BACTERIAL MENINGITIS AND SEPSIS IN CHILDREN

CLINICAL ASPECTS AND HOST RESPONSE

BACTERIËLE MENINGITIS EN SEPSIS BIJ KINDEREN

KLINISCHE ASPECTEN EN GASTHEERRESPONS

René F. Kornelisse

Cover illustration: Gram-stains of cerebrospinal fluid of patients with bacterial meningitis due to *Neisseria meningitidis* (left panel), *Haemophilus influenzae* (middle panel), and *Streptococcus pneumoniae* (right panel).

Bacterial meningitis and sepsis; clinical aspects and host response /
René F. Kornelisse. - Rotterdam, Erasmus Universiteit,
Afdeling Kindergeneeskunde.
Thesis Rotterdam. With ref. - With summary in Dutch.
ISBN 90-75340-07-9
NIGU 743
Subject heading: bacterial meningitis, sepsis, host response

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PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR
AAN DE ERASMUS UNIVERSITEIT ROTTERDAM
OP GEZAG VAN DE RECTOR MAGNIFICUS
PROF. DR P.W.C. AKKERMANS M.A.
EN VOLGENS BESLUIT VAN HET COLLEGE VOOR PROMOTIES

DE OPENBARE VERDEDIGING ZAL PLAATSHEBBEN OP
WOENSDAG 11 DECEMBER 1996 OM 15.45 UUR

DOOR

RENÉ FRANK KORNELISSE

GEBOREN TE ROTTERDAM

PROMOTIE-COMMISSIE:

Promotor:	Prof. Dr. H.J. Neijens
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The studies presented in this thesis were financially supported in part by Glaxo Wellcome B.V. te Zeist. The printing was financially supported by GlaxoWellcome B.V., Hoechst Marion Roussel B.V., Medgenix, Pfizer B.V., Roche Nederland B.V., Schering-Plough B.V., SmithKline Beecham Farma, and Zeneca Farma B.V.

Printed by Offsetdrukkerij Haveka B.V., Ablasserdam

Voor Monique en Anne

CONTENTS

Chapter 1

General introduction

1.1 BACKGROUND

Despite the availability of modern sanitation, public health measures, antibiotics, and vaccines, children still suffer worldwide a sizeable number of infectious diseases before reaching adulthood. Most of these infections are self-limiting. However, the two syndromes studied in this dissertation: bacterial meningitis and septicemia are life-threatening entities in a young child. Early recognition and initiation of adequate treatment are major determinants for a good outcome. The most common causative pathogens of childhood bacterial sepsis and meningitis are *Neisseria meningitidis*, *Streptococcus pneumoniae* and *Haemophilus influenzae*. *N. meningitidis* is also the major cause of fulminant septic shock in previously healthy children. During the 1970's and early 1980's research on sepsis or bacterial meningitis was frequently directed to the development of antimicrobial regimens that achieve high bactericidal titers in cerebrospinal fluid in an attempt to accelerate bacteriological cure and thereby improve outcome. However, despite the availability of excellent antibiotics and highly qualified intensive care treatment, morbidity and mortality did not appreciately change. More recently research on sepsis and meningitis has focused on selected aspects of the host inflammatory response [1-12]. These studies were mainly performed in animal experimental models, and have led to the development of novel therapeutic regimens, which may in the future improve outcome of disease [13-15]. The major scientific achievement, however, has been the development of a new generation of conjugate vaccines against *N. meningitidis*, *H. influenzae* and *S. pneumoniae*. Global implementation of the use of these vaccines will hopefully lead to eradication of these bacterial diseases in the future.

1.2 HISTORICAL PERSPECTIVE

The earliest documentation of patients with meningitis can be found in the writings of Hippocrates. It took however many centuries, before the Englishman T. Willis in 1684 described a clinical picture similar to meningococcal disease [16]. The first description of the clinical and pathological features of bacterial meningitis was only recorded in the early 19th century. Epidemic cerebrospinal meningitis was first described by Gaspard Vieusseux of Geneva in 1806 [17]. Vieusseux observed that many of his patients had purple spots on the skin and marked engorgement of the brain at autopsy. Most victims were infants and children. He noted that the

patients complained of violent headache, stiffness of the spine and convulsions. Elisha North of Goshem, Connecticut, observed a similar epidemic in 1807 which he described in a monograph published in 1811, the first published book on this disease [18]. The puzzling late discovery of meningitis as a clinical entity did not delay a further rapid development of knowledge on its etiology.

The sporadic nature of outbreaks and the apparent absence of transmission of infection by victims led many nineteenth-century writers to believe that the disease was not contagious and was somehow linked to climatic or environmental conditions such as crowding. However by about 1860, it was widely assumed that some sort of poison or agent was involved in the pathogenesis of meningitis. In 1887 an Australian pathologist, Anton Weichselbaum, described the meningococcus under the name *Diplococcus intracellularis meningitidis* [19]. This was followed by the first in vivo diagnosis of meningitis by lumbar puncture performed by Quincke in 1891 and the first identification of the pathogen in a living patient by Heubner in 1896 [20, 21]. Heubner found typical "biscuit shaped" diplococci in pus cells. By 1910 it was recognized that the meningococcus was responsible for epidemics of meningitis and that other bacteria could cause sporadic cases.

H. influenzae was initially identified in 1892, when Pfeiffer described a pleomorphic gram-negative rod and erroneously identified it as the causative agent of influenza [22, 23]. After 1918, when this microorganism was only infrequently isolated from the lungs of patients who died during an influenza pandemic, it was reduced to the role of secondary invader. The organism was given its name "Haemophilus" by Winslow et al. in 1920 [24]. Prior to that time, *H. influenzae* became recognized as the major pathogen responsible for bacteremia and meningitis in children. In 1931, Pittman first reported the presence of encapsulated and unencapsulated forms of *H. influenzae* [25]. Encapsulated forms were classified into six serological types. Invasive disease was subsequently documented to be mainly caused by type b organisms.

Pasteur and Sternberg, working independently in 1880 and 1881 demonstrated pneumococci in human saliva as roughly lancet-shaped pairs of coccoid bacteria [26-29]. Previous reports had already identified similar bacteria [30, 31]. However, Pasteur and Sternberg were the first to demonstrate the pathogenic potential of this microorganism. By 1886 the bacterium was referred to as Pneumococcus because of its ability to cause pneumonia [32]. Later in that same decade, the pneumococcus was clearly demonstrated to be a major cause of meningitis [33] and otitis media [34].

In the pre-antibiotic era, bacterial meningitis was a deadly disease in most cases (90%). Immunotherapy by direct installation of immune serum supplemented with complement was the first effective therapeutic intervention in bacterial meningitis resulting in a reduction of the mortality rate to 25% [35-38]. Immunotherapy remained in use until the introduction of sulfonamides and penicillins the 1930's. Since then bacterial meningitis and meningococcal sepsis have become curable disease entities with variable mortality rates dependent on the individual pathogen and patient characteristics (neonates, immunosuppressed patients, neurosurgical patients, skull trauma, elderly patients).

1.3 PATHOGENESIS

The pathogenesis of bacterial meningitis and meningococcal disease have major similarities. *N. meningitidis*, *H. influenzae* and *S. pneumoniae* are organisms, which colonize the nasopharynx and spread between individuals by the inhalation of droplets [39]. Virulent strains need to overcome the barrier of the nasopharyngeal mucosa in order to become invasive. Favorable circumstances for infection may be provided by climatic conditions (especially a low absolute humidity) as well as by predisposing viral respiratory infections [40, 41].

Advances during the last decade in molecular microbiology and immunology have increased our understanding of the pathogenesis of bacterial meningitis and septic shock. We now know that the host inflammatory response to infection contributes substantially to the clinical evolution of bacterial meningitis and septic shock [2, 42]. Infections begin when microorganisms circumvent or penetrate host barriers such as skin or mucosa. Depending on the infecting agent's virulence and the extent of the host response, microbial invasion of the bloodstream and/or the cerebrospinal fluid compartment may occur. It is not yet completely understood why infection in some patients results in overwhelming septicemia, whereas in others meningitis develops in conjunction with bacteremia. We propose that genetic variability in the host response may play a role in this process as suggested by the data presented in chapter 10.

Changes in the central nervous system occurring during bacterial meningitis include metabolic alterations, an elevated intracranial pressure, and a decrease in the cerebral blood flow. The mechanisms leading to these changes involve bacterial cell-wall components and host factors such as

cytokines, arachidonic acid metabolites, platelet activating factor, complement, granulocytes, and reactive oxygen intermediates [43].

In septicemia, toxic bacterial products present in the circulation activate systemic host defenses, including plasma factors (complement and clotting cascades) and cellular components (neutrophils, monocytes, macrophages, and endothelial cells). Activated cells in turn produce potentially toxic host mediators (cytokines such as tumor necrosis factor-α, and interleukin-1, kinins, eicosinoids, platelet activating factor, and nitric oxide) that augment the inflammatory response. This escalating immune response, in concert with microbial toxins, may lead to shock, multiple organ failure, and death [44].

The outcome of both sepsis and meningitis is clearly dependent on many factors including the virulence of the microorganism, the presence of an adequate immune response, and the extent of the host inflammatory response.

1.4 Aims of the study

In this thesis we studied selected aspects of the clinical outcome and inflammatory host response of children with bacterial meningitis and meningococcal septic shock. Specific aims of our studies were as follows:

1. Analysis of the clinical outcome of the most serious forms of disease: pneumococcal meningitis and meningococcal sepsis.
2. Development of a new prognostic scoring system in patients with septic shock and purpura.
3. Determination of the presence of clonality in pneumococcal strains from children with meningitis.
4. Study of the role and balance between proinflammatory and antiinflammatory mediators in the cerebrospinal fluid compartment of children with bacterial meningitis.
5. Evaluation of the role of nitric oxide in the pathophysiology of bacterial meningitis.
6. Evaluation of the role of proinflammatory and antiinflammatory mediators, and the extent of abnormalities in coagulation and fibrinolysis in patients with meningococcal septic shock.
7. Evaluation of the presence of age-related differences in coagulation abnormalities in patients with septic shock and purpura.
8. Effect of a polymorphism in the promoter of the plasminogen activator inhibitor-gene on outcome of patients with meningococcal septic shock.

1.5 OUTLINES OF THE THESIS

Part I (chapters 2 to 7) contains several studies on the pathophysiological, microbiological and clinical aspects of bacterial meningitis in children. **Chapter 2** briefly reviews novel developments in the pathophysiology and therapy of bacterial meningitis. **Chapter 3** evaluates the outcome in a large group of children with pneumococcal meningitis diagnosed between 1970 and 1994. In **chapter 4**, we investigated whether clonal relatedness could be observed in pneumococcal isolates of these children. The next three chapters contain studies on the involvement of several mediators in the pathophysiology of bacterial meningitis. **Chapter 5** describes the role and balance between proinflammatory and antiinflammatory mediators in the cerebrospinal fluid compartment of children with bacterial meningitis. The possible role of interleukin-12, a novel cytokine with proinflammatory effects, is described in **chapter 6**. In **chapter 7**, we describe the role of nitric oxide in the pathophysiology of bacterial meningitis.

Part II (chapters 8 to 13), contains the results of studies in patients with meningococcal septic shock. **Chapter 8** reviews the epidemiology, microbiology, pathophysiology, clinical features and prevention of meningococcal sepsis. In **chapter 9**, we describe the outcome of patients with meningococcal septic shock. Furthermore, we report the development a new prognostic score that predicts death or survival. In **chapter 10** serum levels of proinflammatory cytokines, counterinflammatory compounds and plasma levels of hemostatic parameters in patients with meningococcal septic shock were measured. Subsequently, the association between these levels and clinical characteristics, such as duration of petechiae were studied. In **chapter 11** the involvement of interleukin-12 in the host defence during septic shock is investigated. In **chapter 12**, we studied the influence of age on mortality and on severity of clotting abnormalities in children with meningococcal sepsis. In **chapter 13** the influence of plasminogen activator inhibitor-gene promoter polymorphism on the outcome of patients with meningococcal septic shock is analyzed. **Chapter 14** discusses the significance of the presented data and their mutual interrelationship in the context of the literature. Recommendations for future research are given. Finally, the results of this dissertation are summarized in English as well as in Dutch.

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PART I

BACTERIAL MENINGITIS

Chapter 2

Bacterial Meningitis: Mechanisms of Disease and Therapy

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European Journal of Pediatrics 1995; 154: 85-96

2.1 ABSTRACT

Bacterial meningitis continues to be a serious infectious disease with a high morbidity and mortality in young children. Early recognition and initiation of adequate treatment are the major determinants for a good outcome. Recent advances in our understanding of the host inflammatory response by cytokines may result in the use of new therapeutic strategies. Such modulation of the inflammatory response may reduce the incidence of sequelae and death. The use of steroids as adjunctive therapy in children with bacterial meningitis probably has beneficial effects although the available data are still controversial. Additionally, studies in experimental meningitis models indicate that non-steroidal anti-inflammatory drugs and monoclonal antibodies against bacterial products, cytokines and CD18 on leukocytes reduce the extent of the meningeal inflammation. Human studies to evaluate the efficacy of these immune modulators are expected to start soon. However, prevention of bacterial meningitis by conjugate vaccines against *Streptococcus pneumoniae* and *Neisseria meningitidis* will be the most promising development in the next decade.

2.2 INTRODUCTION

Despite continuing improvements in therapy bacterial meningitis is still associated with fatality rates of 3%-6% and the development of severe sequelae in approximately 16% of cases [1-6]. Prevention of meningitis by the use of novel conjugate vaccines against *Streptococcus pneumoniae* and *Neisseria meningitidis* will be a point of major interest in the next decade. Recent research has contributed to our understanding of the pathophysiology of bacterial meningitis. This has resulted in new therapeutic approaches. The use of anti-inflammatory drugs in combination with antibiotics in bacterial meningitis has become a topic of major interest. This review focusses on the nature of the inflammatory response to infection in the cerebrospinal fluid (CSF) and the pathophysiological alterations induced by bacterial meningitis. Finally, recent advances in treatment will be discussed.

2.3 EPIDEMIOLOGY

Bacterial meningitis is mainly a disease of young children. During the neonatal period gram-negative enteric bacilli (*Escherichia coli* and *Klebsiella* species), group B streptococci, and *Listeria monocytogenes* are the most common pathogens. These organisms are mainly acquired at delivery. The three major causative organisms in infants above 3 months of age are *N. meningitidis*, *Haemophilus influenzae* and *S. pneumoniae*. They account for more than 90% of bacterial meningitis in children between 3 months and 10 years. The incidence and relative frequencies of these bacteria differ markedly depending on genetic factors, the geographic area, and the recent introduction of efficacious conjugate vaccines against *H. influenzae* type b in many countries.

