19,15a; P1.7,16 and P1.7b,4 in both toddlers and schoolchildren. After 3 vaccinations the highest IgG titers were measured against P1.5c,10 followed by P1.12a,13, P1.7,16 and P1.5a,2c. The isotype distribution pattern to the 6 different PorA was similar: IgG1>IgG3>IgG2>IgG4.

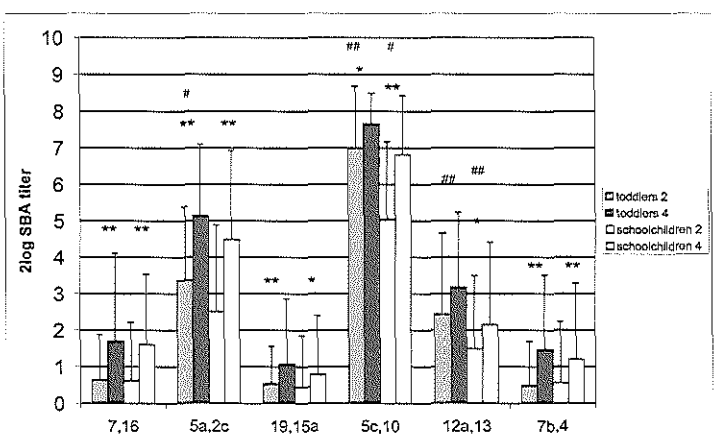

Figure 2 IgG total and isotype concentrations against six different PorA variants of *Neisseria meningitidis* in sera of toddlers and schoolchildren after 2 and 3 immunizations with a hexavalent OMV vaccine.
* significant differences between IgG titers post primary and post boost
# significant differences between IgG titers of toddlers and schoolchildren

The responses were mainly directed against PorA as the SBA and IgG total antibodies titers to HI-5 were very low (data not shown).

The Spearman correlation coefficient (r) was determined to explore whether there was a correlation between the levels of specific antibodies in serum and SBA. Overall a weak correlation was found between the antibodies measured in ELISA and SBA. Mostly IgG total or IgG1 correlated significantly with SBA (see tabel 1). The correlation coefficients were higher in schoolchildren compared to toddlers.

Por A	Serum sample		Total group	Toddlers	Schoolchildren
P1.7,16	post primary	IgG total	0.21 p=0.02	n.s.	n.s.
		IgG1	0.27 p<0.01	0.29 p=0.03	n.s.
		IgG3	n.s.	n.s.	n.s.
P1.7,16	post boost	IgG total	0.24 p<0.01	n.s.	0.40 p<0.01
		IgG1	0.31 p=0.01	n.s.	0.55 p<0.01
		IgG3	n.s.	n.s.	n.s.
P1.5a,2c	post primary	IgG total	0.23 p=0.01	n.s.	n.s.
		IgG1	0.26 p<0.01	n.s.	n.s.
		IgG3	n.s.	n.s.	n.s.
P1.5a,2c	post boost	IgG total	0.26 p<0.01	n.s.	0.29 p=0.02
		IgG1	n.s.	n.s.	n.s.
		IgG3	n.s.	n.s.	n.s.
P1.19,15a	post primary	IgG total	0.37 p<0.01	0.32 p=0.03	0.33 p=0.01
		IgG1	0.46 p<0.01	0.37 p<0.01	0.45 p<0.01
		IgG3	n.s.	n.s.	n.s.
P1.19,15a	post boost	IgG total	0.44 p<0.01	0.38 p<0.01	0.42 p<0.01
		IgG1	0.47 p<0.01	0.34 p=0.01	0.52 p<0.01
		IgG3	n.s.	n.s.	n.s.
P1.5c,10	post primary	IgG total	0.24 p<0.01	n.s.	n.s.
		IgG1	0.37 p<0.01	n.s.	0.27 p=0.04
		IgG3	n.s.	n.s.	n.s.
P1.5c,10	post boost	IgG total	0.48 p<0.01	0.38 p<0.01	0.35 p<0.01
		IgG1	0.25 p<0.01	n.s.	n.s.
		IgG3	0.33 p<0.01	0.28 p=0.04	0.26 p=0.05
P1.12a,13	post primary	IgG total	0.31 p<0.01	0.33 p=0.01	n.s.
		IgG1	0.37 p<0.01	0.31 p=0.02	0.36 p<0.01
		IgG3	n.s	n.s	n.s
P1.12a,13	post boost	IgG total	0.51 p<0.01	0.38 p<0.01	0.55 p<0.01
		IgG1	0.51 p<0.01	0.33 p=0.02	0.64 p<0.01
		IgG3	0.29 p<0.01	0.29 p=0.04	n.s.
P1.7b,4	post primary	IgG total	n.s.	n.s.	n.s.
		IgG1	n.s.	0.27 p=0.04	n.s.
		IgG3	n.s.	n.s.	n.s.
P1.7b,4	post boost	IgG total	0.29 p<0.01	n.s.	0.33 p=0.01
		IgG1	0.41 p<0.01	0.27 p=0.05	0.52 p<0.01
		IgG3	n.s.	n.s.	n.s.

n.s. not significant

Tabel 1 Correlations (Spearman's rho) between SBA and IgG total or IgG1 or IgG3

Discussion

In this study, we measured the SBA and IgG isotype distribution in toddlers and schoolchildren after 2 or 3 immunizations with a hexavalent PorA OMV vaccine. SBA was performed with isogenic strains derived from meningococcal strain H44/76 differing only in their PorA OMP, allowing direct PorA specific comparisons in immune response. The SBA titers were higher in toddlers compared to schoolchildren. De Kleijn et al., showed that the percentage of responders with a fourfold increase in SBA was also higher in toddlers compared to schoolchildren [16]. Furthermore, a study in UK infants [15] showed an even higher percentage of responders after 4 doses of this vaccine. These results indicate that the SBA response after vaccination with hexavalent vaccine is inversely related to age. Earlier studies in Chile and Brazil with monovalent OMV vaccines showed poor responses to these vaccines in young children both for efficacy and for SBA [11, 12, 20]. A randomized controlled trial in Chile demonstrated that infants were capable of inducing SBA after 3 doses of monovalent vaccine developed in either Cuba or Norway to the vaccine strain [21]. Comparison of the various clinical studies indicates that a hexavalent vaccine may be more suitable for infants compared to a monovalent vaccine.

We found differences between toddlers and schoolchildren in the amounts of antibodies but no differences in the isotype distribution, both after the primary series and after the booster vaccination. The IgG response was mainly PorA specific since the titers were significant lower when the PorA negative strain was used as antigen. IgG total, IgG1 and IgG3 responses to the 6 PorAs were higher in toddlers compared to schoolchildren. In these children, the RIVM hexavalent PorA vesicle vaccine was capable of inducing antibodies of the IgG1 and IgG3 isotypes which are considered to be most important for protection against disease. Therefore, the IgG isotype distribution does not seem to provide an explanation for the difference in SBA titres between toddlers and schoolchildren. IgG1 and IgG3 predominance in response was also observed after invasive infection with N. meningitidis [17] and in adult volunteers receiving the Norwegian monovalent vaccine [2]. So it seems that the type of antigen determines the isotype distribution and not the age of the vaccinees. In addition, the age differences in SBA are not related to isotype distribution of the antibody response but probably to the immunogenicity of the antigens in the various age groups.

In contrast to the total IgG, IgG1 and IgG3 responses, IgG2 responses decreased significantly after the third immunization. IgG2 antibodies are often induced against polysaccharide antigens. Probably these antibodies are directed against LPS as most of IgG2 antibody titers remained on HI-5, a PorA deficient strain (data not shown). LPS is considered to be a T-cell independent type 1 antigen, and these antigens are capable of inducing an immune response in neonates but do not induce immunological memory [22]. This is in agreement with the fact no booster response was observed in LPS ELISA after the third dose [23] in contrast to the anti-OMV response.

The hexavalent vaccine induced high SBA against P1.5c,10 and P1.2a,5c, moderate SBA against P1.7,16 and P1.12a,13 and low SBA to P1.7b,4 and P1.19,15a confirming earlier results that the SBA response was subtype specific. The isotype distribution showed a similar pattern for each PorA, indicating that the differences in SBA were not caused by differences in isotype distribution.

The isotype distribution induced by the RIVM hexavalent vesicle vaccine for each subtype of PorA was comparable with the isotype distribution found after immunization with the Norwegian group B meningococcal outer membrane vesicle vaccine in adult volunteers [2, 3, 5, 23]. Using the Norwegian H44/76 vaccine, highly significant correlations (r= 0.7 to 0.9) between levels of IgG antibodies to OMV and SBA against strain H44/76-1 (OpC++) were observed as was previously observed in adults [3, 5, 23]. In this study only weak correlations were observed between the bactericidal titers and the levels of total IgG antibodies or any of the isotype specific titers (r=0.2 to r=0.64, $p< 0.01$). This may be due to the age of the vaccinees, the vaccine or to the differences in ELISA or SBA assay. There are some differences in the used methods. We used whole cells of class 3 and 4 deficient strains to coat the ELISA plates versus OMV in the Norwegian protocol. This may have some influence on the epitope exposure and therefore influence the binding of specific antibodies. Furthermore, our SBA is only directed against PorA and killing is measured at 90% whereas a part of SBA activity induced by the Norwegian vaccine is also directed against Opc while 50% is used as cut-off value [23]. This may influence the titer obtained in the bactericidal assay. The Norwegian and hexavalent OMV vaccines vary with respect to the amount of total protein, the sero-subtypes of proteins and LPS-immunotypes present within the OMVs and the adjuvant used. Monovalent vaccines give good responses in adults with good correlation between induced IgG and functional activity of these antibodies. Only one PorA is present and SBA is directed against VR1 and/or VR2 of p1.7,16. In children the hexavalent vaccine induces a good response but a weak correlation between IgG total, IgG1 or IgG3 and SBA. But the correlation coefficients were higher in the schoolchildren as compared to the toddlers. In the hexavalent vaccine 6 different PorAs are present which only differ in their VR1 and VR2 region. Probably due to the high amount of PorA proteins in the vaccine, the vaccine induces antibodies specific to PorA in children but not all of these antibodies are functionally active. The ratio of total antibodies measured by ELISA and functional antibodies measured by SBA is probably lower in children compared to adults. Milagres et al [12] found a poor relation between the fold increases in antibody levels measured by ELISA and SBA in children immunized with the Finlay vaccine.

This study shows that measuring antibody responses to PorA by ELISA, even an isotype specific ELISA, does not predict the functional activity of these antibodies in children. The used ELISA technique identifies both high and low avidity antibodies and therefore quantitative differences observed in ELISA do not necessarily correlate with differences in functional capacity. For polysaccharides of *Streptococcus pneumoniae* [24] and *Haemophilus influenzae* type b [25], the amount of antibodies needed for functional activity (opsonophagocytosis) was depending on

the avidity of the antibodies. For meningococci of serogroup C, a high avidity ELISA showed to correlate better with SBA than a normal ELISA [26].

In conclusion the RIVM hexavalent PorA vesicle vaccine is capable of inducing bactericidal antibodies in children. The isotypes of the induced antibodies are mainly IgG1 and IgG3 which are considered to be most important for protection against disease. The isotype distribution of the response is not age dependent and does not explain the finding that younger children have a higher SBA response than older children. It is therefore suggested that functional activity of antibodies is epitope specific.

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Chapter 10

Immunogenicity and safety of monovalent P1.7[h],4 meningococcal outer-membrane-vesicle vaccine in toddlers: comparison of two vaccination schedules and two vaccine formulations.

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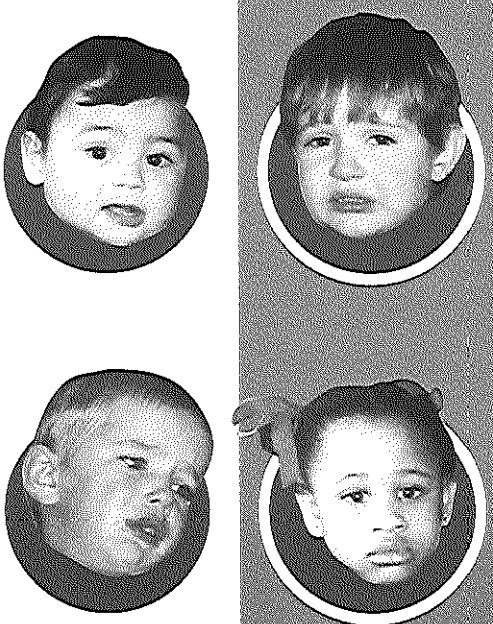


Abstract

The safety and immunogenicity of two PorA-based meningococcal outer membrane vesicle (OMV) vaccines against the P1.4 serosubtype adsorbed with $AlPO_4$ or $Al(OH)_3$ were studied in 134 toddlers. Vaccinations were given 3 times with an interval of 3-6 weeks or twice with an interval of 6-10 weeks. A vaccination was repeated after 20-40 weeks. Pre- and post immunization sera were tested for bactericidal activity against an isogenic strain expressing $P1.7^h$,4 PorA. Both meningococcal OMV vaccines were well tolerated. The percentage of children with a fourfold increase in bactericidal activity was 96% ($AlPO_4$ adjuvated vaccine/ 2+1-schedule), 100% ($AlPO_4$ adjuvated vaccine/3+1-schedule), 93% ($Al(OH)_3$ adjuvated vaccine/2+1schedule) and 97% ($Al(OH)_3$ adjuvated vaccine/3+1-schedule). Adsorption with $AlPO_4$ makes the OMV vaccine more immunogenic than adsorption with $Al(OH)_3$. Bactericidal activity was highest after the 3+1 schedule, although the response shortly after the primary series was higher in the two dose priming group.

Introduction

In the Netherlands as well as in many other countries, *Neisseria meningitidis* serogroup B strains are the major cause of invasive meningococcal disease [1]. Immunization is the best strategy to decrease mortality and morbidity due to this disease, which is predominantly seen in young children. The immune response after exposure to serogroup B meningococci is mainly directed against subcapsular antigens (outer membrane proteins (OMP) and lipooligosaccharides) [2-6]. Previously, OMP vaccines have been tested in efficacy trials. The protective rates varied between 50 and 80% in children older than 5 years of age. None of these vaccines showed adequate protection in infants [7-10]. The National Institute of Public Health and Environment (RIVM) of the Netherlands has developed a meningococcal vaccine based on class 1 outer membrane proteins (PorA) [11-14]. PorA is considered to be one of the major protein antigens which induces serum bactericidal activity (SBA) [2, 6, 15-17]. The RIVM hexavalent meningococcal OMV vaccine with six different PorA proteins has previously been tested in UK infants [18] and in Dutch toddlers and school children [19] for safety and immunogenicity. The vaccine was immunogenic in all age groups. Natural serum bactericidal antibodies (SBA) are associated with protection against clinical disease [20, 21]. A fourfold or higher rise of SBA after vaccination is presumed to be associated with the same protection [22]. More than 90% of vaccinated children had minimal a fourfold rise in titer after 3 or 4 doses of vaccine. However, significant differences were found in the magnitude of SBA responses to different PorA's. Serosubtype P1.4 is the most common serosubtype of the strains isolated from patients in the Netherlands [1] and is also the serosubtype causing an epidemic of meningococcal disease in New Zealand [23]. Anti-P1.4 responses were weaker than responses against other PorA's after administration of the hexavalent meningococcal OMV vaccine [19]. The interference between three different PorA's on one vesicle or a lower immunogenic potential of the P1.4 antigen compared to other antigens in the vaccine may account for this effect. Therefore, the RIVM recently developed a monovalent vaccine, using a meningococcal strain expressing P1.7^{h},4 PorA. In the current study, two monovalent P1.7^{h},4 OMV vaccines adsorbed with aluminium-phosphate or with positive charged aluminium-hydroxide were tested in toddlers. Two different vaccine schedules were evaluated consisting of two or three vaccinations in the primary series followed by a booster vaccination.

Materials and methods

Study population

After approval of the protocol by the Medical Ethical Committee of the University Hospital of Rotterdam, parents or legal representatives living in Rotterdam were asked to participate with their 2-3 years old child in this study. Children were included after a health check and after written informed consent was obtained from their parents or guardians. Participants were excluded on the basis of one of the following criteria. Temporary exclusion: administration of plasma products up to 3 months prior to the study, acute febrile illness (temperature > 38.5°C), evidence of serious disease demanding medical treatment, concurrent use of antibiotics. Definite exclusions: known allergy to one of the vaccine components, history of a severe reaction after vaccination, prior meningococcal disease, prior administration of meningococcal vaccine, immunodeficiency, congenital or chronical illness or a neurological disorder. No other vaccinations were given during this study.

Contra-indicating events following vaccination with the monovalent meningococcal OMV vaccines included: a temperature > 41.0 °C, anaphylactic shock and seizures (except febrile seizures). Those children did not receive a subsequent meningococcal vaccination.

Vaccines

Production of the monovalent meningococcal P1.7^{h},4 OMV vaccine is similar as the production of the hexavalent OMV vaccine as described by Claassen et al [11]. The seed strains for monovalent vaccine contain 2 porA genes encoding for P1.4 instead of 3 different porA genes per production strain encoding for six different serosubtypes as described for the hexavalent vaccine. Class 4 protein was not expressed in the monovalent seed strain; neither was PorB or capsular polysaccharides expressed. In both vaccines LPS of the galE type (a variant of LPS) and Opa and Opc are present at a low level. The monovalent vaccine contained the same amount of P1.4 protein –15 microgram per vaccine- as the high dose hexavalent vaccine. The contents per 0.5 ml dose of the two monovalent meningococcal vaccines (pH 7.4) were: 17 microgram protein of which 88% P1.7h,4 class 1 protein, 50 microgram thiomersal, 50 microgram sucrose and 1.34 mg $AlPO_4$ or 0.86 mg $Al(OH)_3$ in either vaccine.

Study design

A randomized, blinded and comparative study was designed with the two monovalent meningococcal OMV vaccines differing with respect to adjuvant and two different vaccination schedules. The vaccines and vaccination schedules were allocated by a list of random numbers. The vaccines with the two different adsorbents had identical appearance and differ only by colored caps and lot numbers (red: 80003A and blue: 80002A). The investigators did not know the keys to these differences. All children received three or four vaccinations at home from

the research-physician involved in this project (EdK). Vaccines (0.5 ml) were given by injection in the deltoid muscle with a 0.8 x 40 mm needle. One group of children received monovalent meningococcal OMV vaccine with $Al(OH)_3$ as adsorbent in two different vaccination schedules. The other children received monovalent meningococcal OMV vaccine with $AlPO_4$ as adsorbent also in two different vaccination schedules. The two vaccine-schedules used were: 3 vaccinations with a time interval between the vaccinations of 3 to 6 weeks or 2 vaccinations with a time interval of 6 to 10 weeks. A booster vaccination was given 20 to 40 weeks after the last vaccination. Blood samples were collected before each vaccination and 4 to 6 weeks after the last vaccination in the primary series and after the booster vaccination. Blood samples were collected by venipuncture during a home visit and sent to the RIVM, Bilthoven, the Netherlands by regular mail. Serum was stored at –20 °C until distribution of aliquots for blinded specific antibody measurements.

Monitoring for adverse reactions

All participants were monitored 15 minutes following vaccine administration for adverse reactions. The parents were asked to keep a diary for seven days to record local and systemic adverse reactions. Eighteen to 30 hours after each vaccination, the parents were contacted by telephone to assess the occurrence of side effects by a structured interview. When necessary, a child was seen by the research-physician. During the next visit adverse reactions, covering the 7 days period after vaccination, were registered by a structured interview and assessment of the diaries. Monitoring of local adverse reactions included the presence and size of redness, the presence and size of swelling, itching, degree of pain and the degree of not using the arm. Systemic adverse reactions registered were the presence of fever (>38.5 °C), the temperature, headache, listlessness, decreased appetite, nausea, joint complications, cutaneous symptoms, non-attendance to day-care, sleepiness, unusual crying, use of medication, contacts with the health care system and illness in the family.