Epidemiological studies have identified several risk factors for bacterial meningitis. Age is the most important risk factor. Boys are affected more frequently than girls. Several studies have reported the presence of racial differences in the incidence of bacterial meningitis. Black and Hispanic populations are at 2-4 times greater risk than Caucasians [7-10]. Socioeconomic rather than racially determined factors are responsible for this increased risk [8]. The native North American population also has a high incidence of bacterial meningitis. For example, rates of over 200/100,000 per year in the overall population have been described among Canadian Inuits [11]. Another host factor which increases the risk of pneumococcal sepsis and meningitis is splenectomy. Patients with deficiencies in the terminal components of complement (C5-9) also have an increased risk of meningococcal meningitis. Children in day care centres have an increased risk of acquiring invasive bacterial disease in comparison with children in home care.

2.4 DIAGNOSIS OF BACTERIAL MENINGITIS

Nonspecific clinical findings predominate in the newborn and in young infants. These include abnormal temperature, somnolence, irritability, and poor feeding. The presence of a bulging fontanelle is a relatively characteristic sign in this age group but not present early in the disease. More characteristic findings are often seen in children and adults and include alteration of the mental status, nuchal rigidity, and the signs of Kernig and Brudzinski. A lumbar puncture has to be performed if the slightest evidence suggests the presence of bacterial meningitis. Rapid

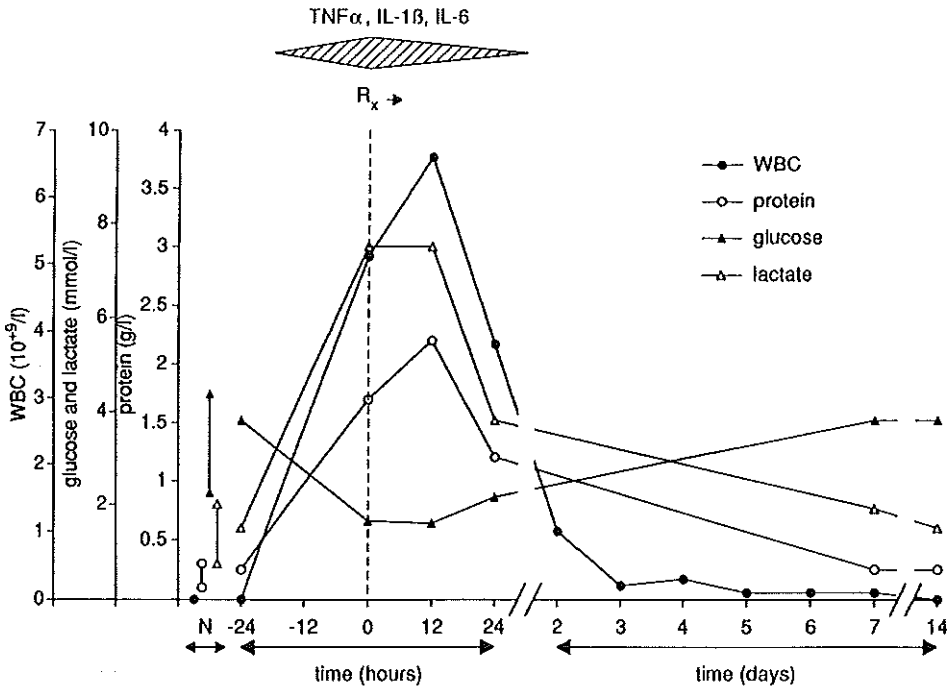

Figure 1. Schematic view of the biochemical changes in the cerebrospinal fluid (CSF) profile and detection of elevated cytokine levels during bacterial meningitis. The normal ranges (N) for white blood cells (WBC), protein, glucose and lactate in the CSF are indicated by vertical bars. Rx marks the timepoint of initiation of antibiotic therapy. The data were derived from the studies of Lebel et al. [93] and Odio et al. [94].

diagnosis and initiation of therapy are the major prognostic factors determining a favourable outcome. Initial management decisions are based largely on the Gram-stain and CSF characteristics (leukocytes, glucose, lactate and protein). The CSF parameters reflect the metabolic and structural alterations, such as increased blood brain barrier (BBB) permeability, which characterize the inflammatory response (Figure 1).

Leukocytes

A characteristic feature of bacterial meningitis is CSF leukocytosis. Before leukocytes reach the CSF a co-ordinated action takes place of adherence to vascular endothelium, and subsequent migration across the endothelial monolayer into the CSF compartment. This process is mediated by adhesion-promoting receptors and ligands located on leukocytes and

endothelium which are activated by exposure to lipopolysaccharide (LPS) and to cytokines: interleukin (IL)-1ß, tumor necrosis factor (TNF)-α and interferon-γ [12-16]. Bacterial replication or lysis in the CSF compartment induces endothelial cells to produce IL-8. IL-8 is a potent chemoattractant [17]. The initial reversible adherence of leukocytes to endothelial cells is mediated by three lectin-like carbohydrate-binding molecules called selectins (granule membrane protein-140 [CD62P], endothelial cell adhesion molecule-1 [CD62E], leukocyte adhesion molecule-1 [CD62L]). Each selectin recognises specific carbohydrate sequences on either leukocytes or endothelial cells. Leukocytes are first observed to roll along endothelial cells adjacent to the extravascular site of inflammation. Studies indicate that selectins are involved in the leukocyte rolling. Strong adhesion is mediated by leukocyte integrins that bind to counter receptors on endothelium. The integrin family of adhesion receptors consists of heterodimeric glycoproteins with an α and ß subunit. They can be classified on the basis of their ß subunit, e.g. $ß_2$ integrins (CD11a/CD18a and CD11b/CD18) and $ß_7$ integrins (CD49d/$ß_7$) [18]. The early selectin-adhesion decreases with continued cytokine stimulation by cleavage of the selectin from the cell surface or inhibition of its binding. At the same time $ß_2$ and $ß_7$ integrin-mediated adherence of the neutrophils to intercellular adhesion molecules (CD54 and CD102) and vascular cell adhesion molecules (CD106) on endothelial cells is induced. Subsequently leukocytes traverse the cerebral capillary endothelium by diapedesis [19].

Glucose and lactate

The changes in glucose and lactate levels are not only induced by living bacteria and leukocytes. The anaerobic brain metabolism in bacterial meningitis contributes to the development of increased CSF lactate concentration and hypoglycorrhachia [20]. It seems likely that local changes in the brain as a result of ischaemia or mediated by humoral factors, induce an increased production of lactate. Another explanation for the development of hypoglycorrhachia was demonstrated in experimental canine meningitis. In these animals low CSF glucose reflected the inhibition of carrier-mediated transport across the BBB [21, 22]. The increased use of glucose by the brain and the abnormal glucose transport across the BBB may further contribute to hypoglycorrhachia [23].

Protein

The elevated concentration of protein in CSF during bacterial meningitis is caused by an increased permeability of the BBB. A uniform response consisting of an early and sustained increase in formation of

pinocytotic vesicles and a progressive increase in the separation of tight junctions between endothelial cells is observed during the course of experimental meningitis [24]. The cytokine-endothelium-leukocyte interaction is probably responsible for the disruption of the barrier by opening intercellular junctions and thereby permitting the passage of serum proteins into the subarachnoidal space.

2.5 PATHOGENESIS OF INFECTION

The three most common microorganisms in bacterial meningitis have several properties that promote adherence, colonization and invasion of the mucous membranes of the nasopharynx. Mucosal attachment is mediated by microbial virulence factors such as pili and nonpilar adhesins. Several host defence mechanisms must be evaded before attachment may occur. The bacteria have to inactivate secretory IgA and escape from the ciliary clearance mechanisms of the nasopharyngeal mucosa. The three major pathogens all secrete a protease capable of cleaving human IgA1. Importantly, IgA1 is the dominant immunoglobulin class in the nasopharyngeal mucosa, which is the site of adherence and invasion by these bacteria [25, 26].

Invasion across the nasopharyngeal mucosa takes place by an endocytotic process (*N. meningitidis*) or through the intercellular route by separations in the apical tight junctions of columnar epithelial cells (*H. influenzae*) [27, 28]. Once the mucosal barrier is crossed, bacteria must overcome additional host defences to survive in the bloodstream and to invade the meninges. The most important virulence factor in this respect is encapsulation. The polysaccharide capsule inhibits neutrophil phagocytosis and prevents classical complement pathway bactericidal activity thus enhancing intravascular bacterial survival and replication.

2.6 PATHOPHYSIOLOGY OF THE INFLAMMATORY RESPONSE

Since CSF defences against infection are very limited, bacteria can proliferate rapidly. Host defence (humoral factors and phagocytes) must be recruited from serum, a process that develops parallel to BBB disruption and alteration of the cerebral metabolism. These abnormalities all arise as part of an inflammatory response mediated by cytokines.

Cell-wall components

Experimental evidence has accumulated that cell-wall components are responsible for triggering the inflammatory response in the subarachnoid space. These are mainly peptidoglycan-teichoic acid in gram-positive micro-organisms and LPS molecules (endotoxins) in gram-negative bacteria. Both are potent inducers of inflammation. Intracisternal inoculation of any of these components into the CSF compartment of animals induces meningeal inflammation [29-32]. Observations in animals with experimental pneumococcal as well as *H. influenzae* meningitis indicate that initiation of antibiotic therapy results in a more pronounced increase in inflammatory indices and cytokine levels in the CSF [33-35]. Additional evidence indicates that LPS does not act alone during gram-negative bacillary meningitis [36]. These bacteria also contain similar amounts of peptidoglycan [37]. LPS is not present in the CSF in sufficiently high concentrations to directly injure the cerebrovascular endothelium. In contrast, clinically relevant concentrations of LPS (1-10 ng/ml) are able to induce CSF leukocytosis and are potent at priming leukocytes for the production of inflammatory mediators. Peptidoglycan is only a weak inducer of inflammatory mediators. However, peptidoglycan can cause cellular separation of endothelia at concentrations 100-fold less than required for LPS and may be responsible for the induction of BBB permeability in gram-negative meningitis [38].

Two studies in infants with gram-negative meningitis have demonstrated increased endotoxin concentrations in CSF and ventricular fluid when intrathecal or intraventricular antibiotic therapy was given in addition to parenteral antibiotics. These increased endotoxin concentrations have been associated with an augmented meningeal inflammation and with a rise in adverse outcome in infants with coliform meningitis treated with intraventricular gentamicin [39-42].

Cytokines

The host responds with the release of cytokines upon recognition of the presence of bacterial products in the subarachnoidal space. TNF-α and IL-1ß appear to play a pivotal role in triggering the cascade of meningeal inflammation [43]. Il-6, another cytokine found in CSF from patients with bacterial meningitis, has also been implicated in the pathogenesis of this infection. Although production of these inflammatory cytokines has been linked to the development of central nervous system injury, the precise mechanism has not been elucidated. It has been suggested that cytokines are produced by glial cells and brain capillary endothelial cells [44]. Experimental studies have indicated that intracisternal inoculation of

endotoxin is followed by detection of TNF-α and IL-1ß activity. Subsequently, leukocytosis and changes in protein, glucose and lactate concentrations are observed within hours. Combined injection of TNF-α and IL-1ß into the CSF has a synergistic effect [35]. Administration of dexamethasone or polymyxin B before or together with *H. influenzae* type b (Hib) lipo-oligosaccharide, inhibits CSF TNF-α and decreases the meningeal inflammatory response [33, 43, 45, 46]. TNF-α and IL-1ß can also be detected in initial CSF samples of children with bacterial meningitis [48]. The presence of IL-1ß is associated with CSF inflammatory abnormalities, an increased TNF-α concentration, and an adverse outcome [49]. Some reports describe an association between indices of inflammation or the clinical course and TNF-α, IL-1ß and IL-6 levels. Infants with culture-proven viral meningitis or with non-infected CSF have low or non-detectable IL-1ß and TNF-α levels [50-55].

Arachidonic acid metabolites

Arachidonic acid metabolites play an important role as mediators and/or modulators of inflammation. These products may be involved in many of the pathological processes in meningitis. They are released after stimulation with bacterial and immunological antigens by a variety of cells, including neutrophils, platelets, and vascular endothelial cells. TNF-α and IL-1ß induce phospholipase A_2 activity thereby triggering the production of these proinflammatory substances [56].

Previous studies have demonstrated a significant increase in prostaglandin E_2 (PGE_2) (cyclo-ogygenase products) but not leukotriene B_4 (lipo-oxgygenase product) concentration in CSF during pneumococcal meningitis in rabbits [57]. Intracisternally administered PGE_2 did not induce detectable CSF leukocytosis, but caused a dose-related increase in protein content [58]. PGE_2 and PGI_2 are elevated in the CSF of infants and children with bacterial meningitis. The concentration of PGE_2 correlates significantly with protein, TNF-α, and IL-1ß concentrations in the initial CSF sample [59].

Platelet activating factor

CSF platelet activating factor (PAF) concentrations are significantly higher in children with *H. influenzae* meningitis than in afebrile and febrile control subjects without meningitis. The concentrations correlate strongly with concomitant bacterial counts and with both LPS and TNF-α concentrations in admission CSF samples. LPS and a variety of cytokines, such as TNF-α and IL-1ß, are potent inducers of PAF by polymorphonuclear leukocytes (PMNL), macrophages/monocytes, endothelial

cells and neuronal cells [60-62]. PAF recruits and activates PMNL and monocytes at the site of inflammation and induces the release of other inflammatory mediators. PAF in high concentrations is toxic to neuronal cells. PAF acts synergistically with LPS and TNF-α in the development of microvascular tissue damage [63, 64].

Leukocytes

Several hours after meningeal infection an intense influx of leukocytes occurs. Cytokines stimulate the function of neutrophils and provoke degranulation superoxide production and increased adherence to the endothelium. It has been demonstrated that products of leukocytes, such as polyunsaturated fatty acids and oxygen-free radicals can induce brain edema, increased lactate production, and energy depletion in cortical brain slices of rats [65, 66]. However, not all studies demonstrate adverse effects of leukocytes. When the effects of experimental pneumococcal meningitis were compared between normal and neutropenic rabbits similar changes of brain water content (brain edema), intracranial pressure, and CSF concentration of lactate and protein were observed [67].

2.7 THERAPY

Antimicrobial therapy

The combination of ampicillin and chloramphenicol has been known for many years as an effective empirical therapy of bacterial meningitis. Ampicillin resistant ß-lactamase producing *H. influenzae* has become a major problem in many European countries and the United States of America. A recent collaborative European study shows a mean rate of resistance of 10% for all participating countries [68]. In addition, sporadic cases of chloramphenicol-resistant *H. influenzae* have been documented. Combined resistance to chloramphenicol and betalactams is increasing [69-74]. *N. meningitidis* resistant to ß-lactam antibiotics is also increasingly encountered. Spain has been a major source for penicillin-resistant *N. meningitidis* [75]. Infections caused by resistant pathogens lead to higher rates of morbidity and mortality than infections caused by susceptible pathogens.

A consensus among experts about the choice of initial empiric therapy of childhood meningitis is still lacking. Third generation cephalosporins have become important antibiotics for the treatment of presumed bacterial meningitis in infants and children [76-79]. Among these cephalosporins,

cefotaxime, ceftriaxone, and ceftazidime have been studied extensively [80-82]. All three agents are extremely active against *H. influenzae* (including ß-lactamase producing strains), *N. meningitidis* and *S. pneumoniae.* However, cephalosporins are inactive against *Listeria monocytogenes*, *Streptococcus faecalis* and methicillin-resistant staphylococci. Empiric therapy of bacterial meningitis in children up to 3 months of age should include ampicillin for activity against *L. monocytogenes* and enterococci. Once the etiological agent has been identified and its susceptibility determined, therapy can be altered to a single drug or a combination of drugs active in vitro. The duration of therapy is dependent on the clinical response of the patients. Seven days is generally considered adequate for meningococcal infections and 10 days for *H. influenzae* and *pneumococcal* meningitis [83]. Despite the availability of active antibiotics against the common meningeal pathogens, the outcome of meningitis has not changed over the last decades. Therefore investigators have been focusing on novel approaches to diminish neurological sequelae of meningitis.

Fluid restriction

It is generally recommended that children with bacterial meningitis receive less than maintenance fluid to treat or prevent the syndrome of inappropriate secretion of antidiuretic hormone (SIADH) [84, 85]. However, a recent study in infants with bacterial meningitis shows that elevated levels of antidiuretic hormone may also be explained as an appropriate response to intravascular volume depletion rather than as the result of hypothalamic-pituitary axis dysfunction [86]. The loss of cerebrovascular autoregulation in bacterial meningitis may be compensated by secretion of antidiuretic hormone to maintain adequate cerebral blood flow. The cerebral blood flow during bacterial meningitis depends on the cerebral perfusion pressure which is the difference between the mean arterial blood pressure and the intracranial pressure. Two studies in rabbits with experimental meningitis compared fluid restriction with a normal fluid regimen. Fluid restriction resulted in reduced mean arterial blood pressure, significantly decreased cerebral blood flow and increased anaerobic glycolysis of the brain. The fluid regimen, however, did not have a major effect on the degree of brain edema [20, 87]. Since cerebral blood flow is often reduced in bacterial meningitis, fluid restriction may even worsen the neurological outcome. Clinical trials to evaluate the effect of fluid regimen on outcome have not been performed. Current recommendations in children with bacterial meningitis may have to be revised in view of these new experimental data. Fluid restriction should possibly only be limited to those patients with meningitis who fulfil the diagnostic criteria for SIADH.

Adjunctive therapy

The improvements in our understanding of the pathophysiology of bacterial meningitis may lead to the development of novel therapeutic approaches. Modulation of the inflammatory cascade may reduce the incidence of sequelae and death in patients with bacterial meningitis. Interventions will be directed against harmful bacterial products (e.g. monoclonal antibodies, polymyxin B), cytokines (e.g. monoclonal antibodies, steroids), leukocytes (e.g. monoclonal antibodies, pentoxiphylline, radical scavengers) or some consequences of the disease, e.g. brain edema, increased intracranial pressure or alterations of the cerebral blood flow (Table 1).

Table 1. Adjunctive anti-inflammatory agents in the treatment of bacterial meningitis

- Corticosteriods
- Monoclonal antibodies
  - Anti-endotoxin
  - Anti-IL-1ß; anti-TNF-α
  - Anti-CD18
- Cyclo-oxygenase inhibitors
- Pentoxifylline
- Radical scavengers
  - Superoxide dismutase (SOD)
  - Catalase

Corticosteroids. In vitro studies indicate that pretreatment of cultured cells with dexamethasone suppresses the synthesis and release of cytokines induced by bacterial products [88-90]. The inflammatory response in the CSF of rabbits after intracisternal inoculation of bacterial products such as endotoxin or live *H. influenzae* may be blocked by dexamethasone. However, this is only effective when dexamethasone is initiated before or at the same time as the antibiotic [33, 45, 46]. TNF-α and IL-1ß levels in the CSF are reduced concomitantly with concentrations of PGE_2 [59]. Dexamethasone also decreases brain water content, intracranial pressure, and CSF lactate in animals with *S. pneumoniae* or *H. influenzae* meningitis [47, 91, 92].

Clinical studies to evaluate the efficacy of dexamethasone in bacterial meningitis have yielded controversial results (Table 2). Lebel et al. [93] demonstrated that conventional antibiotic therapy (cefuroxim or ceftriaxone) plus dexamethasone resulted in improved outcome in the treatment group in comparison with a placebo-treated group. Children treated with dexamethasone had a significantly lower incidence of sensorineural deafness (15% vs 3.3%) and of other neurological sequelae 12 months later [33]. The study of Odio et al., in which dexamethasone administration was started before the initiation of cefotaxime therapy failed to find a significant reduction in hearing impairment in the steroid treated group. However, an overall reduction in the incidence of neurological complications (including hearing loss) was seen in the treatment group in comparison with a placebo

Table 2. Overview of sequelae in recent placebo/dexamethasone therapy studies in children treated for bacterial meningitis.

Study	Antibiotic therapy	No (%) of children with audiological (A), neurological (N) or combined (C) sequelae			*P*
		SEQ	Placebo	Dexamethasone	
Lebel et al. [93]	Cefuroxim	A	16/38 (42)	9/43 (21)	< .05
		N	3/34 (9)	1/38 (3)	NS
		C	NA	NA	NA
Lebel et al. [93]	Ceftriaxone	A	14/46 (30)	7/49 (14)	NS
		N	6/41 (15)	2/43 (5)	NS
		C	NA	NA	NA
Odio et al. [94]	Cefotaxime	A	7/44 (16)	3/50 (6)	NS
		N	15/48 (31)	5/51 (10)	.008
		C	18/48 (38)	7/51 (14)	.007
King et al. [95]	Not uniform	A	4/51 (9)	5/48 (11)	NS
		N	3/51 (7)	3/48 (6)	NS
		C	NA	NA	NA
Wald et al. [96]	Ceftriaxone	A	NA	NA	NA
		N	NA	NA	NA
		C	10/74 (14)	6/68 (9)	NS
Schaad et al. [97]	Ceftriaxone	A	8/55 (15)	3/60 (5)	NS
		N	5/55 (9)	3/60 (5)	NS
		C	9/55 (16)	3/60 (5)	NS
Schaad et al. [97] (meta-analysis)	Ceftriaxone	C	28/175 (16)	12/177 (7)	.007

Abbreviations: SEQ, sequelae; NA, not available; NS, not significant.

group (14% vs 38%) [94]. The majority of cases of meningitis in these two studies were due to *H. influenzae* while *N. meningitidis* and *S. pneumoniae* will become more important in the coming years due to the introduction of Haemophilus conjugate vaccines. In a study from Egypt mortality was reduced in patients (children and adults) with pneumococcal meningitis by using dexamethasone. This corticosteroid also protected against hearing loss in this group of patients [98]. These three studies showed a significant reduction in neurological sequelae with dexamethasone, but benefits were statistically significant only when the sequelae were unusually common as result of suboptimal therapy (cefuroxim), supportive, and diagnostic management (in developing countries). The results of these studies could not be confirmed in more recent trials, which detected no significant difference between steroid-treated patients and control subjects [95-97]. However, a meta-analysis of sequelae in ceftriaxone-treated bacterial meningitis in children indicated that dexamethasone as adjuvans therapy is superior to placebo [97]. The American Academy of Pediatrics advises to

use dexamethasone in patients with proven or strongly suspected bacterial meningitis. However, the Canadian Pediatric Society has not yet decided to recommend routine use of dexamethasone therapy [99, 100].
Furthermore, the use of corticosteroids is associated with potential side-effects supporting the need for more information to assess the risks or benefits [101]. In the report by Lebel et al. [93], 2% of the patients had gastro-intestinal bleeding. None of the patients receiving placebo had this complication. At present, the frequency and severity of this complication of dexamethasone are unknown. Other studies indicate that glucocorticoids potentiate ischaemic injury to neurons. Since decreased cerebral blood flow appears to be one of the pathophysiological features of bacterial meningitis, it is important to know whether dexamethasone has beneficial effects on hearing but worsens cerebral cortical function [102]. These important questions concerning the place of corticosteroids should be addressed further in carefully conducted clinical trials in homogeneous populations.

Monoclonal antibodies against endotoxin. Inhibition of the effects of endotoxin may have beneficial effects on gram-negative bacterial meningitis, since the toxic moiety of endotoxins (lipid A) can be neutralized by polymyxin B or by monoclonal antibodies to lipid A [103-106]. Indeed, cures of bacterial meningitis were achieved in the pre-antibiotic era by direct installation of immune serum supplemented with complement [107-110]. Direct CSF inoculation was needed for this therapy. Experimental studies in rats demonstrate that intravenous administration of an IgG monoclonal antibody results in low CSF levels ($\leq$ 5.5%) [111]. Intracisternal inoculation of an IgM monoclonal antibody or polymyxin B directed against the lipid A moiety of *E. coli* LPS reduced the cefotaxim-induced increase in CSF LPS concentration and brain water content. The monoclonal antibody was clearly more effective in reducing brain water content although no differences were observed in the capacity to neutralize endotoxin [34].

Monoclonal antibodies against cytokines. Intracisternal administration of TNF-α and IL-1ß in rabbits induces a brisk inflammatory response, which may be blocked by antibodies against the cytokines. Moreover, simultaneous intracisternal administration of anti-TNF-α polyclonal antibody with Hib lipooligosaccharides neutralizes CSF TNF-α activity and is associated with substantial attenuation of the meningeal inflammatory changes [46].

Monoclonal antibodies against CD18. Agents that attenuate the augmented granulocyte-endothelial interaction followed by leukocyte influx into CSF could be beneficial in preventing brain damage. Monoclonal antibodies against the CD18 family of adhesion-promoting receptors on

leukocytes have been studied. Animals receiving intravenous mAb IB4 (CD18 antibody) before intracisternal inoculation with living bacteria (*S. pneumoniae*, *H. influenzae*, *N. meningitidis*), endotoxin or cell-wall demonstrate a dramatic reduction in CSF leukocyte density and protein concentration. Cerebral edema was absent in mAb-treated animals [112, 113].

Inhibition of prostaglandins. Non-steroidal anti-inflammatory agents inhibit the cyclo-oxygenase pathway and reduce meningeal inflammation in bacterial meningitis. Cyclo-oxygenase inhibitors (indomethacin, diclofenac, oxindanac) block the development of brain edema and decrease the number of leukocytes in comparison with non-treated animals. In addition, the influx of protein in the CSF compartment is prevented by these inhibitors. Oxindanac has a stronger activity than other cyclo-oxygenase inhibitors (indomethacin, diclofenac). A study in rabbits with experimental meningitis demonstrated a dramatically decreased mortality by the use of a combination of ampicillin plus oxindanac in comparison with treatment with ampicillin alone or ampicillin plus dexamethasone or indomethacin. Trials of selected nonsteroidal anti-inflammatory agents hold much promise [35, 57, 58, 114, 115].

Pentoxifylline. Recent studies suggest that pentoxifylline, a xanthine-derived phosphodiesterase inhibitor, can inhibit the inflammatory process. Pentoxifylline can reverse or counteract many of the effects of endotoxin and endotoxin-induced cytokines on leukocyte function [116]. Studies in rabbits with experimental meningitis indicate that continuous intravenous infusion of pentoxifylline compared with saline significantly reduces CSF concentrations of leukocytes, protein, and lactate [117]. Pentoxifylline also reduces the release of cytokines from primary murine microglial cell cultures. When added concomitantly with LPS, pentoxifylline blocked the release of TNF-α and IL-1ß but not IL-6, while dexamethasone inhibited the release of TNF-α and IL-6. Pentoxifylline, but not dexamethasone, inhibited TNF-α release from microglia previously stimulated with LPS [118].

Radical scavengers: superoxide dismutase and catalase. Reactive oxygen species in experimental meningitis are capable of inducing vasodilation of cerebral arterioles, increased BBB permeability, increased lactate production and brain edema. Pfister et al. studied the effects of two radical scavengers in experimental meningitis. Superoxide dismutase completely blocked the increase of regional blood flow, intracranial pressure, and brain water content during the early phase of experimental pneumococcal meningitis. Catalase, another radical scavenger, only partly attenuated the increase of regional cerebral blood flow, intracranial pressure

and brain water content. These results suggest that the increase of regional cerebral blood flow, brain water content, and intracranial pressure is mainly caused by superoxide or its products [119, 120].

2.8 VACCINATION

Recently, conjugate vaccines against *H. influenzae* type b (Hib) have been developed. These vaccines, but not the previous non-conjugated ones, are immunogenic and effective during the age of the highest incidence of meningitis caused by Hib. A very low rate of side-effects was noted [121-128]. Hence, Hib vaccination has been introduced in childhood vaccination programmes in an increasing number of countries. Subsequently a 90% reduction in *H. influenzae* meningitis has been observed [123, 129]. However, a substantial number of children have bacterial meningitis caused by *N. meningitidis* and *S. pneumoniae*. Meningococcal disease is caused by several serogroups *N. meningitidis*; serogroup B is the most common cause in Europe, North-America and several countries in Latin America. A meningococcal non-conjugate tetravalent A, C, Y, and W135 polysaccharide vaccine is available and has been shown to be safe and immunogenic in adults, but not in young infants. The serogroup B polysaccharide, however, is poorly immunogenic in humans and has not been useful for development of a vaccine. Therefore, vaccines based on outer membrane proteins are currently being evaluated for their efficacy [130, 131]. Recent field trials with such vaccines have demonstrated only partial protection against group B infection [132, 133]. Several pneumococcal conjugate vaccines combining the most relevant serotypes coupled to different protein antigens are already available [134].

The final aim would be a combined conjugate vaccine, including adequate immunogenic structures of Hib, meningococci and pneumococci to provide a protective antibody response at an early age.