Serum bactericidal activity assay

Bactericidal activity of the sera against the serosubtype P1.7b,4 isogenic variant of *N. meningitidis* type B strain H44/76 was determined as described by Peeters [12] and Rouppe van der Voort [24].

In short, the strain was spread on a GC-agar plate with 1% isovitalex (SVM, Bilthoven, the Netherlands) and incubated at 37°C for 18-20 hours in 5% CO_2. Single colonies are picked from this plate 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 A_{620} of 0.07-0.08. The bacteria were grown for approx. 1½ hours at 140 rpm in an orbital shaker until an A_{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% BSA (ICN, Irvine, USA) to $\approx 10^5$ cfu/ml. To each well of the microtiter plate 6 μl (2.5-5.0 x 10^2 cfu) of this dilution was added to 12 μl of 2-fold dilutions of heat inactivated sera

(56°C for 30 min) 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 performed in triplicate: 7 μl of a well with only bacteria, complement and GBSS/BSA was spread on a GC agar plate with 1% isovitalex and incubated overnight.

The microtiter plates were then incubated at 37 °C, 5% CO_2 for 60 minutes. Subsequently 7μl of the suspension in each well was spotted onto GC agar plates with 1% isovitalex. After 18-20h incubation at 37°C in 5% CO_2, the number of cfu on time zero plates was counted. The average number of cfu at time zero 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 $\geq$90% killing. The percentage of children with fourfold rise in SBA titer was determined.

Statistics

Statistical analysis was done using SPSS (version 9.0). Chi-squared tests were used to evaluate the significance of the differences of local and systemic reactions between the two different monovalent meningococcal vaccines. The Mann-Whitney U test was used to determine the significance of differences between bactericidal GMT titers. A p-value <0.05 was considered to be statistically significant.

Results

Study population

In total, 134 toddlers (58 boys / 76 girls) participated in this study. The mean age at inclusion in the study was 2.8 years (range 2.3-3.4; SD 0.3 years). The monovalent vaccine with $AlPO_4$ as adsorbent was administered three times to 32 children and four times to 35 children. The monovalent vaccine with $Al(OH)_3$ as adsorbent was administered three times to 32 children and four times to 35 children. Six participants dropped out during the study, without any adverse event as a reason for withdrawal (3 times $Al(OH)_3$: 2 times 2 + 1schedule, 1 time 3+1schedule and 3 times $AlPO_4$: 3 times 3+1 schedule). The children, who received two vaccinations in the primary series, had their vaccinations with a mean of 44 days (range 41-56) after the first vaccination. The children, who received three vaccinations in the primary series, were vaccinated with mean time intervals of 29 days (range 26-41) between the vaccinations. The booster vaccination was given after a mean interval of 162 (range 130-211) days after the last vaccination.

Adverse reactions

No serious adverse events were observed. In general only mild local and systemic reactions occurred (table 1). 31% of the toddlers complained about a mild painful arm, with redness around the injection side in 7.2% of the children. Systemic reactions were less common; 8.6% of the toddlers felt listlessness the day after vaccination and 2.3% of the toddlers had fever (>38.5 °C). All reactions peaked within 48 hours after vaccination and declined rapidly within one week. The reactions were not aggravated by subsequent vaccinations. Local and systemic reactions were alternately present in the total group of children. Pain, redness and listlessness were more frequently seen after the monovalent meningococcal OMV vaccine with $AlPO_4$ as adsorbent than with $Al(OH)_3$ as adsorbent (Table 1).

Serum bactericidal activity

In a small number of children the volume of blood obtained was too small to permit completion of the serological tests (n=15).

The summarized results of the serum bactericidal activity (SBA) tests against the meningococcal vaccine strain $P1.7^h,4$ are shown in Figure 1a and 1b and Table 2. Bactericidal titers were statistically significant increased after the second vaccination with monovalent $P1.7^h,4$ meningococcal OMV vaccine in comparison with prevaccination titers ($p<0.01$) in both vaccine groups and both schedules. After the second vaccination, 66% of the toddlers showed a fourfold or higher increase in SBA (Figure 2a and 2b). There was no important increase in SBA after the third vaccination in the three plus one vaccination schedule. The SBA titers decreased significantly after the 2d vaccination (Figure 1a) or the 3d vaccination (Figure 1b) within 20 to 40 weeks, but the prebooster titers were still significantly higher than prevaccination titers ($p<0.01$). The highest titers were

Table 1 Adverse reactions

Toddlers	Vaccination 1 Day 1				Vaccination 2 Day 1				Vaccination 3 Day 1				Vaccination 4 Day 1			
Reaction	$AlPO_4$ (N=67)		$Al(OH)_3$ (N=67)		$AlPO_4$ (N=65)		$Al(OH)_3$ (N=66)		$AlPO_4$ (N=65)		$Al(OH)_3$ (N=65)		$AlPO_4$ (N=31)		$Al(OH)_3$ (N=34)	
	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%
Systemic reactions																
Fever (> 38.5 °C)	1	1.5	2	3.0	3	4.6	1	1.5	1	1.5	3	4.6	1	3.2	0	
Headache	1	1.5	0		0		1	1.5	1	1.5	0		0		0	
Listlessness	10	14.9	3	4.5 *	9	13.8	2	3.0 *	6	9.2	3	4.6	5	16.1	1	2.9
Unusual crying	2	3.0	3	4.5	0		0		0		1	1.5	0		0	
Less appetite	5	7.5	1	1.5	1	1.5	1	1.5	2	3.1	0		1	3.2	1	2.9
Nausea	1	1.5	1	1.5	0		0		0		0		0		0	
Joint complication	0		0		0		0		0		0		0		0	
Cutaneous symptoms	0		1	1.5	0		0		0		0		0		0	
Absence in daycare	0		0		0		0		0		0		0		0	
Medication	1	1.5	1	1.5	0		0		0		0		0		0	
Health Care contact	0		0		0		0		0		0		0		0	
Family illness	0		1	1.5	0		1	1.5	0		0		0		0	
Local reactions																
Redness <2.5 cm	8	11.9	5	7.5	6	9.2	5	7.6	5	7.7	1	1.5	4	12.8	0	*
Redness >2.5cm	0		0		0		0		0		0		0		0	
Swelling <2.5 cm	3	4.5	5	7.5	3	4.6	0		2	3.1	0		2	6.5	2	5.9
Swelling >2.5cm	1	1.5	0		0		0		0		0		0		0	
Itching	0		0		0		0		1	1.5	0		0		0	
Pain mild	23	34.3	8	11.9 **	21	32.3	16	24.2	22	33.8	18	27.7	10	32.3	17	50.0
Pain severe	0		0		0		0		0		0		0		0	
Not using arm	1	1.5	0		0		0		1	1.5	0		0		0	

** $p < 0.01$
* $p < 0.05$

Vaccination schedule 2+1				
Blood sample	**Vaccine**	**GMT (P1.7h,4)**	**% SBA>=4**	**N**
Prevaccination 1	$AlPO_4$	1	0	32
	$Al(OH)_3$	1	0	30
Prior to 2^d vaccination	$AlPO_4$	1.02	0	32
	$Al(OH)_3$	1	0	30
After 2^d vaccination	$AlPO_4$	8.72	81.2	32
	$Al(OH)_3$	5.08	62.1	29
Prebooster	$AlPO_4$	1.27	9.4	32
	$Al(OH)_3$	1.17	6.5	31
Postbooster	$AlPO_4$	25.6	96.4	28
	$Al(OH)_3$	25.0	92.9	28

Vaccination schedule 3+1				
Blood sample	**Vaccine**	**GMT (P1.7h,4)**	**% SBA>=4**	**N**
Prevaccination 1	$AlPO_4$	1	0	35
	$Al(OH)_3$	1	0	34
Prior to 2^d vaccination	$AlPO_4$	1.05	0	30
	$Al(OH)_3$	1.04	3.1	32
Prior to 3^d vaccination	$AlPO_4$	7.82	68.7	32
	$Al(OH)_3$	2.95 *	53.1	32
After 3^d vaccination	$AlPO_4$	7.66	78.1	32
	$Al(OH)_3$	4.90	58.8	34
Prebooster	$AlPO_4$	1.43	16.1	31
	$Al(OH)_3$	1.18	6.1	33
Postbooster	$AlPO_4$	70.4	100.0	29
	$Al(OH)_3$	46.3	96.9	32

* $p<0.05$

Table 2 Geometric mean titres (GMTs) for serum bactericidal antibodies and percentage of children with at least a fourfold increase in bactericidal antibody titres after monovalent meningococcal P1.7h,4 OMV vaccine

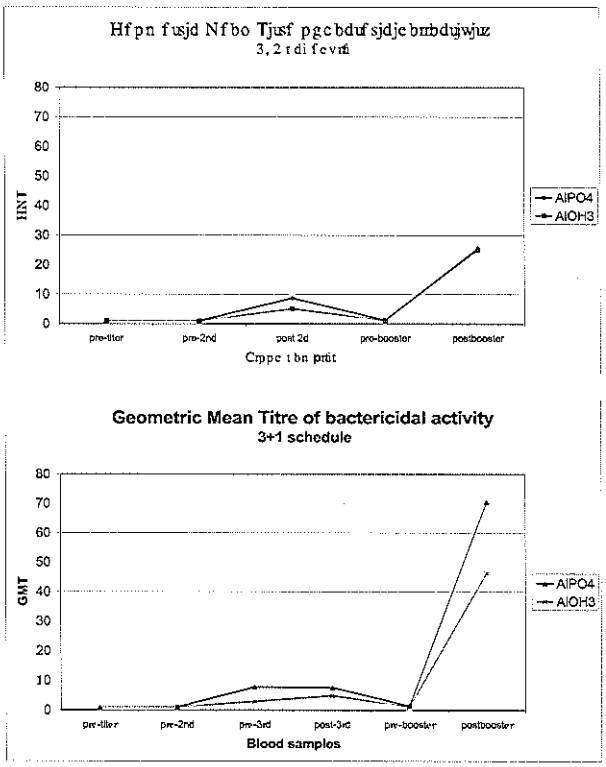
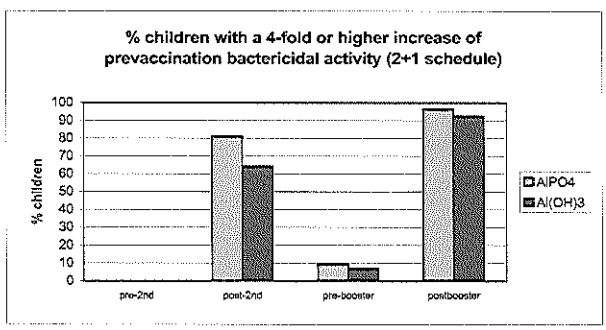


Figure 1a and 1b

Geometric mean titers of serum bactericidal antibodies against the vaccine strain for the 2+1 schedule (Figure 1a) and for the 3+1 schedule (Figure 1b). The two lines represent the two different vaccine adsorbents. AlPO4: adsorbent aluminium phosphate. Al(OH)3: adsorbent aluminium hydroxide.