2.9 CONCLUSIONS

Brain damage in patients with bacterial meningitis results from the combined deleterious effects of the micro-organisms and its products and of the host inflammatory response. A number of pathophysiological alterations have been demonstrated in animal models. These include brain edema, elevation of intracranial pressure, changes in CSF outflow resistance,

morphological changes of the BBB, and changes in cerebral blood flow. In addition, mediators of pathophysiological changes have been identified, including cytokines, cyclo-oxygenase metabolites, and PAF. Several adjunctive therapeutic interventions have been developed to modulate the damaging host response to invading micro-organisms. The new treatment strategies are directed against bacterial products, cytokines, and white blood cells. Most of these adjunctive therapies have only shown advantageous effects when administered before or simultaneously with the induction of experimental meningitis. However, pentoxifylline has been shown to attenuate the inflammatory process by reducing the release of cytokines in a model previously exposed to endotoxin. The use of corticosteroids as adjunctive treatment in human studies shows the presence of a possible beneficial effect. Nevertheless, routine use of steroids in children with bacterial meningitis is still a subject of intense debate, because recent studies did not demonstrate a significant difference in the percentage of neurological or audiological sequelae. Although improvement of treatment strategies has given encouraging results, prevention of bacterial meningitis by development and introduction of a combined conjugate vaccine against the three common causative pathogens will be the major challenge of the next decade.

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Chapter 3

Pneumococcal Meningitis in Children: Prognostic Indicators and Outcome

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Clinical Infectious Diseases 1995; 21: 1390-1397

3.1 ABSTRACT

We studied the outcome of pneumococcal meningitis in 83 children admitted to a referral hospital whose meningitis was diagnosed between 1970 and 1994. The median age of the children was 8 months (range, 3 days to 12.3 years). The most frequently isolated capsular serotypes and/or serogroups of *Streptococcus pneumoniae* were 6, 14, 18, 19, and 23. Twenty-nine children (35%) were referred by other hospitals. A mortality rate of 17% (primary referrals, 7%; secondary referrals, 35%) was observed. At discharge, 25 survivors (36%) had sequelae: hearing loss (≥ 30 dB) in 19% and neurological sequelae in 25%. During admission, the presence of coma, respiratory distress, shock, a cerebrospinal fluid (CSF) protein level of ≥ 2.5 g/L, a peripheral white blood cell count of < $5x10^9$/L, and a serum sodium level of < 135 mmol/L were associated with mortality. Sequelae were associated with the presence of coma and a CSF glucose level of < 0.6 mmol/L. We conclude that the mortality rate of pneumococcal meningitis is lower among children than among adults. Children often die of neurological sequelae while adults frequently die of cardiorespiratory failure due to underlying diseases. For children, coma, respiratory distress, and shock during admission were clinical findings with the strongest predictive value for sequelae or death.

3.2 INTRODUCTION

Streptococcus pneumoniae is a frequent cause of pneumonia, otitis media, and bacteremia and the third most common pathogen of meningitis in infants and children. The recent introduction of the *Haemophilus influenzae* type b vaccine in many countries increases the relative importance of *S. pneumoniae* as a major cause of acute bacterial meningitis in children. Epidemiologic studies in the Netherlands have estimated that the annual incidence of pneumococcal meningitis is ~ 1.5 cases per 100,000 population [1, 2]. The incidence of pneumococcal meningitis in children younger than 5 years of age is substantially higher (7 cases per 100,000 population) [3, 4].

Despite continuing improvements in antibiotic and supportive therapy, pneumococcal meningitis is still associated with a high mortality rate and substantial morbidity. The mortality rate among children ranges between 4% and 16% [4-9]. The mortality rate among adults is substantially higher. Among the elderly older than 70 years of age, the mortality rate may be >

50% [6, 10-13]. Neurological sequelae, including hearing impairment, hydrocephalus, convulsions, and mental retardation, have been described in 29%-56% of children with pneumococcal meningitis [4, 7, 14-16]. In follow-up studies of pneumococcal meningitis [17, 18], the rate of sensorineural hearing impairment has ranged from 21% to 50%. The incidence of sequelae in adults at discharge in some studies was as high as 72% [11].

The purpose of the present study was to evaluate the epidemiology and outcome of pneumococcal meningitis in children admitted to the Sophia Children's Hospital/University Hospital Rotterdam (Rotterdam, The Netherlands) between 1970 and 1994. The outcome for children was compared with the outcome for adult patients with pneumococcal meningitis in other studies. In addition, the prognostic significance of several clinical features and laboratory parameters was evaluated.

3.3 Patients and methods

Patients with pneumococcal meningitis who were admitted to our hospital were identified retrospectively by discharge diagnosis from 1 January 1970 to 30 April 1994. The medical records of all patients younger than 18 years of age were abstracted with use of a standard form. Pneumococcal meningitis was defined as a bacterial culture of CSF positive for *S. pneumoniae*, the presence of gram-positive cocci in CSF, or a blood culture positive for *S. pneumoniae* in combination with clinical evidence of meningitis and a CSF WBC count of > 10 mm^3. Patients who received antibiotic therapy before transfer to our institution were also included in the study. In addition, two patients with a clinical history of recurrent pneumococcal meningitis were included in the study; however, only their first episodes were examined. Medical records from our hospital as well as those from referring hospitals were analyzed for demographic parameters, clinical features, laboratory data, antibiotic therapy, complications, and outcome. Clinical and laboratory data on the day of admission to the first hospital were used for statistical analysis. In some medical records, laboratory data were missing or not determined, but this never occurred for > 13 patients for a given characteristic.

Bacteriologic methods

Microorganisms were identified according to standard procedures [19]. Pneumococcal isolates from blood and/or CSF were sent for serotyping to

the Netherlands Reference Laboratory for Bacterial Meningitis (Amsterdam) and to the World Health Organization's Collaborating Centre for Reference and Research on Pneumococci (Copenhagen). The available isolates were serotyped by the capsular swelling (quellung reaction) procedure with use of type-specific antisera obtained from the Statens Seruminstitut (Copenhagen). Since the Netherlands Reference Laboratory for Bacterial Meningitis started to collect *S. pneumoniae* strains from CSF and/or blood in 1975, serotyping was not performed on strains isolated before that year. Susceptibility to penicillin G was determined by the disk diffusion test with use of oxacillin disks according to the methods of the National Committee for Clinical Laboratory Standards [20].

Definitions

Fever was defined as a temperature of > 38.0° C. Consciousness levels during admission were graded as alert or irritable, somnolent or lethargic, and comatose. Shock was defined as a systolic blood pressure that was < 2 SDs of the age-related mean value or severely decreased peripheral perfusion during physical examination. Respiratory distress was defined as a condition that required mechanical ventilation because of one or more episodes of apnea or respiratory failure. Nonsurvivors included patients who died during the acute phase of infection as well as during the follow-up period as a result of sequelae of meningitis. Patients were examined at discharge to detect neurological sequelae. Spasticity or paresis was defined as hypertonia, slight of incomplete paralysis, or paralysis of one or more limbs. Seizures were defined as a convulsive disorder of any type that was present after hospitalization. Hearing impairment was established by audiometric measurements: pure tone audiogram or brain stem auditory evoked potentials (BAEP). Assessment of hearing in young children with BAEP was available from 1981 onward. A hearing loss of ≥ 30 dB that was determined by an audiologist as a probable sensorineural loss was defined as hearing impairment. Severe deafness was diagnosed when a bilateral hearing impairment of > 70 dB was present [17]. The scoring sytem of Herson and Todd was used to assess the clinical severity of pneumococcal meningitis [21]. Patients were divided in different groups for statistical analyses. Survivors were compared with nonsurvivors. In addition, a comparison was made between survivors with and without sequelae and between children younger and older than 2 years of age. The referred patients were compared with the patients who were primarily admitted to our hospital.

Statistical analysis

Results are expressed as means ± SD unless otherwise noted. Differences between groups of variables were tested with the Student's *t*-test or the Mann-Whitney test. Differences in frequencies of various findings between groups were analyzed by the Fisher's exact test. An exact trend test was used to analyze a difference between groups of categorical variables with three ordered categories (e.g., levels of consciousness). Two-tailed *P* values < .05 were considered statistically significant. The relative risk and the 95% confidence interval for the presenting signs and initial laboratory measurements relation to mortality and morbidity were also determined. A significance level of .01 was used for the prognostic indicators because of multiple post hoc hypothesis testing (Bonferroni procedure). The data processing was performed with SPSS-PC+ version 4.0 (SPSS, Chicago).

3.4 RESULTS

Patients

A total of 83 children were included in the study. The diagnoses of pneumococcal meningitis was confirmed by a positive CSF culture in 78 cases. The remaining five children had a blood culture positive for *S. pneumoniae* and an elevated CSF WBC count. Seventy-six percent (53 of 70) of the available blood cultures were positive for *S. pneumoniae*, and 94% of the gram-stains revealed gram-positive diplococci.

Serotypes of *S. pneumoniae*

A total of 55 strains were available for typing. The strains isolated from the 28 remaining patients were missing for a variety of reasons. Eleven of these 28 isolates were not available because they were cultured before 1975. Seventeen strains were not sent to the reference laboratory. The most prevalent serotypes and/or serogroups in descending order of frequency were 19, 14, 18, 6, and 4, which together accounted for 62% of all cases of pneumococcal meningitis diagnosed between 1975 and 1994 (Table 1).

Baseline characteristics

The median age of all patients was 8 months (range, 3 days to 12.3 years). Fifty-four percent of the patients were younger than 1 year of age. Only 22% of the patients were older than 4 years of age. The male-to-

female ratio was 2.1. Approximately one-third (29 of 83) of all patients were referred from another hospital, usually for intensive care treatment because of respiratory distress or mental status that required careful monitoring. The average duration of preceding general symptoms of illness before initiation of intravenous antibiotic therapy was 2.5 ± 2.7 days (range, < 12 hours to 14 days). In four patients, the disease developed during hospitalization.

During admission, 41 of the 83 patients had the classical triad of signs of fever, nuchal rigidity, and change in mental status. The other 42 children had a least one of these signs. Fever was the only presenting sign in six patients. Neck stiffness was present in 67% of patients during initial examination. Most patients had abnormal mental status at the time of presentation: 34% were alert or irritable, 54% were somnolent or lethargic, and 12% were comatose. Seizures occurred in 30% of the patients prior to or within 24 hours after admission to the first institution. A high proportion (34%) of children needed respiratory support. Thirteen patients (16%) were in shock on arrival at the hospital. Eleven patients died during the acute phase of meningitis. Three patients died later as a consequence of neurological sequelae. Twenty-five survivors (36%) had one or more sequelae at discharge from the hospital. The outcome of pneumococcal meningitis did not improve during the study period. The mortality and morbidity rates remained similar between 1970 and 1994.

Serotype and/or serogroup	No (%)
19/19A/19F	10 (18)
14	9 (17)
18/18C	6 (11)
6/6A	5 (9)
4	4 (7)
3	3 (5)
7F	3 (5)
8	3 (5)
Others	12 (23)

Table 1. Capsular serotypes and/or serogroups of 55 strains isolated from children with pneumococcal meningitis.

Predisposing factors

Twelve patients had an underlying disorder: malignancies (n = 4), hydrocephalus (n = 3), asplenia (n = 1), AIDS (n = 1), X-linked

hypogammaglobulinemia (n = 1), short bowel syndrome (n = 1), and type III glycogenosis (n = 1). Three patients had one or more previous episodes of bacterial meningitis. Predisposing conditions directly related to the episode of pneumococcal meningitis were infections (33 patients) or prior head injury and/or surgery (8 patients). The most common associated infection was otitis media (23 patients). Other infections, including pneumonia, sinusitis, mastoiditis, or a combination of these infections, were present in 10 patients. The possible portal of entry remained unknown for 51% of all patients.

Treatment

The antibiotics most frequently used at the time of admission were a combination of penicillin and chloramphenicol (39 patients), a combination of cefotaxime and amoxicillin (26 patients), and penicillin alone (4 patients). For the remaining 14 children, most of whom were admitted before 1975, other initial antibiotic regimens were prescribed. All isolates were susceptible to penicillin. Antibiotic therapy was changed to penicillin monotherapy after the results of susceptibility testing became available.

Survivors vs. nonsurvivors

The clinical features of and laboratory data for survivors and nonsurvivors at the time of admission are summarized in Tables 2 and 3, respectively. The overall mortality rate was 17%. The clinical conditions (level of consciousness, hemodynamic condition, and respiratory status) of the nonsurvivors were worse than those of the survivors. The Herson and Todd severity scores were higher for the nonsurvivors, but the difference was not significant. Most of the deaths were a result of complications of the CNS. Three patients probably died as a consequence of fulminant septic shock. The peripheral WBC counts and the serum sodium levels in the nonsurvivors were significantly lower than those in the survivors. The CSF protein concentrations were higher in the nonsurvivors (P = .012). There was a significant difference in mortality between patients referred from another hospital and patients who were primarily admitted to our hospital (10 [34%] of 29 vs. 4 [7%] of 54, respectively; P = .017). A comparison of the referred patients and the children who were primarily admitted to our hospital revealed that the level of consciousness (8 [28%] of 29 vs. 2 [4%] of 54, respectively; P = .003), the hemodynamic status (8 [28%] of 29 vs. 5 [9%] of 54, respectively; P = .054), and the respiratory condition (20 [69%] of 29 vs. 8 [15%] of 54, respectively; P <.001) during admission were worse in the referrals.

Table 2. Clinical features at the time of admission of 83 patients with pneumococcal meningitis.

Feature	Outcome of all episodes			Sequelae in survivors		
	Nonfatal (n = 69)	Fatal (n = 14)	P	Absent (n = 44)	Present (n = 25)	P
Age (months)*	28 ± 37	39 ± 41	.54	27 ± 36	30 ± 38	.79
Male	46 (67)	10 (71)	1.00	29 (66)	17 (68)	1.00
Duration of symptoms (days)*	2.6 ± 2.8	1.8 ± 1.5	.48	2.4 ± 2.7	2.9 ± 3.1	.44
Clinical score*†	2.7 ± 1.7	4.1 ± 2.9	.063	2.3 ± 1.6	3.3 ± 1.7	.032
Level of consciousness						
Alert or irritable	25 (36)	3 (21)		18 (41)	7 (28)	
Somnolent or lethargic	39 (57)	6 (43)	.024	24 (55)	15 (60)	.22
Comatose	5 (7)	5 (36)		2 (5)	3 (12)	
Neck stiffness	46 (67)	6 (43)	.49	31 (70)	15 (60)	.43
Seizures	19 (28)	5 (36)	.33	12 (27)	7 (28)	1.00
Vomiting	13 (19)	2 (14)	1.00	12 (27)	1 (4)	.023
Shock	5 (7)	8 (57)	<.001	0 (..)	5 (20)	.005
Respiratory distress	15 (22)	13 (93)	<.001	10 (23)	5 (20)	1.00

NOTE: Data are number (%) of patients with feature unless otherwise specified.
*Mean ± SD.
†Herson and Todd severity score [21].

Table 3. Laboratory parameters at the time of admission for 83 patients with pneumococcal meningitis.

Analysis, parameter		Outcome of all episodes			Sequelae in survivors		
		Nonfatal (n = 69)	Fatal (n = 14)	P	Absent (n = 44)	Present (n = 25)	P
Hematologic							
Hb level (mmol/L)		6.7 ± 1.1	6.7 ± 1.0	.96	6.7 ± 1.1	6.6 ± 0.9	.96
Peripheral WBC count (10^9/L)		19.8 ± 11.1	11.2 ± 12.8	.001	22.3 ± 11.7	15.7 ± 8.8	.021
Platelet count (10^9/L)		383 ± 206	233 ± 138	.079	431 ± 221	296 ± 147	.053
Serum chemistry							
Sodium level (mmol/L)		135 ± 5	129 ± 7	.002	135 ± 3	134 ± 7	.77
Potassium level (mmol/L)		4.3 ± 0.7	4.2 ± 0.8	.71	4.4 ± 0.7	4.0 ± 0.7	.11
Glucose level (mmol/L)		7.4 ± 3.1	7.7 ± 4.0	.47	7.8 ± 2.9	6.5 ± 3.4	.10
pH		7.39 ± 0.09	7.31 ± 0.19	.21	7.40 ± 0.08	7.36 ± 0.10	.17
PCO_2 (kPa)		4.7 ± 1.0	5.0 ± 1.7	.61	4.7 ± 0.8	4.6 ± 1.5	.60
BE (mmol/L)		-2.5 ± 4.0	-5.8 ± 8.0	.35	-1.9 ± 3.7	-4.0 ± 4.3	.06
CSF							
WBC count (/mm^3)	Median	1530	525	.14	1770	800	.02
	Range	6-34,000	29-30,000		6-40,000	15-35,000	
Protein level (g/L)	Median	1.4	3.9	.012	1.4	1.4	.89
	Range	0.1-8.0	0.5-25.0		0.1-8.0	0.1-8.0	
Glucose (mmol/L)	Median	0.9	0.5	.27	2.7	0.5	.005
	Range	<0.2-6.8	<0.2-4.1		<0.2-6.8	<0.2-4.3	
CSF/serum glucose ratio	Median	0.26	0.07	.21	0.34	0.09	.11
	Range	0-1.09	0-0.55		0-1.09	0-0.88	

NOTE: Data are means ± SD unless otherwise specified. Abbreviations: WBC, white blood cell; CSF, cerebrospinal fluid; Hb, Hemoglobin; BE, base excess.

Neurological and audiological sequelae

The clinical features of and the laboratory findings for the survivors with and without sequelae are shown in Tables 2 and 3, respectively. Twenty-five survivors (36%) had one or more neurological sequelae at discharge. Sensorineural hearing loss was diagnosed for 13 survivors (19%). A severe bilateral hearing loss was detected in six children. Audiological examination of four patients revealed a unilateral sensorineural hearing loss of > 80 dB. Other neurological sequelae were present in 17 survivors (25%): seizures (n = 7), paresis or spasticity (n = 7), and cranial nerve abnormalities (n = 5). Two patients had hydrocephalus. Forty-four patients recovered without any sequelae.

Clinical features and CSF characteristics according to age

The clinical features of and laboratory data for patients older and younger than 2 years of age at the time of admission are depicted in Table 4. The presence of neck stiffness was significantly different between the two groups, whereas CSF characteristics were similar. The mortality rate was higher among the children older than 2 years of age, but this difference was not significant (8 [25%] of 32 vs. 6 [12%] of 51, respectively; P = .14). The occurrence of sequelae was similar in both age groups.

Prognostic indicators

A number of clinical findings and laboratory measurements were associated with a poor outcome (Table 5). Quantitative variables were dichotomized. The cutoff values for quantitative prognostic variables were based either on the results of previous studies or on the value for which the highest relative risk could be calculated. The relative risk for mortality and morbidity and the 95% confidence interval for the prognostic indicators were calculated. The presence of an underlying disorder and predisposing conditions were not associated with sequelae or death. A difference in CSF glucose concentration between survivors and nonsurvivors was not observed. A limited number of parameters were associated with the development of sequelae in survivors: shock during admission (RR = 3.2; 95% CI = 2.2-4.6; P = .005), a CSF WBC count of < 1000/mm^3 (RR = 1.6; 95% CI = 1.0-2.7; P = .031), and a CSF glucose level of < 0.6 mmol/L (RR = 2.9; 95% CI = 1.4-6.0; P = .004).

Table 4. Clinical features and CSF characteristics at the time of admission according to age of 83 patients with pneumococcal meningitis.

Feature or characteristic	Patients younger than 2 years of age (n = 51)	Patients older than 2 years of age (n = 32)	P
Age (months)*	6 ± 5	67 ± 36	<.001
Sex No. (%) of males	33 (65)	23 (72)	
No. (%) of females	18 (35)	9 (28)	.63
Duration of symptoms (days)*	2.5 ± 2.7	2.4 ± 2.6	.68
No. (%) with indicated level of consciousness			
Alert or irritable	9 (37)	9 (28)	
Somnolent or lethargic	29 (57)	16 (50)	.11
Comatose	3 (6)	7 (22)	
No. (%) with neck stiffness	24 (47)	28 (88)	<.001
No. (%) with seizures	18 (35)	6 (19)	.14
No. (%) with shock	8 (16)	5 (16)	1.00
No. (%) with respiratory distress	15 (29)	13 (41)	.34
CSF characteristic			
WBC count (/mm^3)			
Median	1170	1530	.098
Range	6-34,000	92-32,000	
Protein level (g/L)			
Median	1.4	1.7	.60
Range	0.1-25.0	0.1-12.5	
Glucose level (mmol/L)			
Median	0.5	2.2	.27
Range	<0.2 - 6.8	<0.2 - 5.0	
CSF/serum glucose ratio			
Median	0.13	0.34	.68
Range	0-1.09	0-0.90	

*Mean ± SD. Abbreviations: CSF, cerebrospinal fluid; WBC, white blood cell.

3.5 Discussion

S. pneumoniae is a common pathogen of childhood meningitis. Groups at high risk for pneumococcal meningitis are the very young (younger than 5 years old) and the elderly (65 years of age of older) [2, 22]. The outcome for patients with pneumococcal meningitis is less satisfactory than that for patients with bacterial meningitis caused by other microorganisms. Reviews

on pneumococcal meningitis have predominantly included cases in adult patients or a combination of cases in pediatric and adult patients [4, 6, 11, 13, 23-28]. We evaluated 83 cases of pneumococcal meningitis in children admitted to Sophia Children's Hospital/University Hospital Rotterdam between 1970 and 1994. We compared our data with those from other studies.

Most (54%) of the patients were younger than 1 year of age. A predominance of male patients was seen, as has been reported by other researchers [6, 26]. Recent studies of pneumococcal disease in children in North America and western Europe demonstrated that certain serotypes and/or serogroups (6, 14, 18, 19 and 23) are responsible for most cases of bacteremia and meningitis [2, 3, 8, 29-33]. The distribution of serotype and/or serogroups of *S. pneumoniae* in the present study is remarkably similar.

Despite the progress in medical treatment, the outcome of pneumococcal meningitis in the present study remained similar in the period between 1970 and 1994. The overall mortality rate among the patients was 17%. Because of the substantial number of patients referred by other hospitals, this rate is relatively high in comparison with rates reported in previous studies [4-6, 9, 14, 34]. The mortality rates of pneumococcal meningitis in children who were either primarily admitted or referred to our hospital were 7% and 34%, respectively ($P = .017$). The higher mortality rate among the secondary referrals was expected since this group of patients had significantly worse levels of consciousness and worse hemodynamic and respiratory conditions during admission. Bohr et al. [6] also reported a significant difference in mortality rates between patients (all ages) referred by other hospitals and patients primarily admitted to their hospital (27% vs. 9%, respectively; $P < .05$). The mortality rate among adult patients with pneumococcal meningitis is higher than that among children. A recent retrospective study of pneumococcal meningitis in Dutch adults reported a mortality rate of 33% [11], which was higher than our rate ($P = .06$). The fatal outcome of pneumococcal meningitis in adults, most of whom are elderly, is frequently due to the development of cardiorespiratory failure caused by underlying disorders. In contrast, children often die as a result of neurological complications. It is interesting that pneumococcal meningitis in adults was more frequently associated with pneumonia (range, 16%-39%) [11, 24-26, 35, 36]. This finding is in contrast with our observation that only three (4%) of 83 children had pneumonia. The presence of underlying disorders and predisposing conditions in the patients in the current study was not associated with a bad outcome. Age-specific differences in outcome were also not observed. However, neck stiffness was seen

Table 5. Mortality rate of pneumococcal meningitis according to presenting signs and initial laboratory measurements.

Sign or measurement	No. of cases tested	No. (%) of nonsurvivors	RR (95% CI)	*P*
Level of consciousness				
Comatose	10	5 (50)	4.1 (1.7-9.7)	.01
Noncomatose	73	9 (12)		
Respiratory distress				
Yes	28	13 (46)	25.5 (3.5-185.4)	<.001
No	55	1 (2)		
Shock				
Yes	13	8 (62)	7.2 (3.0-17.3)	<.001
No	70	6 (9)		
Peripheral WBC (10^9/L)				
< 5.0	8	5 (63)	5.6 (2.4-13.1)	.002
≥ 5.0	72	8 (11)		
Serum sodium (mmol/L)				
< 135	37	12 (32)	11.0 (1.5-80.4)	.002
≥ 135	34	1 (3)		
CSF WBC (/mm^3)				
< 1,000	31	8 (26)	2.9 (1.0-8.8)	.06
≥ 1,000	45	4 (9)		
CSF glucose (mmol/L)				
< 0.6	34	6 (18)	1.7 (0.5-5.6)	.50
≥ 0.6	39	4 (10)		
CSF protein (g/L)				
≥ 2.5	25	8 (32)	7.7 (1.8-33.5)	.002
< 2.5	48	2 (4)		

Abbreviations: CI, confidence interval; RR, relative risk; WBC, white blood cells; CSF, cerebrospinal fluid.

significantly more often in patients older than 2 years of age. This observation is in agreement with the finding that nonspecific symptoms predominantly occur in newborns and young infants [37].

We observed that one or more sequelae were present at discharge in 36% of the survivors. It is interesting that the rate of sequelae was not significantly higher among the referred patients. The incidence of neurological sequelae has ranged between 25% and 56% in several reports [4, 7, 14-16]. Many of the neurological abnormalities observed in the survivors will probably resolve within 1 year [38]. A hearing impairment, which is usually permanent, was observed in 19% of the patients. In a recent review [18] the incidence of permanent sensorineural hearing

impairment after pneumococcal meningitis was 32% (range, 21%-50%). We may have underestimated the percentage of patients with hearing loss since an audiological assessment was performed in only 52% of all survivors. Assessment of hearing was performed in 74% of the patients with pneumococcal meningitis who were admitted since 1980, when the measurement of BAEP became available. The percentage of children with hearing impairment in that period remained similar (21%).

Several clinical findings were associated with a high mortality rate among children in the present study. An altered level of consciousness at the time of admission was associated with an increased rate of mortality. This association was highly significant in patients who were comatose. The presence of shock was a bad prognostic indicator for mortality and morbidity. The mortality rate was also significantly increased among patients who needed artificial ventilation. These findings were also reported previously [6, 24, 26, 39].

Various investigators have evaluated the prognostic importance of laboratory parameters in fatal cases of pneumococcal meningitis and in cases in survivors with sequelae. An unfavorable outcome has previously been associated with a decreased CSF WBC count [24, 25, 35], a low CSF glucose level (e.g., < 0.6 mmol/L) [14, 24, 27, 39], a high CSF protein concentration (e.g., > 1.5 g/L) [11, 24, 25, 27, 28, 40, 41], high CSF levels of lactate or fibrin-degradation products, high CSF bacterial counts, or high CSF bacterial antigen titers [25, 35, 42]. However, other investigators could not confirm these associations [6, 36]. In the present study, the following laboratory measurements were associated with a poor outcome: a decreased CSF glucose level (< 0.6 mmol/L), an elevated CSF protein level (≥ 2.5 g/L), a decreased peripheral WBC count (< 5 x 10^9/L), and a low serum sodium level (< 135 mmol/L). Patients with a low CSF WBC count (< 1,000/mm^3) tended to have worse outcomes. These data were similar to the data in most other studies, although the cutoff values of the variables were slightly different. Bohr et al. [6] observed that pleocytosis was not associated with significant differences in mortality rates; however, an increased rate of sequelae was found among children with high CSF WBC counts (> 5,000/mm^3). In the present study, the CSF glucose level was significantly decreased only in the survivors with sequelae, while in other reports higher mortality rates were also observed [14, 24, 27]. We conclude that the mortality rate of pneumococcal meningitis is lower among children than among adults. Children often die of neurological sequelae, whereas adults frequently die of cardiorespiratory failure due to underlying diseases. For children, coma, respiratory distress, and shock at the time of admission were clinical findings with the strongest predictive value for a bad outcome.

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Chapter 4

Molecular Epidemiological Characteristics of Pneumococcal Meningitis in Children

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European Journal of Clinical Microbiology & Infectious Diseases 1996; 15: 635-638

4.1 ABSTRACT

The molecular epidemiological characteristics of pneumococcal meningitis in children were studied. Pneumococcal isolates were characterized by serotyping and two genotyping methods, BOX fingerprinting and restriction fragment end labeling, to evaluate whether clonal strains were present within the serotypes or serogroups. During a 17-year period, 68 children admitted to the Sophia Children's Hospital were diagnosed with meningitis due to *Streptococcus pneumoniae*. Pneumococcal isolates from 44 patients were still available for analysis. All strains were susceptible to penicillin. Serotypes/serogroups 14, 19, 6, and 8 represented 56% of all isolates. The results of the molecular typing methods demonstrate the absence of clonal relatedness between isolates from patients with pneumococcal meningitis.

4.2 INTRODUCTION

Streptococcus pneumoniae remains a major cause of morbidity and mortality and is the third most common microorganism worldwide in children with bacterial meningitis. Pneumococci can be divided into 84 serotypes on the basis of differences in structure and antigenicity of capsular polysaccharides [1]. A continuing survey of capsular types of *S. pneumoniae* from cerebrospinal fluid (CSF) or blood remains necessary to determine if the current and future multivalent (conjugate) polysaccharide vaccines still contain the predominant serotypes. Serotyping is a useful technique to study the epidemiological relatedness of *S. pneumoniae* isolated during outbreaks [2]. However, capsular serotyping does not give an accurate estimation of genetic relatedness between isolates [3, 4]. Several new phenotypic and genotypic methods have been developed and applied to study relationships among isolates from clinical outbreaks, mainly with drug-resistant *S. pneumoniae*. These techniques include penicillin-binding protein profile analysis, multilocus enzyme electrophoresis, DNA fingerprinting by pulsed-field gel electrophoresis, polymerase chain reaction (PCR) fingerprinting, and ribotyping [4-8]. Application of these techniques has revealed that penicillin-resistant clones from a limited number of serotypes (6B, 19A, 23F) are responsible for the rapid worldwide dissemination of antibiotic-resistant pneumococci. Recently, our group reported that BOX fingerprinting and restriction

fragment end labeling (RFEL) are very suitable molecular techniques for typing pneumococcal strains [9].