2+1: vaccination schedule with two primary vaccinations followed by a booster vaccination. 3+1: vaccination schedule with three primary vaccinations followed by a booster vaccination.



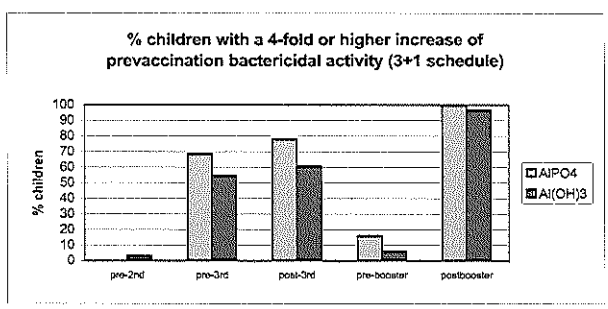


Figure 2a and 2b

Percentage of children with at least a fourfold increase of prevaccination serum bactericidal titer against the meningococcal vaccine strain. Figure 2a: children receiving two primary vaccinations followed by a booster. Figure 2b: children receiving three primary vaccinations followed by a booster.

seen after the booster vaccination: the ratio between the post- and pre-booster titers had a mean of 75. Ninety-seven percent of the toddlers had a fourfold or higher increase of SBA after the last vaccination irrespective the vaccine used. However, after the booster vaccination, a significant difference was observed in bactericidal activity between the children who received three versus two vaccinations before the booster vaccination. SBA titers increased more after three vaccinations than after two vaccinations ($p<0.01$). However, no significant difference was seen in the percentage of children with at least a fourfold increase of SBA titer between the two vaccination schedules: 98% after three vaccinations versus 95% after two vaccinations before the booster vaccination. Children vaccinated with monovalent meningococcal OMV vaccine adsorbed to $AlPO_4$ showed a significant higher increase of bactericidal antibodies in the post primary titers than those who received the $Al(OH)_3$ adsorbed vaccine ($p=0.02$).

Discussion

This study showed that the current monovalent meningococcal OMV vaccine is able to induce bactericidal antibodies in toddlers and did not elicit serious adverse reactions. Adsorption to aluminium phosphate made the PorA expressing vesicles more immunogenic than adsorption to aluminium hydroxide. Three vaccinations before the booster vaccination resulted in higher bactericidal titers than two vaccinations. However, the percentage of children with a fourfold increase in SBA in both groups was more than 90%.

The most interesting observation in this study is the finding that the immunogenicity of the monovalent meningococcal OMV vaccine against P1.4 is superior to that of the previous used hexavalent vaccine [19]. In both trials toddlers were recruited from the same population ([19] and this study). After two hexavalent meningococcal vaccinations and a booster, only 38% of the toddlers had a putative protective SBA level against the P1.7^{h},4 meningococcal strain. However in this study 96% of the children showed at least a fourfold increase in SBA against the same strain after the two plus one vaccination schedule with monovalent vaccine with the same adjuvant as the hexavalent vaccine ($AlPO_4$). The increased immunogenicity of the monovalent vaccine compared to the previously tested hexavalent vaccine, may be due to the absence of interference with other PorA's or class 4 protein on the vesicle, a better production method, or a better immune response to a vaccine containing less antigens. Serum bactericidal activity against the P1.4 monovalent strain was quite similar to that reported by Tappero et al. [15]. In that study 78% of the children vaccinated with Finlay-Institute-produced meningococcal vaccine and 98% of the children vaccinated with National Institute of Public Health, Oslo produced meningococcal vaccine responded with at least a fourfold increase of SBA against the homologous vaccine strain after a schedule with three vaccinations. In our study, 98% of the children responded with a fourfold increase in SBA after four vaccinations and 95% had a fourfold increase in SBA after three vaccinations. This indicates that the higher immunogenicity of the P1.4 vaccine may not be due to the absence of class 4 protein or differences in production method. The hexavalent vaccine used in toddlers, schoolchildren and infants was older then the monovalent vaccine used in this study, however stability experiments showed that the hexavalent vaccine was stable even over a longer period than it was used in this study. It is more likely that the presence of multiple PorA proteins within one vesicle and vaccine affects the immunogenicity for at least the P1.4 antigen. Immunization experiments in mice have shown that interference occurs independent of the presence of various PorA's in the hexavalent formulation or as a mixture of monovalent vaccines. Mice vaccinated with the hexavalent formula or with a mixture of 6 monovalent vaccines showed lower titers against strain P1.4, than mice vaccinated with only the P1.4 monovalent vaccine (Germie van den Dobbelsteen, Loek van Alphen; unpublished observations). Thus, the lower immunogenicity when six serosubtypes are involved in one vaccination may be caused by interference between the vesicles or by the higher number of antigens administered.

The effect of the adjuvants on the extent of the immunogenicity is intriguing. Adsorption to $Al(OH)_3$ was expected to be more efficient than to $AlPO_4$ because of the negative charged vesicles at neutral pH. However, immunogenicity of $AlPO_4$ adjuvated vaccine was better. This suggests that presentation of the vaccine to the immune system is dependent on the interaction with various aluminium-salts.

Serum bactericidal titers after four vaccinations were significantly higher than bactericidal titers after three vaccinations, although the percentage of children with a fourfold rise in SBA was similar after the two vaccination schedules. Also in other studies it was shown that at least three vaccinations are required to obtain high SBA [15, 25]. Whether four vaccinations are necessary is currently not clear. Although high SBA was present, it is not known whether protection against meningococcal disease is achieved. Phase 3 studies with RIVM meningococcal OMV vaccine are needed to demonstrate whether the fourfold increase in SBA confers protection against invasive disease by meningococci.

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Chapter III

Prevention of meningococcal serogroup B infections in children: a protein based vaccine induces immunological memory

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Abstract

The immunological memory against meningococci in children was studied in 177 children (10-11 years: 100, 5-6 years: 77), 2.5 years after vaccination with hexavalent meningococcal outer membrane vesicle (OMV) vaccine or Hepatitis B vaccine. Children were (re)vaccinated with monovalent P1.7^{h},4 meningococcal OMV vaccine. Serum bactericidal antibodies (SBA) were measured before (re)vaccination and 4-6 weeks afterwards. A minimal fourfold increase in SBA against serosubtype P1.7^{h},4 was detected in 48.5% of the children after hexavalent meningococcal vaccine and in 8.9% after hepatitis B vaccine. Seventy-eight percent of the initial responders on hexavalent meningococcal vaccine showed again a minimal fourfold increase in SBA against strain P1.4.

We conclude that immunological memory is present in toddlers and schoolchildren, who previously received three hexavalent meningococcal vaccinations. The booster vaccination with monovalent P1.7^{h},4 meningococcal OMV vaccine induces a significant increase in SBA against serosubtype P1.7^{h},4 and cross reactivity against other serosubtypes present in the hexavalent vaccine.

Introduction

Meningococcal disease is a major cause of sepsis and meningitis especially in young children. Serogroups A, B and C meningococci are responsible for 90% of the cases. Vaccines based on polysaccharides or a combination of polysaccharides with a carrier protein do protect against infection by serogroups A, C, Y and W135. In contrast, capsular polysaccharides of serogroup B are poorly immunogenic and have structural homology with human embryonal neural tissue. Outer membrane proteins have been used as alternative immunogenic components in a vaccine against serogroup B meningococci. Immunization of adults with meningococcal outer-membrane vesicle vaccines results in an antibody response, in which the serum bactericidal activity (SBA) is predominantly associated with class 1 proteins [1-4]. Class 1 protein (PorA) is heterogeneous, accounting for multiple serosubtypes. Comparison of porA genes shows that this diversity is based on two distinct variable regions, VR1 and VR2, located to the apices of loops 1 and 4 of the protein [5]. Bactericidal antibodies are directed to these regions [6-8]. At the moment, SBA forms the best serologic correlate to estimate the protective potential of OMP serogroup B meningococcal vaccines [2, 9-11].

We previously performed a randomized, controlled study in 337 Dutch children with hexavalent meningococcal OMV vaccine composed of six different class 1 OMP's expressing serosubtypes P1.7^{h},4 / P1.12,13 / P1.5^{c},10 / P1.5,2 / P1.7,16 and P1.19,15 or with hepatitis B vaccine (controlgroup) [12]. After three vaccinations, the percentage of children with a minimal fourfold increase of SBA varied depending on the serosubtype between 16% (P1.7^{h},4) and 100% (P1.5^{c},10). In the present study we revaccinated 177 children with a monovalent P1.7^{h},4 OMV vaccine to investigate whether immunological memory against meningococcal vaccine strains would still be present 2.5 years after the primary series with a hexavalent meningococcal vaccine.