We previously reviewed our clinical experience with pneumococcal meningitis in children [10]. Forty-four of 68 pneumococcal isolates taken between 1975 and 1992 were still available for further analysis. The aim of the current study was to investigate by RFEL and BOX fingerprinting whether clonal isolates were present within the different serotypes/ serogroups.

4.3 Materials and methods

Identification of pneumococcal strains

We surveyed the medical records of children < 18 years of age admitted to the Sophia Children's Hospital/University Hospital Rotterdam, The Netherlands between 1975 and 1992 and diagnosed with pneumococcal meningitis. Meningitis was defined by the isolation of *S. pneumoniae* from CSF or blood in combination with clinical evidence of meningitis and a CSF white blood cell count > 10 cells/mm^3. Only one isolate per disease episode was analyzed. Sixty-eight children were diagnosed with pneumococcal meningitis. Forty-four strains were available for serotyping and genotyping.

Bacteriological methods

Pneumococcal isolates from blood and/or CSF were sent for identification to the Netherlands Reference Laboratory for Bacterial Meningitis (University of Amsterdam/National Institute of Public Health and the Environment) and to the WHO Collaborating Center for Reference and Research on Pneumococci (Copenhagen, Denmark). Microorganisms were identified according to standard procedures. All strains were serotyped by the capsular swelling procedure (quellung reaction) using type-specific antisera obtained from the Statens Seruminstitut, Copenhagen. Susceptibility to penicillin G was determined by the disk diffusion method using oxacillin disks according to the National Committee for Clinical Laboratory Standards [11].

DNA isolation, restriction endonuclease digestion, and DNA probes

Bacterial cultures were grown in Todd-Hewitt broth containing 0.5% yeast extract at 37°C to an optical density of 0.5 at 560 nm. Bacterial cells from 5 ml cultures were harvested by centrifugation and genomic DNA was

extracted by the cetyl-trimethyl-ammoniumbromide (CTAB) method [12]. The restriction enzymes PvuII and EcoRI were used to digest the CTAB-purified chromosomal DNA samples [13]. The oligonucleotides BR-A (5'ATACTCTTCGAAAATCTCTTCAAAC) and BR-C (5'TATACTCAAT-GAAAATCAAAGAGCA) were derived from the BOX repeat sequence of *S. pneumoniae* [14]. The oligonucleotide primer pair BR-A/BR-C was used in PCR to generate the 151 base pair (bp) BOX repeat probe. Amplification of the DNA probe by PCR was performed as previously described [15]. The PCR-amplified probe was labeled by the enhanced chemiluminescence gene detection system (Amersham International, Bucks, UK) according to the manufacturer's recommendations.

Restriction fragment end labeling

Restriction fragment end labeling was performed as described by Van Steenbergen et al. [16], and modified by Hermans et al. [9]. Briefly, purified pneumococcal DNA was digested by EcoRI. DNA restriction fragments were labeled at 72°C with [α-^{32}P] dATP using Taq DNA polymerase. The radio-labeled fragments were denatured and separated electrophoretically on a 6% polyacrylamide sequencing gel containing 8 M urea. Subsequently the gel was transferred onto filter paper, vacuumdried, and exposed for variable lengths of time at room temperature to radiographic films (Fuji RX film; Fuji Medical Systems, The Netherlands).

BOX fingerprinting

Molecular analysis of the pneumococcal isolates was also performed by BOX fingerprinting [9]. By this method, purified chromosomal DNA of *S. pneumoniae* strains was digested with PvuII. Internal markers were added to the digested DNA samples as described previously [12]. The restriction fragments were separated electrophoretically on a 0.8% agarose gel, denatured, and transferred onto a nylon membrane (Hybond N+; Amersham International) by vacuum blotting (VacuGene; Pharmacia LKB Biotechnology, Sweden). Hybridization analysis was performed using the 151 bp BOX repeat probe. Hybridization and detection was carried out by the chemiluminescence gene detection system as recommended by the manufacturer (Amersham).

Computer-Assisted analysis of DNA fingerprints

The fingerprints displayed by RFEL were analyzed using the Windows version of the Gelcompar software, version 3.10 (Applied Maths, Belgium) after imaging of the autoradiograms with a scanner (HP Scanjet IIcx/T; Hewlett Packard, USA) at 190 dots per inch. Fingerprint patterns were

Table 1. Capsular serotypes of pneumococci isolated from cerebrospinal fluid during the period 1975-1992.

Order of frequency	Serotype	No. (%)	Cumulative No. (%)
1	14	9 (20)	9 (20)
2	19/19A/19F	6 (14)	15 (34)
3	6/6A	5 (11)	20 (45)
4	18/18C	5 (11)	25 (56)
5	4	4 (9)	29 (65)
6	7F	3 (7)	32 (72)
7	8	3 (7)	35 (79)
8	3	2 (5)	37 (84)
9	1/2/13B/15B/23/27/33F	7 (16)	44 (100)

normalized using pneumococcus-specific bands that were present in the fingerprints of all strains. Comparison of the fingerprints displayed by RFEL was performed by unweighted pair group method using arithmetic averages (UPGMA) [17], and using the Jaccard similarity coefficient applied to peaks [18].

The BOX fingerprints were analyzed according to the software manufacturer's instructions. The mobilities of the DNA fragments hybridizing with the DNA probe were compared with a set of internal molecular weight markers by superimposing autoradiograms containing the DNA fingerprints and autoradiograms containing the internal markers of known molecular size [12]. This procedure enabled us to normalize the position of each of the hybridizing fragments, irrespective of autoradiogram and/or gel distortions. Comparison of the BOX fingerprints was performed using the Pearson correlation coefficient.

4.4 Results

Susceptibility testing and serotyping of the pneumococcal strains

Susceptibility testing was performed using the 44 available isolates. All isolates were susceptible to penicillin. The isolates were also characterized by serotyping. Serotype distribution is depicted in Table 1. *S. pneumoniae* belonging to the eight most common groups comprised 84% of all infections. The most prevalent serogroups were 14, 19, 6, and 18,

accounting for 56% of all cases. Geographical or temporal clustering of serotypes could not be demonstrated.

Molecular analysis of the pneumococcal strains

The collection of pneumococcal isolates were analyzed by RFEL. The fingerprint patterns of 43 isolates were suitable for computerized analysis. Fingerprints were all different from each other (Figure 1). However, one pair of fingerprints (891308 serotype 19F and 910105 serotype 14) was more than 95% identical, but these isolates were less than 70% identical in the analysis by BOX fingerprinting. BOX fingerprinting resulted in 44 distinct fingerprint patterns (Figure 2) using a cut-off value of 95% similarity (data not shown). These data demonstrate the enormous genetic heterogeneity of the pneumococcal meningitis isolates.

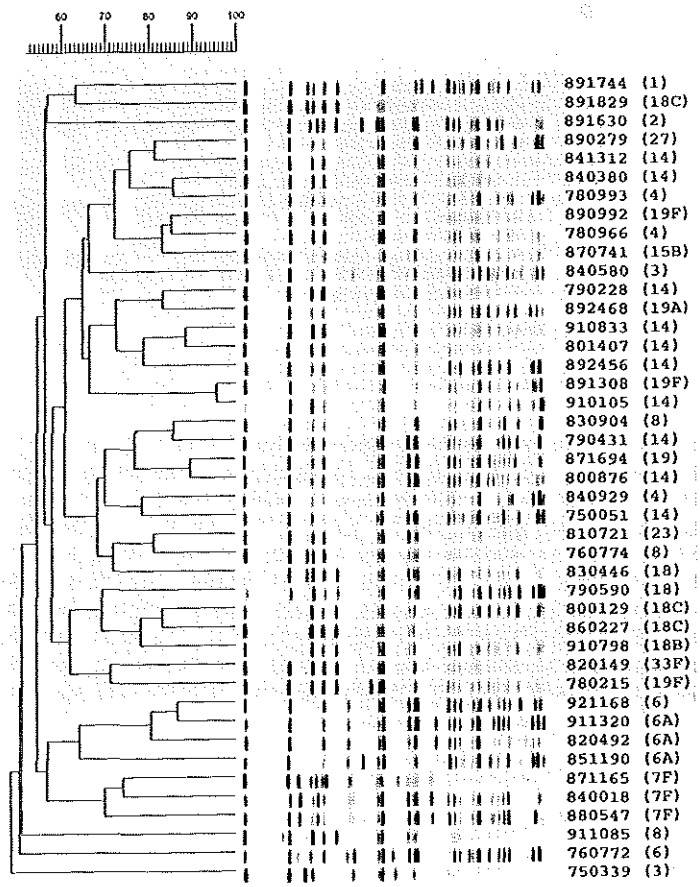

Figure 1. DNA fingerprint analysis of 43 pneumococcal isolates. The analysis was performed by restriction fragment end labeling. A dendrogram of the fingerprints showing the genetic relatedness of the strains is also depicted. The scale indicates similarity values in percentages. The fingerprint codes represent strain number and serotype (in parentheses).

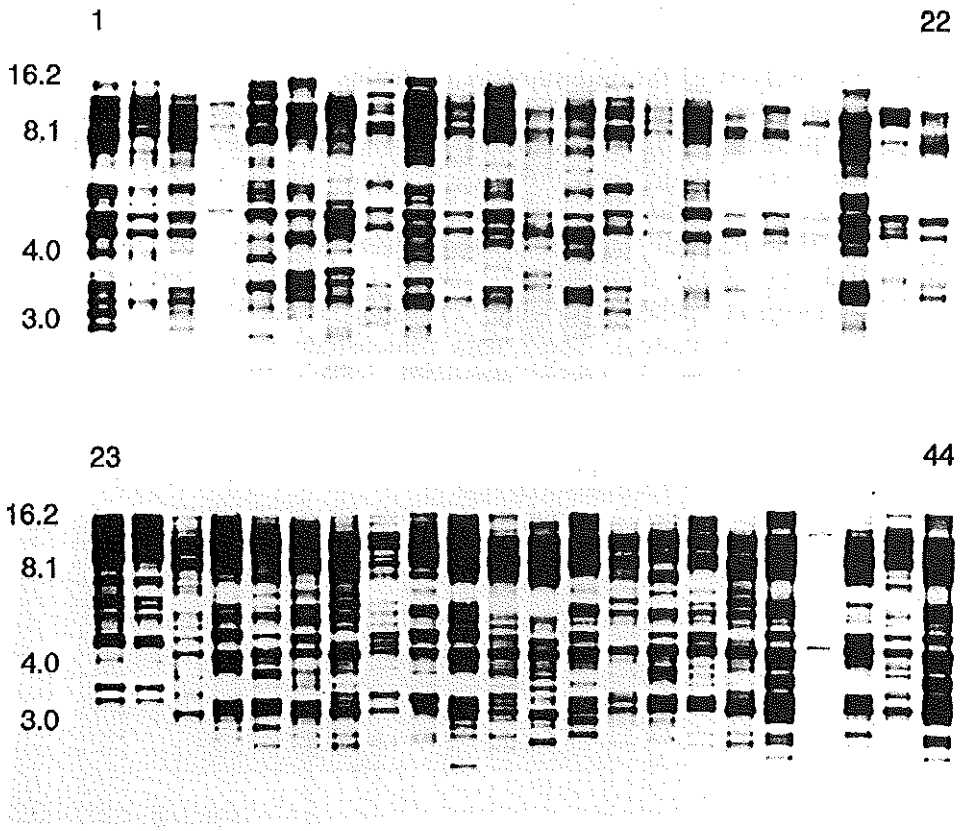

Figure 2. Molecular epidemiological analysis of 44 *Streptococcus pneumoniae* strains by BOX fingerprinting. The lane numbers correspond with the following strains: lane 1, strain 760774; lane 2, 840380; lane 3, 820149; lane 4, 780993; lane 5, 851190; lane 6, 891744; lane 7, 871165; lane 8, 830904; lane 9, 790228; lane 10, 910833; lane 11, 790590; lane 12, 840580; lane 13, 850531; lane 14, 890279; lane 15, 841312; lane 16, 910798; lane 17, 800876; lane 18, 871694; lane 19, 801407; lane 20, 891630; lane 21, 880547; lane 22, 891829; lane 23, 820492; lane 24, 911320; lane 25, 892465; lane 26, 800129; lane 27, 892468; lane 28, 911085; lane 29, 790431; lane 30, 840018; lane 31, 921168; lane 32, 860227; lane 33, 780215; lane 34, 750339; lane 35, 830466; lane 36, 870741; lane 37, 890992; lane 38, 760772; lane 39, 810721; lane 40, 840929; lane 41, 891308; lane 42, 750051; lane 43, 910105; and lane 44, 780966. Numbers at left

4.5 DISCUSSION

S. pneumoniae is a major pathogen in meningitis in children and adults. The aim of the present study was to identify the molecular epidemiological characteristics of pneumococcal meningitis in children. We questioned whether clonal types would be present within the limited number of serotypes/serogroups.

Serotype distribution of *S. pneumoniae* varies with age, geographic area, time, and site of infection [19]. Pneumococcal types 6, 7F, 14, 18C, 19F, and 23F have previously been reported to be responsible for the large majority of pneumococcal infections in children [19]. The distribution of serotypes in our study was similar, with a limited number of serotypes (14, 19, 6, and 18) accounting for 56% of all isolates. However, clustering observed by serotyping was not confirmed by the two genotypic methods, RFEL and BOX fingerprinting. Since the strains were sampled over a 17-year period, the epidemiological relatedness of these strains is unlikely, and subsequently, the high degree of genetic diversity is not surprising. Furthermore, genetic diversity has been demonstrated to be higher in drug-susceptible pneumococci compared with drug-resistant isolates [20]. The drug-susceptible phenotype of the strains used in this study might therefore also contribute to the observed high degree of genetic diversity within this group.

Analysis by RFEL and BOX fingerprinting of ten multiply resistant serotype 23F *S. pneumoniae* strains isolated from different locations during a five-year period in the USA [7] displayed identical fingerprints (unpublished observations). These data support the power of these two methods to analyze clonality among pneumococci presumably over decades. Therefore, these techniques may allow the investigation of long-term epidemiological issues, such as global spread or global persistence of certain pneumococcal serotypes.

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Chapter 5

Interleukin-10 and Soluble Tumor Necrosis Factor Receptors in Cerebrospinal Fluid of Children with Bacterial Meningitis

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Journal of Infectious Diseases 1996; 173: 1498-1502

5.1 ABSTRACT

The antiinflammatory mediators interleukin (IL)-10 and soluble tumor necrosis factor (TNF) receptors p55 (sTNFR-55) and sTNFR-75 in cerebrospinal fluid (CSF) from 37 children with bacterial meningitis were studied. CSF concentrations of IL-10, sTNFR-55, and sTNFR-75 and of the proinflammatory cytokines TNF-α, IL-6, and IL-8 were markedly elevated and were, with the exception of the sTNFRs, significantly higher in CSF than in serum. CSF concentrations of sTNFR-55 and sTNFR-75 were only associated positively with IL-10 levels. CSF glucose levels correlated highly with levels of IL-10, sTNFR-55, and sTNFR-75 and weakly with TNF-α and IL-6. Cytokine levels in CSF decreased rapidly, while sTNFR levels remained elevated for at least 24 hours.

5.2 INTRODUCTION

Proinflammatory cytokines (tumor necrosis factor-α [TNF-α], interleukin [IL]-6, IL-8) are released within the cerebrospinal fluid (CSF) compartment of patients with bacterial meningitis upon recognition of bacterial products. The production of proinflammatory cytokines and the extent of the inflammatory response are partly controlled by antiinflammatory compounds, such as IL-10, and naturally occurring antagonists of TNF-α, including soluble extracellular domains of the 55- and 75-kDa membrane-bound TNF receptors (sTNFR-55 and sTNFR-75).

IL-10 is produced by monocytes/macrophages, the Th2 subset of T helper lymphocytes, and B lymphocytes and suppresses the synthesis of proinflammatory cytokines by T cells [1], polymorphonuclear leukocytes [2], and monocytes/macrophages [3]. IL-10 protects against TNF-mediated lethality in murine models of endotoxemia [4]. CSF levels of IL-10 are significantly increased in patients with bacterial meningitis, suggesting a role for IL-10 in the control of the inflammatory response in the CSF compartment [5].

The biologic activity of TNF-α is also neutralized by sTNFR-55 and sTNFR-75 [6]. Release of sTNFR-55 and sTNFR-75 may provide a mechanism for modulation of excessive TNF-α activity in response to injury or infection. sTNFRs are shed from the cell surface of polymorphonuclear cells and monocytes in response to many of the same inflammatory stimuli that are known to induce TNF-α [7]. Of interest, IL-10 induces in vitro an increased release of sTNFRs from monocytes [8, 9].

To investigate the role of antiinflammatory compounds (IL-10, sTNFR-55, sTNFR-75) and their relation to proinflammatory cytokines (TNF-α, IL-6, IL-8) in children with bacterial meningitis, serum and CSF levels of these mediators were measured in 37 patients. The kinetics of these mediators in the CSF compartment and the association between mediators and CSF characteristics were also studied.

5.3 METHODS

Patients and controls

Patients between the ages of 3 months and 18 years diagnosed with bacterial meningitis between August 1992 and September 1994 were included. The patients were admitted to the Departments of Pediatrics of Sophia Children's Hospital, Zuiderziekenhuis, Reinier de Graaf Gasthuis, or Juliana Children's Hospital. Bacterial meningitis was defined as the presence of a positive bacterial culture from CSF or a positive blood culture in combination with clinical evidence of meningitis and a CSF white blood cell (WBC) count >10 x 10^6/L. Patients with prior antibiotic treatment were excluded.

Initial CSF and serum samples were collected in most patients. A subset of patients had a second lumbar puncture done in a randomized fashion at 6, 12, or 24 h after initiation of antibiotic treatment. These patients were treated with intravenous ceftazidime (150 mg/kg/day in three doses). The remaining patients were treated with ceftazidime or cefotaxime, but a second lumbar puncture was not done.

Paired control samples of serum and CSF were obtained from pediatric cancer patients who were in remission. The lumbar punctures in these children were done as part of diagnostic protocols.

Laboratory studies

CSF samples from patients and controls were examined for WBC count and levels of glucose and protein. Samples of blood and CSF were cultured before antibiotic treatment and were processed according to standard procedures. Antibiotic susceptibility was determined by disk diffusion method. CSF and serum samples were stored at -70 °C until used for different assays.

Serum and CSF levels of TNF-α, IL-6, IL-8, IL-10, sTNFR-55, and sTNFR-75 were assayed with ELISA kits (Medgenix, Fleurus, Belgium), according to the manufacturer's instructions, with the following detection limits (lowest positive standard): TNF-α, 15 pg/mL; IL-6, 30 pg/mL; IL-8, 7 pg/mL; IL-10, 11 pg/mL; sTNFR-55, 0.4 ng/mL; and sTNFR-75, 1.0 ng/mL.

Statistical analysis

Differences between groups in continuous variables were tested for significance with the Mann-Whitney test. Pearson's (r) and Spearman's (r_s) correlation coefficients were used to evaluate the relation between variables. A regression analysis for repeated measurements evaluated the change in concentration of the mediators with time. P ≤.05 (two-tailed) was considered significant.

5.4 RESULTS

Patients and controls

The mean age of the 37 patients was 3.3 years (range, 0.3-13.0). There were 17 boys and 20 girls. The causative pathogens were *Haemophilus influenzae* (*n* = 16) *Neisseria meningitidis* (*n* = 14), *Streptococcus pneumoniae* (*n* = 6), and *Fusobacterium necrophorum* (*n* = 1). Twenty-one patients had a second lumbar puncture at 6 (*n* = 8), 12 (*n* = 4), or 24 h (*n* = 9) after initiation of antibiotics. All strains were susceptible to ceftazidime and cefotaxime.

Control subjects were 10 patients who were in remission from acute lymphatic leukemia. All CSF samples from the controls were sterile and had normal CSF characteristics. Chemotherapy had been discontinued for at least 3 months. The children in the control group were 2.7-16.5 years old.

Proinflammatory cytokines (TNF-α, IL-6, IL-8)

Median CSF concentrations of TNF-α, IL-6, and IL-8 during the acute phase of bacterial meningitis were 555, 89,590, and 2604 pg/mL, respectively. These levels were significantly (*P* < .001) elevated compared with those of control subjects (Figure 1). Levels of TNF-α, IL-6, and IL-8 correlated with each other by significantly positive (*P* < .001) correlation coefficients between .57 and .77.

IL-10 in initial serum and CSF samples

IL-10 was detected in 33 of 35 initial CSF samples from patients with bacterial meningitis (Figure 1). CSF IL-10 levels were below the detection limit (11 pg/mL) in all control subjects. The median CSF concentration of L-10 in patients was 701 pg/mL (range, <11-4000), which was significantly higher than in the control population ($P < .001$). CSF levels of IL-10 correlated positively with TNF-α ($r = .71$, $P < .001$), IL-6 ($r = .60$, $P < .001$), sTNFR-55 ($r = .53$, $P = .003$), and sTNFR-75 ($r = .61$, $P < .001$) but not with IL-8 ($r = .28$, $P = .10$). Serum levels of IL-10 in 22 patients were also significantly elevated (median, 78 pg/mL; range, <11-20,000) in comparison with levels in the control subjects (median, <11 pg/mL; $P < .001$).

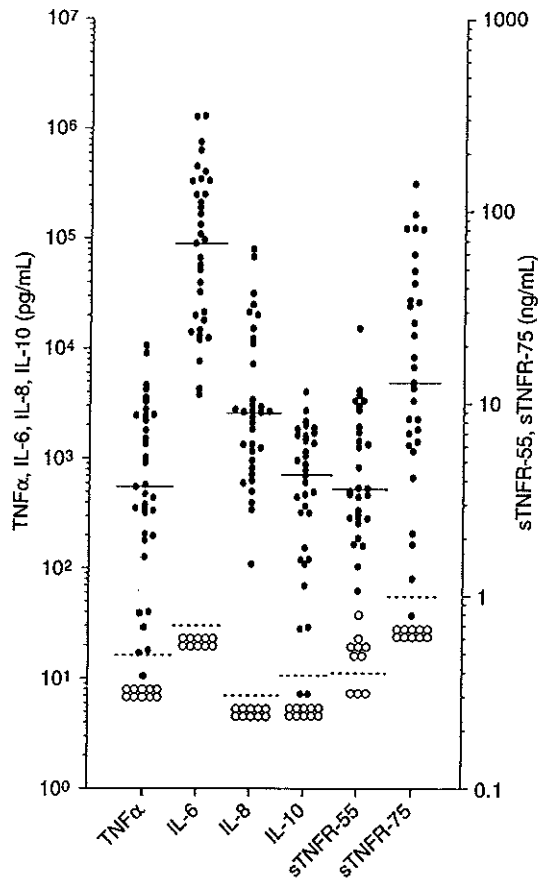

Figure 1. Cerebrospinal fluid concentrations of proinflammatory cytokines tumor necrosis factor-α (TNF-α, n = 35), interleukin (IL)-6 (n = 35), and IL-8 (n = 35) and antiinflammatory compounds IL-10 (n = 35), soluble TNF receptor p55 (sTNFR-55, n = 31), and sTNFR-75 (n = 31) during acute phase of bacterial meningitis (●) and in control subjects (○). Horizontal bars = medians in patients; dashed lines = detection limits of each mediator.

sTNFR-55 and sTNFR-75 in initial serum and CSF samples

CSF concentrations of both sTNFRs were measured in 31 patients (Figure 1). The median CSF level of sTNFR-55 was 3.6 ng/mL (range, 1.1-25.0) versus 0.6 ng/mL (range, <0.4-0.9) in control subjects ($P < .001$). Median CSF sTNFR-75 concentrations were 13.0 ng/mL (<1.0-140.4) in patients and below the detection limit in control subjects ($P < .001$).

A positive correlation was observed between sTNFR-55 and sTNFR-75 levels ($r = .88$, $P < .001$). CSF levels of sTNFR-55 and sTNFR-75 correlated significantly with IL-10 concentrations ($r = .53$, $P = .003$, and $r = .61$, $P < .001$, respectively) but not with TNF-α, IL-6 or IL-8 levels. Median serum levels of the sTNFRs were significantly higher in patients ($n = 23$) than in control subjects (sTNFR-55, 6.0 ng/mL [range, 2.3-18.3] vs. 1.5 ng/mL [range, 1.2-2.1], $P < .001$; sTNFR-75, 19.7 ng/mL [range, 5.3-126.0] vs. 4.1 ng/mL [range, 3.7-4.4], $P < .001$).

TNF-α, IL-6, IL-8, IL-10, sTNFR-55, and sTNFR-75 in initial paired serum-CSF samples

The median concentrations of TNF-α, IL-6, IL-8, and IL-10 were significantly higher in initial CSF specimens than in serum. In contrast, median concentrations of both sTNFRs were similar in the 23 available pairs of serum and CSF samples on admission (sTNFR-55, 6.0 ng/mL [range, 2.3-18.3] vs. 3.7 ng/ML [range 1.1-25.0], $P = .43$; sTNFR-75, 19.7 ng/mL [range, 5.3-126.0] vs. 13.0 ng/mL [range, < 1.0-140.4], $P = .88$).

Correlation between CSF inflammatory parameters and levels of TNF-α, IL-6, IL-8, IL-10, sTNFR-55, and sTNFR-75

CSF glucose concentrations were highly correlated with levels of IL-10 ($r_s = -.87$, $P < .001$), sTNFR-55 ($r_s = -.81$, $P < .001$), and sTNFR-75 ($r_s = -.85$, $P < .001$), whereas correlations with TNF-α and IL-6 were relatively low. In contrast, IL-8 concentrations were not significantly associated with CSF glucose concentrations. The CSF concentrations of the mediators were, with the exception of IL-8, significantly correlated with the WBC count. CSF levels of protein were significantly associated with concentrations of TNF-α ($r_s = .54$, $P = .002$), IL-6 ($r_s = .52$, $P = .002$), IL-8 ($r_s = .48$, $P = .006$), IL-10 ($r_s = .53$, $P = .002$), and sTNFR-75 ($r_s = .37$, $P = .05$) but not with sTNFR-55.

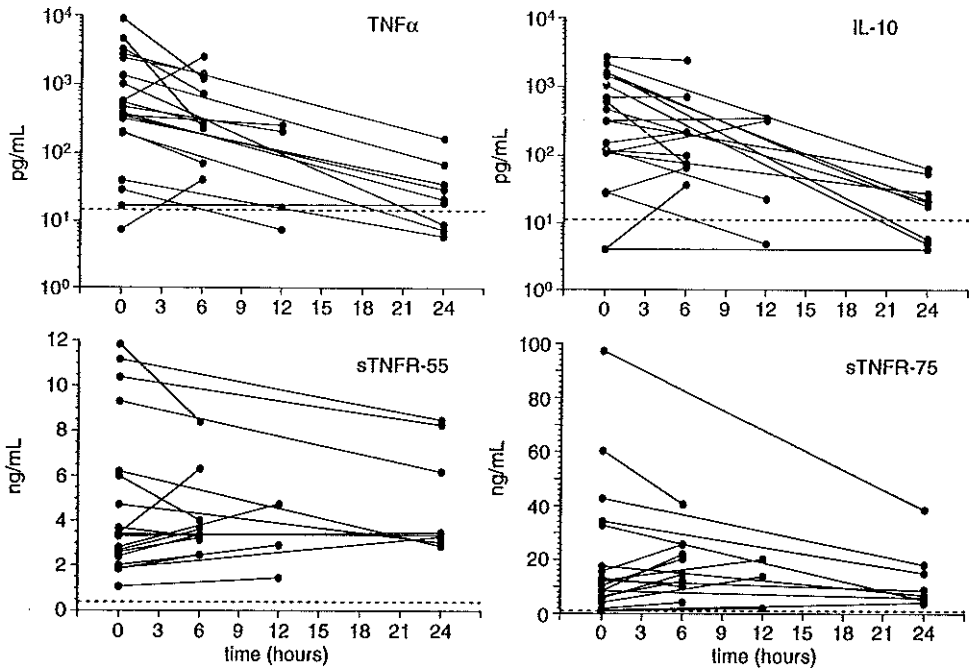

Figure 2. Kinetics of tumor necrosis factor α (TNF-α, n = 20) and antiinflammatory compounds interleukin (IL)-10 (n = 20), soluble TNF receptor p55 (sTNFR-55, n = 19), and sTNFR-75 (n = 19) in cerebrospinal fluid compartments of patients with bacterial meningitis. Dashed lines = detection limits of some mediators.

Kinetics of TNF-α, IL-6, IL-8, IL-10, sTNFR-55, and sTNFR-75

CSF levels of TNF-α, IL-6, IL-8, and IL-10 declined 46% (95% confidence interval [CI], 39%-57%; $P < .001$), 52% (95% CI, 39%-63%; $P < .001$), 58% (95% CI, 45%-67%; $P < .001$), and 50% (95% CI, 35%-61%; $P < .001$) per 6 h, respectively. However, levels of the sTNFRs did not significantly decrease but remained markedly elevated, at least during the initial 24 h of treatment (Figure 2).