Materials and methods

Study population

Healthy children (5-6 and 10-11 years old), who previously enrolled in a study on the safety and immunogenicity of hexavalent RIVM meningococcal OMV vaccine [12] were asked to participate. Children were included after written informed consent and a health check. Exclusion criteria were: administration of plasma products 3 months prior to the study, acute febrile illness (temperature > 38.5°C), serious disease demanding medical treatment, concurrent use of antibiotics, allergy to vaccine components, severe reaction after previous vaccination, immunodeficiency and congenital or chronical illnesses or neurological disorders (except febrile seizures). The study was approved by the Medical Ethical Committee of the University Hospital, Rotterdam.

Study design

Participants were (re)vaccinated once with a monovalent meningococcal P1.7^{h},4 OMV vaccine. Blood samples were obtained before vaccination and 4 to 6 weeks afterwards. Serum was stored at -20 °C until distribution of aliquots for blinded specific antibody measurements.

Vaccines

The production of the monovalent meningococcal P1.7^{h},4 OMV vaccine was similar to the production of the hexavalent OMV vaccine [13]. The seed strains for monovalent vaccine contain duplicate copies of the porA P1.4 gene. Class 4 protein was not expressed in the monovalent seed strains. The monovalent vaccine strains did not express PorB or capsular polysaccharides, and produced galE LPS. Minor amounts of Opa and Opc were present. The vaccine contains 15 microgram P1.4 protein. The contents per monovalent meningococcal vaccine (pH 7.4) are: 17 microgram protein, 88% of which is P1.7^{h},4 class 1 protein, 50 microgram thiomersal, 50 microgram sucrose and 1.34 mg $AlPO_4$.

The vaccines used in the previous study are described by de Kleijn et al. [12] and Claassen et al. [13].

Monitoring for adverse reactions

Local and systemic adverse reactions were registered by a structured telephone interview between 18 to 30 hours after each vaccination. The children and their parents were asked to keep a diary until 7 days after vaccination in order to record adverse reactions. Local adverse reactions were documented as presence of redness, size of redness and/or swelling (<2.5 cm, 2.5 cm – 5.0 cm, > 5.0 cm), itching, pain and inability to use the arm. Systemic adverse reactions included the presence of fever (>38.5 °C), headache, listless, unusual crying, decreased appetite, nausea, joint complaints, cutaneous symptoms, school non-attendance, use of medication, contacts with the

health care system and illness in the family.

Serum bactericidal antibody assay

The serum bactericidal antibody (SBA) response, as described by Peeters [14] and Rouppe van der Voort [3], was assessed against six isogenic variants of strain H44/76 in which each PorA of the hexavalent vaccine (subtypes P1.7,16 - P1.19,15 - P1.5,2 - P1.5^{c},10 - P1.12,13 & P1.7^{h},4) was expressed individually. As control strain for Por A specificity the Por A negative mutant strain H1-5 was used. The serum bactericidal titre was reported as the reciprocal of the serum dilution yielding $\geq$ 90% killing.

Statistics

Differences in bactericidal geometric mean titers between the various groups were determined by the Mann-Whitney U test. Wilcoxon Signed Ranks test was used to measure differences in increase of serum bactericidal activity. Correlations were measured with the Spearman Correlation test.

Results

Study population

177 children (89 boys, 88 girls) participated in this study. The 77 children, who previously received hexavalent meningococcal vaccine (low dose n=29, high dose n=24) or hepatitis B vaccine (n=24) at toddler-age (2-3 years old) had a mean age of 5.7 years (range 5.2-6.1). The 100 children, who previously at the age of 7-8 years received hexavalent meningococcal vaccine (low dose n=43, high dose n=35) or hepatitis B vaccine (n=22), had a mean age of 10.7 years (range 10.2-11.3). All children received one monovalent meningococcal P1.7^{h},4 vaccination. Blood samples were taken before vaccination and 29.5 (28-42) days afterwards.

Reactogenicity

No serious adverse events occurred. Only mild local reactions and less frequently mild systemic reactions were registered. All reactions peaked within 48 hours and declined rapidly within one week. During the first day 80% of the children complained about a mild painful arm with 2.8% of the children not fully using the vaccinated arm. Local redness was most frequently registered on the second day by 25% of the children. Systemic reactions peaked in the second or third day with 8.0% of the children feeling listless. Fever was registered in 2.3% of the children.

Bactericidal antibody response

Sera from the children were tested against 6 different isogenic meningococcal strains expressing the serosubtype specific class 1 outer membrane proteins represented in the hexavalent vaccine (P1.7^{h},4 / P1.12,13 / P1.5^{c},10 / P1.5,2 / P1.19,15 / P1.7,16) and the H1.5 control strain (without class 1 OMP) (Table 1).

SBA against the strains expressing serosubtype P1.5^{c},10 and P1.5,2 in the prevaccination sera was significantly higher in children previously vaccinated with hexavalent meningococcal vaccine in comparison with children vaccinated with hepatitis B vaccine ($p<0.01$).

All children showed a significant increase in SBA against serosubtype P1.7^{h},4 upon vaccination with monovalent P1.7^{h},4 meningococcal vaccine (meningococcal group $p<0.01$, hepatitis B group $p=0.03$). Serum bactericidal titers after vaccination with the monovalent P1.7^{h}, 4 meningococcal vaccine were significantly higher in children who were primed with hexavalent meningococcal vaccine than in children with prior vaccination against hepatitis B ($P<0.01$). The P1.7^{h},4 serum bactericidal titers were significantly increased after the vaccination with monovalent meningococcal vaccine in comparison with the SBA 4-6 weeks after the three hexavalent meningococcal vaccines 2.5 years before ($p<0.01$). These titers (4 to 6 weeks after three hexavalent vaccinations in comparison with 4 to 6 weeks after the monovalent booster) showed a significant correlation (Spearman's rho $r=0.45$, $p<0.01$).

Table 1 Geometric mean titres and percentage of children with at least a bactericidal titre of 1:4 against the meningococcal strains expressing the six serosubtypes included in the hexavalent meningococcal vaccine and one cotrol strain (H1.5). Samples are taken pre- and post boosting with one monovalent P1.4 meningococcal vaccine

		Children 5-6 years of age						Children 10-11 years of age					
	Blood sample	Prevaccination			Post vaccination			Prevaccination			Post vaccination		
	Vaccine	Men Low	Men high	HepB	Men Low	Men high	HepB	Men Low	Men high	HepB	Men Low	Men high	HepB
Strain													
P1.7h,4 (TR4)	N	29	24	23	28	24	24	43	35	23	43	35	22
	GMT	1.3	1.02	1.0	3.2	10.1	1.02 **	1.15	1.08	1.06	3.35	3.03	1.55 *
	range	0.92-1.94	0.96-1.09		1.73-5.72	4.65-20.9	0.96-1.09	0.97-1.37	0.97-1.06	0.93-1.21	2.14-5.23	1.89-5.22	1.03-2.33
	%>=1:4	10.3	0.0	0.0	42.9	75.0	0.0	7.0	2.9	4.3	48.8	40.0	18.2
P1.12,13 (TR1213)	N	29	24	23	28	24	24	43	35	23	43	34	22
	GMT	1.2	1.0	1.0	1.37	1.4	1.0 *	1.2	1.2	1.0	1.6	1.35	1.03 *
	range	0.84-1.82			0.90-2.20	1.05-1.96		0.99-1.47	0.89-1.38		1.19-2.27	1.08-1.76	0.96-1.10
	%>=1:4	6.9	0.0	0.0	10.7	16.7	0.0	7.0	5.7	0.0	16.3	11.8	0.0
P1.5c,10 (TR10)	N	28	24	23	28	23	24	43	35	23	43	34	22
	GMT	7.6	9.5	1.31 **	17.2	25.9	0.13 **	4.6	6.4	1.03 **	12.36	14.7	1.24 **
	range	4.02-12.8	5.28-19.6	0.79-2.16	9.74-27.7	16.1-41.6		2.99-6.90	3.61-8.86	0.96-1.10	8.06-18.9	8.54-23.1	0.88-1.76
	%>=1:4	78.6	75.0	4.3	92.9	100	8.3	60.5	74.3	0.0	86.0	88.2	9.1
P1.5,2 (TR52)	N	28	24	23	27	24	24	43	35	23	43	33	22
	GMT	1.64	1.99	1.03 *	3.25	5.03	1.29 **	1.7	1.7	1.0 **	3.5	3.8	1.17 **
	Range	0.96-2.86	1.22-3.47	0.96-1.09	1.85-5.92	2.92-8.92	0.88-1.93	1.25-2.30	1.18-2.18		2.24-5.51	2.07-6.19	0.89-1.52
	%>=1:4	14.3	25.0	0.0	40.7	70.8	8.3	25.6	22.9	0.0	46.5	48.5	4.5
P1.7,16 (H44/76)	N	28	24	23	28	24	24	43	35	23	43	34	22
	GMT	1.34	1.0	1.0	1.64	1.77	1.0 **	1.08	1.2	1.03	1.7	2.35	1.2
	Range	0.84-2.23			1.14-2.53	1.22-2.71		0.96-1.21	0.93-1.38	0.96-1.10	1.23-2.42	1.39-3.55	0.95-1.10
	%>=1:4	7.1	0.0	0.0	21.4	29.2	0.0	4.7	8.6	0.0	18.6	32.4	9.1
P1.19,15 (TR1915)	N	29	24	23	28	24	24	43	35	22	43	34	22
	GMT	1.31	1.0	1.0	1.52	1.09	1.06	1.17	1.35	1.0	1.84	2.21	1.07 *
	Range	0.85-2.02			0.97-2.40	0.96-1.24	0.97-1.16	0.97-1.43	0.90-1.68		1.27-2.69	1.35-3.65	0.93-1.21
	%>=1:4	6.9	0.0	0.0	14.3	4.2	0.0	7.0	8.6	0.0	20.9	26.5	4.5
H1.5	N	29	24	23	28	23	24	43	35	23	43	33	22
	GMT	1.15	1.0	1.0	1.13	1.0	1.0	1.0	1.1	1.0	1.0	1.0	1.0
	range	0.84-1.63			0.86-1.50								
	%>=1:4	3.4	0.0	0.0	3.6	0.0	0.0	0.0	2.9	0.0	0.0	0.0	0.0

Mann Whitney U between the combined group high / low dose meningococcal vaccine and the hepatitis B group
** p<0.01 * p<0.05

	Low *dose* MenB						High *dose* MenB					
	5-6 yrs			10-11 yrs			5-6 yrs			10-11 yrs		
Strain	*n*	*N*	*%*	*n*	*N*	*%*	*n*	*N*	*%*	*n*	*N*	*%*
P1.7[h],4	7	9	78	8	12	67	12	12	100	2	4	50
P1.12,13	1	20	5	4	20	20	4	17	24	1	18	6
P1.5[c],10	9	26	35	16	38	42	10	23	43	14	34	41
P1.5,2	7	25	28	11	32	34	12	23	52	12	34	35
P1.7,16	2	9	22	5	16	31	6	14	43	4	13	31
P1.19,15	2	9	22	6	8	75	1	9	11	2	10	20

	overall		
Strain	*n*	*N*	*%*
P1.7[h],4	29	37	78.4
P1.12,13	10	75	13.3
P1.5[c],10	49	121	40.5
P1.5,2	42	114	36.8
P1.7,16	17	52	32.7
P1.19,15	11	36	30.6

Table 2 Percentage participants with ≥4-fold rise in SBA after vaccination with monovalent meningococcal vaccine of participants showing a ≥4-fold rise after primary series with hexavalent meningococcal vaccine

48.5% of the children in the hexavalent meningococcal group and 8.9% in the hepatitis B group showed at least a fourfold increase in bactericidal activity against serosubtype P1.7[h],4 after vaccination with monovalent P1.7[h],4 meningococcal vaccine (Table1). Prior vaccination with hexavalent meningococcal vaccine followed by revaccination with a monovalent P1.7[h],4 booster resulted also in a significant increase in bactericidal titers against serosubtype P1.5[c],10 / P1.5,2 / P1.7,16 / P1.19,15 and P1.12,13 ($p<0.01$). The responses against the heterologous strains were lower than the response against the homologous P1.4 strain. There was no increase in SBA against the meningococcal strain H1.5 (without class 1 OMP). Children who previously received hepatitis B vaccine only had a significant increase against meningococcal strain P1.7[h],4 ($p=0.03$). The percentage of children with a SBA of at least 1:4 against the serosubtypes P1.12,13 / P1.19,15 / P1.7,16 / P1.5,2 and P1.5[c],10 after monovalent vaccine varied from 14% to 90.6% in the hexavalent group and from 0% to 8.7% in the hepatitis B group (Table1).

The results of revaccination in children with a minimal fourfold increase in SBA after the primary vaccine series are depicted in Table 2. After the monovalent booster, 78.4% of the previous responders showed again a minimal fourfold increase of SBA against the homologous strain P1.4. This is significant higher than the 48.4% in the group of non-responders after hexavalent meningococcal vaccination ($P<0.01$). Lower percentages of responders were seen against the heterologous serosubtypes present in the hexavalent vaccine.

Discussion

The most important finding in this study is the persistence after 2.5 years of immunological memory after three vaccinations with hexavalent meningococcal vaccine. The SBA activity in the previous study showed the kinetics of priming with a booster response after the third hexavalent meningococcal vaccination with 16% of the children showing a minimal fourfold increase in SBA against serosubtype P1.7^{h},4 [12]. Revaccination with monovalent P1.7^{h},4 meningococcal vaccine 2.5 years later results in 48.5% of the children with a minimal fourfold increase in SBA against the meningococcal strain representing serosubtype P1.7^{h},4. 78.4% of the responders after hexavalent vaccine again reacted with at a minimal fourfold increase in SBA when revaccinated with the monovalent vaccine. The antibody titers against serosubtype P1.4 were significantly higher after the monovalent booster than 4 to 6 weeks after the third hexavalent vaccination. Immunological memory maturated even against serosubtype P1.4, one of the weakest immunogenic PorAs in the hexavalent vaccine. In the control group, 8.9% of the children responded with at least a fourfold increase in SBA against serosubtype P1.7^{h}, 4 after their first dose of monovalent meningococcal vaccine, suggesting induction of memory by natural exposure of meningococci.

To our surprise SBA against meningococcal strains expressing the serosubtypes present in the hexavalent meningococcal vaccine other than P1.4 were significantly increased after the monovalent P1.7^{h},4 meningococcal vaccination, although lower than for the homologous serosubtype P1.4. These antibodies were PorA specific since no reaction was seen with meningococcal strain H1.5 lacking PorA. Previous studies indicated that antibodies against class 1 outer membrane proteins were expected to be serosubtype specific [4, 11]. However, in this study crossreactivity was found against the heterologous meningococcal strains expressing the serosubtypes present in the hexavalent meningococcal vaccine. The crossreactivity occurred only in the group of children, who previously received hexavalent meningococcal vaccine. We speculate that T-cell memory is present for specific T-cell epitopes in the 'conserved parts' thereby also boosting antibody responses against the heterologous serosubtypes. However, the heterologous responses were weaker than the response against the P1.4 epitope present in the monovalent vaccine. The heterologous booster effect varied by serosubtype. Another explanation may be that the variable regions in loop 1 or 4 are of different size and that antibodies cross-react with little parts of the variable regions of other class 1 OMP's.

In the previous study in which the hexavalent meningococcal OMV vaccine was administered in three doses, SBA were preferentially induced against vaccine strains expressing serosubtype P1.5^{c},10 and P1.5,2 [12]. Comparable differences in SBA against the different serosubtypes were seen in UK infants after administration of this vaccine [15]. Serum bactericidal titers against the serosubtypes P1.5^{c},10 and P1.5,2 were again the highest of the heterologous subtypes conforming their higher immunogenicity, although lower than the homologous serosubtype P1.4. Before revaccination, 71% of the children (hexavalent meningococcal group) had at least a

serum bactericidal titer of 1:4 against serosubtype P1.5^{c},10 two and a halve years after their last hexavalent vaccination in comparison with only 2.2% in the control group. This may be due to a slow decrease of antibodies in the 2.5 years after hexavalent vaccination, to natural boosting by meningococcal carriage, or to cross-reactivity by other bacterial species.

We conclude that the hexavalent meningococcal OMV vaccines induces serum bactericidal antibodies against PorA containing meningococci in children and persistence of immunological memory for at least 2.5 years. We currently prepare an efficacy trial to elucidate whether this promising vaccine is able to prevent meningococcal disease.

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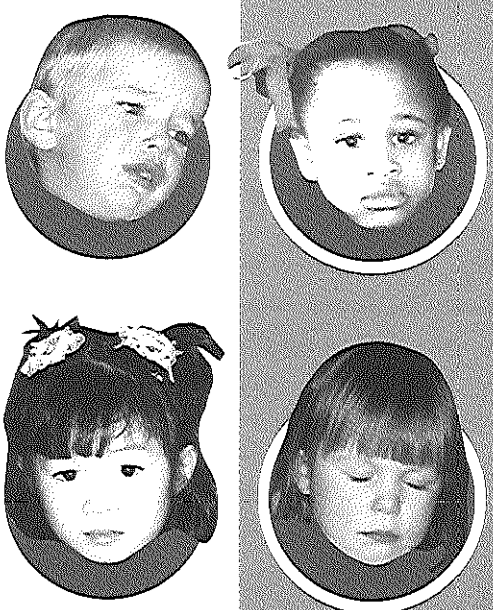
Chapter 12

Summary and future perspectives



Meningococcal sepsis

Infection with *Neisseria meningitidis* may cause severe illness in previously healthy children. The clinical picture varies from self-limiting bacteremia to meningitis or fulminant sepsis. Meningococcal sepsis is characterised by a rapid onset. The diagnosis becomes obvious when petechiae develop in a seriously ill child with high fever. Meningococcal disease is predominantly seen in children with a peak incidence around 2 years of age. A second peak is noted among teenagers. The overall mortality rate of meningococcal septic shock varies between 20% and 50%, is higher in infants than in older children and has not changed significantly over the past three decades despite improvements in management and therapy. The overwhelming character of this disease in a considerable number of patients is related to the virulence characteristics of meningococci, environmental factors and the genetic constitution of the patient. The systemic inflammatory response in patients with the disease aims to neutralise micro-organisms and their toxic products, but may also overreact and induce serious damage to the host. The pathogenesis of meningococcal disease is discussed in **Chapter 2**. The inflammatory changes are mediated by the induction of cytokines, activation of the complement system, activation of the coagulation and fibrinolytic systems and also by changes of hormonal systems. **Chapter 3** describes the endocrine and metabolic changes in a group of children with meningococcal septic shock. In this study, we show that children who do not survive meningococcal sepsis have an impaired adrenal response, altered thyroid hormones and decreased levels of non-esterified fatty acids. These factors are all associated with a higher severity of disease score on admission.

Further exploration of the hypothalamic-pituitary-adrenal axis in **Chapter 4** indicates that low cortisol concentrations and high ACTH concentrations on hospital admission are strongly associated with a more severe clinical picture and a poor outcome of meningococcal disease. On the basis of these relatively low levels of cortisol, one may question whether this group of patients may benefit from corticosteroid therapy. However, at this moment it is unknown whether the observed hormonal changes are critical factors in the outcome of acute meningococcal sepsis or merely represent secondary effects of the severity of disease. Further research is needed to provide answers to the question whether corticosteroid treatment may improve outcome in patients with meningococcal sepsis.

Meningococcal sepsis is associated with clinical and laboratory evidence of disseminated intravascular coagulation (DIC). The highly activated coagulation process may result in depletion of hemostatic proteins. In meningococcal sepsis levels of protein C are significantly more decreased than those of other hemostatic proteins. Treatment with protein C is one possibility to interfere in the dysbalance of coagulation and fybrinolysis in meningococcal sepsis. The results of a randomised, placebo-controlled, dose finding study with protein C concentrate in children with an early stage of presumed meningococcal septic shock are described in **Chapter 6**. Administration of protein C concentrate appears to be safe in children with meningococcal septic shock and

results in increased serum levels of protein C and activated protein C (APC). No beneficial clinical effects could be observed in these forty patients. Increasing dosages of protein C resulted in a more rapid normalisation of coagulation as reflected by a significantly more rapid decrease in serum levels of D-dimers. This inhibition of the activated coagulation by APC may theoretically result in a decrease of complications such as amputations and skin transplantations. Additional large randomised trials are needed to study the efficacy of protein C in children with severe meningococcal disease. Such studies with great logistic and ethical challenges can only be performed by large-scale, international collaborative study groups. In this respect it is important to have a simplified objective mortality prediction score for patients with meningococcal sepsis. These scores generally facilitate in stratifying cases according to severity and allow accurate descriptions of the casemix in individual units. The Rotterdam score includes four objective laboratory values and is simple, objective and highly specific for patients with meningococcal septic shock (**Chapter 5**).

During the last decade, the management of meningococcal disease has been directed to apply new immuno-therapeutic strategies. Interventions to neutralise endotoxin with recombinant bactericidal/permeability–increasing protein may be effective given in a very early phase of the disease. Recent studies have tried to modulate the immune response by the use of natural anticoagulants with the specific attention to antithrombin III, tissue factor pathway inhibitor (TFPI) and protein C. From a theoretical point of view, a combination of substitutive therapies may be more effective. Clinical studies using different combinations of immunomodulatory agents cannot be undertaken without obtaining knowledge on the safety and efficacy of the single agent.

Prevention of meningococcal disease

Protection against meningococcal disease has been associated with the presence of bactericidal antibodies. Therefore, the best approach to prevent diseases is to induce these antibodies by vaccination. Vaccines based on polysaccharides or polysaccharides conjugated to a carrier protein are already available against meningococci of serogroup A, C, Y and W135. For meningococci of serogroup B other surface structures are needed as vaccine candidates, because the polysaccharides of this serogroup are poorly immunogenic. **Chapter 7** provides an overview of the different vaccine candidates and their results. Outer membrane proteins (OMP) are the most promising structures in the development of a vaccine against serogroup B. Clinical trials based on OMP-based vaccines demonstrated an efficacy rate between 50% and 80%, but no protection was seen in infants. The RIVM constructed a hexavalent meningococcal outer membrane vesicle vaccine based on different class 1 outer membrane proteins. With this vaccine a study was performed as described in **Chapter 8**. Administration of Hexavalent meningococcal OMV vaccine is considered to be safe in children aged 2-3 and 7-8 years. Mild local (in 74% of the children) and systemic (in 27% of the children) reactions were reported after hexavalent meningococcal OMV vaccination. After 3 hexavalent meningococcal vaccinations increased levels of IgG and increased bactericidal activity were measured with the highest levels in the youngest children. The percentage of children with a fourfold rise in bactericidal antibody titre, which is assumed to be indicative for protection, varied between 11% and 95% for the different meningococcal serosubtypes. To explain the differences in response between the various age groups and the different serosubtypes, the IgG isotype distributions were measured (**Chapter 9**). In all children, the vaccine was capable to induce antibodies of the IgG1 and IgG3 isotypes, which are considered to be most important for protection against disease. We did not detect differences in isotype distribution between the toddlers and schoolchildren or between the different serosubtypes, which could provide an explanation for the differences in bactericidal activity.

The class 1 OMP corresponding with serosubtype P1.4 is one of the weakest antigens in the hexavalent vaccine and the most prevalent serosubtype in the Netherlands. The interference between three different PorA's on one vesicle or a lower immunogenic potential of the P1.4 antigen compared to other antigens in the vaccine may account for this effect. Therefore the RIVM developed a monovalent vaccine expressing serosubtype P1.7^{h},4. Two different formulas and two different vaccine schedules were evaluated (**Chapter 10**). This study shows that the monovalent meningococcal OMV vaccine is safe and able to induce bactericidal antibodies in toddlers. The vaccine formula with aluminium phosphate as adsorbent made the PorA expressing vesicles more immunogenic than adsorption to aluminium hydroxide. Three vaccinations in the primary series resulted in higher bactericidal titres than two vaccinations. However in both groups above 90% of children responded with a fourfold increase in SBA after the booster vaccination.

A clinical trial was performed with the same monovalent P1.7h,4 OMV vaccine, to study the

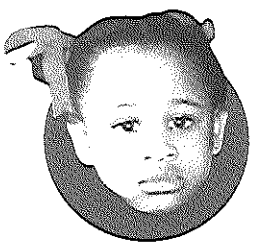
immunological memory and persistence of bactericidal antibodies 2.5 years after a primary series of hexavalent meningococcal vaccinations (**Chapter 11**). The children from the previous study with hexavalent vaccine were asked to participate again and received one monovalent P1.7h,4 vaccination. This study confirms the presence of persistence of immunological memory in toddlers and schoolchildren since bactericidal antibodies were still present 2.5 years after vaccination with hexavalent meningococcal vaccine. Forty-nine percent of the children who previously received hexavalent meningococcal vaccine had at least a fourfold increase in specific bactericidal antibodies against one of the weakest antigens of the hexavalent vaccine (serosubtype P1.4) after a booster dose with monovalent P1.7^{h},4 meningococcal vaccine. Bactericidal activity was highest in the youngest children. A cross-reactive class 1 OMP dependent immune response against other serosubtypes was found in the children who were previously vaccinated with hexavalent meningococcal vaccine.

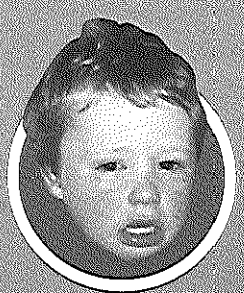
To date, the hexavalent RIVM OMV vaccine is one of the most promising vaccines against meningococci of serogroup B. Bactericidal activity is present in young children and crossreactivity can result in a broader efficacy. The fourfold rise in SBA after vaccination is the best correlate for protection up to now. A large clinical trial has to be undertaken to demonstrate the efficacy of this hexavalent meningococcal vaccine for the Netherlands.

The search for the solution against meningococcal disease has not yet ended, but hopefully the solution comes closer step by step.

Chapter 13

Samenvatting en toekomst





Meningokokken sepsis

Infectie met *Neisseria meningitidis* kan bij gezonde kinderen een ernstig ziektebeeld veroorzaken. De klinische presentatie kan variëren van een zelf genezend "griepje" tot hersenvliesontsteking en in de meest ernstige vorm tot bloedvergiftiging (sepsis) gepaard gaande met shock. Meningokokken sepsis is vooral bekend door het zeer snelle beloop van de ziekte. De diagnose kan veelal à vue gesteld worden wanneer petechiën (kleine huidbloedinkjes) ontstaan bij een ernstig ziek kind met hoge koorts. Infectie door meningokokken komen voornamelijk voor bij kinderen onder de vijf jaar. De piek incidentie ligt rond de leeftijd van twee jaar. Een tweede piek wordt gezien bij adolescenten tussen 15 en 17 jaar. De mortaliteit ten gevolge van meningokokken sepsis varieert tussen de 20% en 50% en is aanzienlijk hoger bij zuigelingen dan bij oudere kinderen. Ondanks verbeteringen in de ondersteunende behandeling is de mortaliteit in de afgelopen 30 jaar weinig verminderd. Het overweldigende karakter van de ziekte bij een aanzienlijk aantal patiënten wordt mede bepaald door virulentie factoren van de meningokok, omgevingsfactoren en de genetische constitutie van de patiënt. De systemische ontstekingsreactie in patiënten met de ziekte is erop gericht het micro-organisme en zijn toxische producten zoals endotoxine op te ruimen. Echter, de overmatige reactie van het immuunsysteem leidt bij veel patiënten tot ernstige schade op cel- en orgaan niveau. De pathogenese van infectie door meningokokken wordt besproken in **Hoofstuk 2**. De omvang van de ontstekingsreactie wordt bepaald door de productie van pro- en anti-inflammatoire cytokinen, de mate van activatie van het complement systeem, de stolling en van de fibrinolyse en door veranderingen in het hormonale systeem. In **Hoofdstuk 3** worden de endocriene en metabole veranderingen beschreven in een groep kinderen met door meningokokken veroorzaakte septische shock. De resultaten van deze studie tonen aan dat kinderen die overlijden aan de ziekte een verminderde bijnierrespons, veranderde spiegels van schildklierhormonen en lagere spiegels van vrije vetzuren hebben.

Uitgebreider onderzoek naar het functioneren van de hypothalamus-hypofyse-bijnier-as bij kinderen met een door meningokokken veroorzaakte sepsis in **Hoofdstuk 4** toont aan dat lage cortisol in combinatie met hoge ACTH spiegels bij binnenkomst in het ziekenhuis geassocieerd zijn met een ernstiger beloop van het klinische beeld. De bevinding, dat cortisol spiegels sterk verlaagd zijn, leidt tot de vraag of patiënten met een meningokokken sepsis mogelijk baat zouden hebben bij een behandeling met corticosteroiden. Op dit moment is het nog niet duidelijk of de door ons gevonden hormonale veranderingen een belangrijke factor vormen in het beloop van de ziekte of slechts een bijkomende factor, die een reflectie vormt van de ernst van de ziekte. Verder onderzoek is noodzakelijk om een antwoord te kunnen geven op de vraag of behandeling met corticosteroiden een gunstig effect heeft bij patiënten met een meningokokken sepsis.

Bij veel patiënten met meningokokken sepsis worden klinische en laboratorium kenmerken vastgesteld van diffuse intravasale stolling. De sterke activatie van het stollingsproces leidt tot het verbruik van stollingseiwitten. Bij patiënten met meningokokken sepsis zijn de bloedspiegels van

het stollingseiwit 'proteïne C' significant lager dan die van andere stollingseiwitten. Behandeling met proteïne C biedt een mogelijkheid om de balans te herstellen tussen stolling en fibrinolyse (ontstolling). De eerste resultaten van een gerandomiseerde, placebo gecontroleerde, doserings-studie met proteïne C bij kinderen met een verdenking op meningokokken septische shock worden beschreven in **Hoofdstuk 6**. Toediening van proteïne C bij kinderen met een meningokokken septische shock lijkt niet gepaard te gaan met ernstige bijwerkingen en resulteert in een toename van de bloed spiegels van proteïne C en van geactiveerd proteïne C (APC). Wij vonden in deze studie met 40 patiënten geen positieve effecten op het klinisch beloop. Hogere doses van proteïne C leiden tot een snellere normalisatie van de stolling aangetoond door een snellere daling van de spiegel van D-dimeren in het bloed. Deze remming van de geactiveerde stolling door APC zou theoretisch kunnen leiden tot een vermindering van het aantal complicaties zoals amputaties en huidtransplantaties. Verdere studies zijn nodig om de effectiviteit van proteïne C bij kinderen met een meningokokken sepsis vast te stellen. Zulke studies vormen een logistieke en ethische uitdaging en zijn slechts mogelijk binnen het kader van een internationaal samenwerkingsverband. In dit opzicht is het belangrijk te beschikken over een eenvoudige en objectieve score om de kans op overlijden te voorspellen voor patiënten met een door meningokokken veroorzaakte sepsis. Deze score maakt het mogelijk om groepen te onderscheiden naar ernst van ziekte zodat een betere patiënten selectie gemaakt kan worden. De Rotterdam score maakt gebruik van vier objectieve laboratorium waarden, is eenvoudig uitvoerbaar en is ontwikkeld voor patiënten met een door meningokokken veroorzaakte septische shock (**Hoofdstuk 5**).

In de laatste decennia heeft de behandeling van meningokokken sepsis zich vooral ontwikkeld in de richting van het gebruik van nieuwe immuno-therapeutische strategieën. Interventies om het endotoxine (de toxische deeltjes van de bacterie) te neutraliseren met recombinant-BPI (bactericidal/permeability increasing protein) kunnen effectief zijn in een vroege fase van de ziekte. In recente studies is daarnaast geprobeerd de immuun respons te moduleren door het gebruik van natuurlijke anti-stollers zoals antithrombine III, TFPI (tissue factor pathway inhibitor) en proteïne C.

Preventie van meningokokken ziekte

Bescherming tegen infecties door meningokokken is geassocieerd met de aanwezigheid van bactericide antistoffen. Het is mogelijk door middel van vaccinatie bactericide antistoffen te genereren. Vaccins waarin polysacchariden (suikers van het kapsel van de bacterie) gebruikt worden of polysacchariden gekoppeld aan een drager-eiwit zijn reeds beschikbaar tegen meningokokken van serogroep A, C, Y en W135. Voor meningokokken van serogroep B zijn andere oppervlakte structuren nodig als vaccin kandidaat aangezien de polysacchariden van deze serogroep slecht immunogeen zijn. In **Hoofdstuk 7** wordt een overzicht gegeven van de veiligheid, immunogeniteit en samenstelling van verschillende meningokokken vaccins. Buiten membraan eiwitten zijn de meest veelbelovende structuren voor incorporatie in een vaccin tegen meningokokken van serogroep B. Eerder klinisch onderzoek waarin vaccins met buiten membraan eiwitten gebruikt zijn tonen een effectiviteit tussen de 50% en 80%. Echter, deze vaccins geven helaas onvoldoende bescherming bij zuigelingen. Het RIVM construeerde een hexavalent meningokokken buiten membraan vaccin dat samengesteld is uit verschillende klasse 1 buiten membraan eiwitten in de vorm van "blaasjes". Met dit vaccin werd de studie verricht zoals beschreven in **Hoofdstuk 8**. Toediening van dit hexavalente OMV vaccin is veilig bij kinderen in de leeftijd van 2-3 jaar en 7-8 jaar. Na vaccinatie met het hexavalente meningokokken vaccin werden milde lokale (in 74% van de kinderen) en systemische (in 27% van de kinderen) reacties gerapporteerd. Na een serie van drie vaccinaties werd in het serum een toename gemeten van de spiegels van IgG en van de bactericide activiteit tegen meningokokken. De hoogste waarden werden gevonden bij de jongste kinderen. Het percentage kinderen met een viervoudige stijging van bactericide antistof titers, hetgeen wordt beschouwd als beschermend, varieerde tussen de 11% en 95% voor de verschillende serosubtypen. De IgG isotype distributie werd onderzocht om de verschillen in respons te verklaren tussen de verschillende leeftijdsgroepen en de verschillende serosubtypen (**Hoofdstuk 9**). In alle kinderen was het vaccin in staat om IgG1 en IgG3 antistoffen op te wekken. Dit zijn de belangrijkste isotypen in bactericide testen en waarschijnlijk ook de belangrijkste isotypen voor bescherming tegen infecties door meningokokken. De distributie patronen van de isotypen waren vergelijkbaar tussen de leeftijdsgroepen en de verschillende serosubtypen. Er kon geen verklaring gevonden worden voor het verschil in bactericide activiteit. Het klasse 1 buiten membraan eiwit dat correspondeert met serosubtype P1.4 is het meest voorkomende serosubtype in Nederland en vormt helaas één van de zwakste antigenen in het hexavalente vaccin. De interferentie tussen de drie verschillende eiwitten op één vaccin-blaasje of de lagere immunogeniteit in vergelijking met andere antigenen in het vaccin kunnen een mogelijke verklaring vormen voor dit effect. Het RIVM heeft daarom ook een monovalent vaccin ontwikkeld waarbij alleen serosubtype P1.7^{h},4 tot expressie gebracht is. De immunogeniteit en veiligheid van twee verschillende formules van dit vaccin en van twee verschillende vaccinatie-schema's worden beschreven in **Hoofdstuk 10**. Deze studie laat zien dat het monovalente vaccin

evenals het hexavalente vaccin veilig is en bactericide antistoffen induceert bij peuters. Het vaccin met aluminium fosfaat als adjuvans is meer immunogeen dan het vaccin waarbij aluminium hydroxide als adjuvans gebruikt wordt. Toediening van drie vaccinaties in de primaire serie leidde tot hogere bactericide titers dan het geven van twee vaccinaties. Echter in beide groepen respondeerden meer dan 90% van de kinderen met een viervoudige titerstijging van bactericide antistoffen na de booster vaccinatie.

Een vervolg studie met hetzelfde monovalente P1.4 OMV vaccin werd uitgevoerd om het immunologisch geheugen en de residuele aanwezigheid van bactericide antistoffen te onderzoeken 2.5 jaar na vaccinatie met het hexavalente meningokokken vaccin **(Hoofdstuk 11).** De kinderen uit de eerste studie werden opnieuw gevraagd om deel te nemen en kregen éénmalig een monovalent P1.7^{h},4 vaccin. In deze studie wordt de aanwezigheid van immunologisch geheugen bij kinderen bevestigd. Bactericide antistoffen tegen 2 van de 6 serosubtypen waren nog aanwezig 2.5 jaar na vaccinatie met het hexavalente vaccin. Negenenveertig procent van de kinderen die voorheen hexavalent meningokokken vaccin hadden gekregen respondeerden met een viervoudige titerstijging van specifieke bactericide antistoffen tegen één van de zwakste antigenen van het hexavalente vaccin (serosubtype P1.4). De bactericide activiteit was het hoogst bij de jongste groep kinderen. Een kruisreactieve immuunrespons tegen de andere serosubtypen (niet aanwezig in het monovalente P1.7^{h},4 vaccin) werd gevonden bij de kinderen die 2.5 jaar eerder gevaccineerd waren met hexavalent meningokokken vaccin.

Tot nu toe is het hexavalente RIVM OMV vaccin één van de meest veelbelovende vaccins tegen meningokokken van serogroep B. Het vaccin induceert bactericide activiteit in jonge kinderen terwijl de door ons gevonden kruisreactiviteit leidt tot bredere effectiviteit. Naar verwachting zal binnen de komende 5 jaar in een internationale multicenter studie (Nederland, Engeland) de effectiviteit onderzocht kunnen worden van de nieuwe generatie buiten membraan eiwit vaccins tegen meningokokken. Hiermee is het perspectief voor eradicatie van infecties door meningokokken sterk verbeterd in vergelijking met de situatie van een decade geleden.

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ESTER

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Curriculum Vitae

Ester Doret de Kleijn werd op 29 oktober 1969 geboren in Groningen. In Juni 1988 werd het einddiploma VWO behaald aan het Onze Lieve Vrouwe Lyceum te Breda. Datzelfde jaar werd de studie medicijnen aangevangen aan de Faculteit der Geneeskunde en Gezondheidswetenschappen van de Erasmus Universiteit, Rotterdam. In 1995 werd het arts-examen behaald. Tijdens de medische studie werd een bijdrage geleverd aan de productie van de geneeskunde almanak en werd 2 maanden stage gelopen op de afdeling kinderchirurgie in het Zevende Ziekenhuis in Riga, Letland. Als keuze co-assistent werd er onderzoek verricht naar de klinische en epidemiologische aspecten van kinderen met een meningokokken ziekten in het interconfessioneel ziekenhuis de Baronie te Breda (drs AJCM van der Velden). Vanaf december 1995 tot oktober 2000 was zij onder leiding van Prof dr R de Groot werkzaam als arts-onderzoeker bij de afdeling Kindergeneeskunde, subafdeling Infectieziekten/Immunologie. Hierbij werden in samenwerking met de subafdeling Intensive Care Pediatrie (Dr JA Hazelzet) diverse onderzoeksprojecten opgezet en uitgevoerd op het gebied van pathogenese en preventie van infecties door meningokokken. Tijdens de promotie periode was zij van begin 1998 tot september 2000 lid van de Medisch Ethische Commissie van het Academisch Ziekenhuis Rotterdam. Vanaf 1 oktober 2000 is zij assistent in opleiding tot kinderarts in het Academisch Ziekenhuis Vrije Universiteit in Amsterdam (opleider Prof dr JJ Roord).

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