5.5 DISCUSSION

In the present study, proinflammatory cytokines (TNF-α, IL-6, IL-8) and antiinflammatory mediators (IL-10, sTNFR-55, sTNFR-75) were markedly elevated in serum and CSF of children with bacterial meningitis. The concentrations of the proinflammatory cytokines were significantly higher in CSF, suggesting a compartmentalized release in the subarachnoid space of patients with bacterial meningitis. This has been also reported by others [10].

Antiinflammatory compounds IL-10, sTNFR-55, and sTNF-75 down-regulate the host response. Administration of recombinant IL-10 effectively inhibits systemic LPS-induced synthesis of proinflammatory cytokines. In addition, IL-10 suppresses the synthesis of nitric oxide and reactive oxygen intermediates, which are involved in the pathophysiology of bacterial meningitis [11]. In agreement with Lehmann et al. [5], we observed significantly elevated levels of IL-10 in CSF of children with bacterial meningitis. IL-10 is mainly produced in the CSF compartment, since CSF levels are significantly higher than serum levels [5]. Endogenous IL-10 production in the CSF compartment may thus play an important role as a natural regulatory and antiinflammatory cytokine.

We observed significantly elevated levels of sTNFR-55 and sTNFR-75 in CSF and serum of patients with bacterial meningitis, as reported by others [12]. TNF-α induces release of sTNFR-55 and sTNFR-75 from neutrophils in suspension in a time- and dose-dependent manner [13]. The shedding of these receptors and the resultant acute decrease of TNFRs on the cell surface may serve to transiently desensitize cells, thereby providing a mechanism for inhibition of TNF-α activity. This process may have implications in vivo, since soluble receptors may inhibit TNF-α bioactivity by binding to the molecule and preventing ligand binding to the cellular TNFRs. Of interest, levels of sTNFRs in this study were exclusively associated with concentrations of IL-10 but not with concentrations of TNF-α, IL-6, or IL-8. This is in agreement with the recent observation that IL-10 induces an increase in the synthesis and cell-surface turnover of sTNFRs from monocytes [8, 9]. IL-10 thus may suppress TNF activity in the CSF compartment by an inhibition of TNF-α secretion, stimulation of the release of sTNFRs, and down-regulation of the expression of surface TNFRs [8, 9].

In our study, serum and CSF levels of sTNFR-55 and sTNFR-75 were similar. The relatively low CSF levels of sTNFRs may be partly explained by a release of sTNFRs from polymorphonuclear leukocytes before entry of these cells into the CSF compartment. Alternatively, shedding of sTNFRs in the CSF compartment may be lower because of the 10-fold lower WBC

count in CSF than in peripheral blood in these patients. The significantly higher stimulus by TNF-α and IL-10 for release of sTNFRs in the CSF compartment apparently does not compensate for this inequality.

CSF inflammatory characteristics were significantly associated with the levels of inflammatory mediators. CSF glucose levels correlated strongly with levels of IL-10, sTNFR-55, and sTNF-75 and weakly with levels of TNF-α.

The CSF levels of TNF-α, IL-6, IL-8, and IL-10 decreased rapidly. This feature is similar to that observed in consecutive serum samples of experimental models of bacteremia or endotoxemia and in patients with sepsis [14]. The elevated levels of sTNFR-55 and sTNFR-75 in the present study persisted for a remarkably long time (at least 24 h), as is observed in bacteremia [15]. This may be due to slow clearance from the CSF compartment or to continuing release.

We conclude that IL-10, sTNFR-55, and sTNFR-75 were released along with proinflammatory cytokines in the CSF compartment of patients with bacterial meningitis. IL-10 may have been partly responsible for the release of sTNFRs in the CSF compartment. CSF levels of TNF-α, IL-6, IL-8, and IL-10 rapidly decreased, while CSF levels of the sTNFRs remained elevated.

5.6 Acknowledgements

We thank H.M. van Vuuren, H. Koning, M.R.M. Baert and A.E.C.M. Vredendaal for technical assistance.

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Chapter 6

Intrathecal Production of Interleukin-12 and Interferon-γ in Patients with Bacterial Meningitis

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Submitted for publication

6.1 ABSTRACT

To assess the role of interleukin (IL)-12 and interferon (IFN)-γ in children with bacterial meningitis, bioactive IL-12 (p70) and the inactive IL-12 subunit p40 as wll as IFN-γ were measured in serum and cerebrospinal fluid (CSF) from 35 children with bacterial meningitis and 10 control subjects. The production of IFN-γ is induced by IL-12 with TNF-α as a costimulator and inhibited by IL-10. CSF concentrations of IL-12 p40 as well as those of IFN-γ were markedly elevated whereas IL-12 p70 was hardly detectable. Detectable CSF levels of IFN-γ correlated positively with IL-12 p40 (r = .40, P = .02) and TNF-α (r = .46, P = .04), but not with IL-6, IL-8 or IL-10. In contrast to CSF levels of TNF-α, IL-12, and IL-10, the IFN-γ levels were significantly higher in patients with pneumococcal meningitis than in children with meningitis by *Haemophilus influenzae* and *Neisseria meningitidis*, which presumably was due to a high CSF TNF-α / IL-10 ratio in the former. We suggest that IFN-γ production induced by IL-12 and TNF-α may contribute to the natural immunity against microorganisms in the CSF compartment during the acute phase of bacterial meningitis.

6.2 INTRODUCTION

Proinflammatory cytokines (tumor necrosis factor [TNF]-α, interleukin [IL]-6, IL-8 and interferon [IFN]-γ are involved in the pathophysiology of bacterial meningitis [1]. Recently, a novel cytokine with proinflammatory effects has been described [2-5]. This cytokine, IL-12, augments the host defence against bacteria, bacterial products, and parasites. Bioactive IL-12 is a heterodimeric protein of 70 kDa (p70) which consists of 40 kDa (p40) and 35 kDa (p35) subunits linked by a disulfide bond [3]. IL-12 is predominantly produced by macrophages and monocytes, but also by B cells [6] and polymorphonuclear leukocytes [7]. It has been shown to enhance the cytolytic activity of a number of effector cells including T cells, natural killer (NK) cells, lymphokine activated killer cells, and macrophages. Furthermore, IL-12 also increases the proliferation of activated NK and T cells and stimulates the production of cytokines, such as IFN-γ, granulocyte-macrophage colony-stimulating factor, and TNF-α [8-10]. The release of IFN-γ is costimulated by TNF-α and IL-1ß, and inhibited by IL-10 [11, 12].

It has been proposed that IL-12 is involved in the early development of an immune response against infectious agents. Production of IL-12 contributes to the control of the host response against infections with intracellular organisms such as *Listeria monocytogenes* [13] *Toxoplasma gondii* [14] and *Leishmania major* [15]. IL-12 mediated protection may be invoked through its ability to stimulate IFN-γ, which inhibits the parasite [16]. Furthermore, IL-12 is also a key cytokine modulating the endotoxin-induced inflammatory process in mice [17]. Metzger et al. have suggested that IL-12 augments the natural immune response due to innate or inflammatory components of the host defense system prior to the development of the humoral or cellular immune response [18]. This initial response may be important in bacterial meningitis since CSF defense mechanisms against infection are limited.

We questioned whether IL-12 is involved in the early phase of the inflammatory response in the CSF of children with bacterial meningitis. To this purpose the presence of IL-12 and its relation to other proinflammatory (TNF-α, IL-6, IL-8, IFN-γ) as well as anti-inflammatory (IL-10) cytokines were studied in serum and CSF of 35 children with bacterial meningitis.

6.3 METHODS

Patients and controls

Patients between the age of 3 months and 18 years who were diagnosed with bacterial meningitis between August 1992 and September 1994 were eligible for inclusion. The patients were admitted to the Departments of Pediatrics of the Sophia Children's Hospital and Zuiderziekenhuis both in Rotterdam, Reinier de Graaf Gasthuis in Delft and Juliana Children's Hospital in The Hague. Bacterial meningitis was defined as the presence of a positive bacterial culture from CSF or the presence of a positive blood culture in combination with clinical evidence of meningitis and a CSF white blood cell (WBC) count above $10x10^6$/L. Patients with prior antibiotic treatment were excluded. Paired control samples of serum and CSF were obtained from 10 pediatric cancer patients who were in remission. The lumbar punctures in these children were done for diagnostic reasons. CSF samples from patients and controls were examined for WBC count and levels of glucose and protein.

The Medical Ethics Committee of each of the participating centers approved the study protocol. Written informed consent was obtained from the parents or the child's legal representatives.

Collection of samples

Samples of blood and CSF were obtained on admission prior to the initiation of antibiotic treatment. Blood was collected into sterilized siliconized vacutainer glass tubes (Becton Dickinson, Meylan Cedex, France) and allowed to clot at room temperature. CSF samples were collected into pyrogen-free polystyrene tubes (Falcon^R, Becton Dickinson, Franklin Lakes, NJ). The samples were centrifuged at 2800 g at 4 °C for 10 minutes. The supernatants were stored at -70 °C until used for different assays. Samples were never thawed and refrozen before assay.

Laboratory studies

Cultures of blood and CSF were processed according to standard procedures [19]. The CSF WBC count, glucose and protein concentration were determined by routine laboratory procedures in each of the participating hospitals.

IL-12 p40: IL-12 p40 antigen was measured with an ELISA as described [20]. Results were related to a dose-response curve of recombinant IL-12 p40 and expressed as pg/mL. The lower limit of sensitivity was 50 pg/mL.

IL-12 p70: IL-12 p70 antigen was measured using an ELISA with mAb 20C2, which preferentially reacts with IL-12 p70, and mAb C8.6 which reacts with IL-12 p40 and p70 antigens equally well [6]. Concentrations of p40 antigen up to 20 ng per ml were not detected by this ELISA. Observations in septic baboons showed a good correlation ($r = .87$) between results obtained with this ELISA and a bioassay for IL-12 [21]. Recombinant human IL-12 p70 was used as a standard. The lower limit of sensitivity of this assay was 2.5 pg/mL.

IFN-γ: IFN-γ was measured using an ELISA obtained from the Department of Immune Reagents, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service according to manufacturer's instructions. The lower limit of sensitivity of this assay was 10 pg/mL.

TNF-α, IL-6, IL-8 and IL-10: Serum and CSF levels of TNF-α, IL-6, IL-8 and IL-10 were measured with ELISA kits (Medgenix, Fleurus, Belgium) according to the manufacturer's instructions with the following detection limit (lowest positive standard): TNF-α, 15 pg/mL; IL-6, 30 pg/mL; IL-8, 7 pg/mL; IL-10, 11 pg/mL.

Statistical analysis

Differences between groups in continuous variables were tested for significance with the Mann-Whitney test. Differences in frequencies of findings between groups were analyzed by the Fisher's Exact test. Pairwise

Table 1. Clinical and laboratory characteristics of 35 children with bacterial meningitis and 10 control subjects.

	Patients (n = 35)	Controls (n = 10)
Age (years)	1.8 (0.3-13.0)	9.9 (4.1-17.9)
Sex (No.)		
male	15	5
female	20	5
Micro-organisms (No.)		
H. influenzae	15	-
N. meningitidis	13	-
S. pneumoniae	6	-
Others	1	-
Cerebrospinal fluid		
WBC count (/mm^3)	3330 (15-34,000)	1 (0-2)
glucose (mmol/L)	1.7 (<0.1-5.3)	3.0 (2.7-3.5)
protein (g/L)	1.5 (0.2-8.0)	0.3 (0.3-0.6)

Except as indicated, data are expressed as median (range). Abbreviations: WBC, white blood cell.

comparisons regarding the percentage of CSF levels above the detection limit were assessed with McNemar's test. The Spearman correlation coefficient (r) was used to evaluate the relation between specific variables. Two-tailed P values $\leq .05$ were considered significant.

6.4 Results

Patients and controls

The clinical and laboratory characteristics of patients and control subjects are shown in Table 1. Thirty-five patients with a proven bacterial meningitis were enrolled. CSF samples from the controls were sterile and showed normal characteristics. Chemotherapy had been discontinued in all of the control subjects for at least 3 months. None of the control subjects underwent craniospinal irradiation or bone marrow transplantation. The controls were still in remission at the next follow up visit. The median age

(range) of the children in the control group was significantly higher than that of the patients (9.1 yrs [4.1-17.9] versus 1.8 yrs [0.3-13.0], $P < .001$).

IL-12 and IFN-γ in serum and CSF samples on admission

The biologically active form of IL-12 p70 was detected in CSF in 2 of the 35 (6%) patients on admission while the subunit p40 of IL-12 was detectable in 28 of the 35 (80%) patients with bacterial meningitis. CSF levels of IL-12 p40 and p70 were below the detection limit in all 10 control subjects. The percentage of detectable CSF levels of IL-12 p40 in the patients was significantly higher in comparison with those of control subjects ($P < .001$). In contrast, CSF levels of IL-12 p70 were not different between patients and control subjects.

CSF levels of IFN-γ were above the detection limit in 20 of the 35 (57%) patients with bacterial meningitis whereas none of the control subjects had detectable IFN-γ levels ($P < .001$).

Pathogen-dependent differences in CSF levels of cytokines

The pathogen-dependent CSF levels of TNF-α, IL-12, IFN-γ IL-10 and the ratio between TNF-α and IL-10 are shown in Table 2. Children with meningitis by *Haemophilus influenzae* or *Streptococcus pneumoniae* had comparably high CSF levels of IL-12 p40 (median 503 pg/mL vs. 509, $P = .50$). CSF levels of IL-12 p40 were lowest in patients with meningococcal meningitis (median 86 pg/mL). Detectable CSF levels of IFN-γ were observed significantly more frequently in patients with pneumococcal meningitis in comparison with those in children with meningitis due to *H. influenzae* and *N. meningitidis*. Pathogen-dependent differences in CSF levels of TNF-α and IL-10 were not observed. The CSF ratio of TNF-α / IL-10 was significantly higher in patients with pneumococcal meningitis in comparison with that in children with meningitis by *H. influenzae* and *N. meningitidis*. Comparison between the ratio of TNF-α / IL-10 of *H. influenzae* against the other two pathogens and the ratio of TNF-α / IL-10 of *N. meningitidis* against the other two pathogens did not yield signficant results.

Serum versus CSF levels of IL-12 and IFN-γ on admission

Paired samples of CSF and serum were available in 20 patients with bacterial meningitis. The median (range) serum levels of IL-12 p40 were comparable to those of control subjects (165 pg/mL [72-1042] vs. 109 [59-299], $P = .52$). The proportion of detectable serum levels of IL-12 p70 and IFN-γ was also comparable between patients and control subjects (IL-12 p70: 2 of 20 [10%] vs. 0 of 10 [0%], $P = .54$; IFN-γ: 6 of 20 [30%] vs. 1 of 10 [10%], $P = .37$)

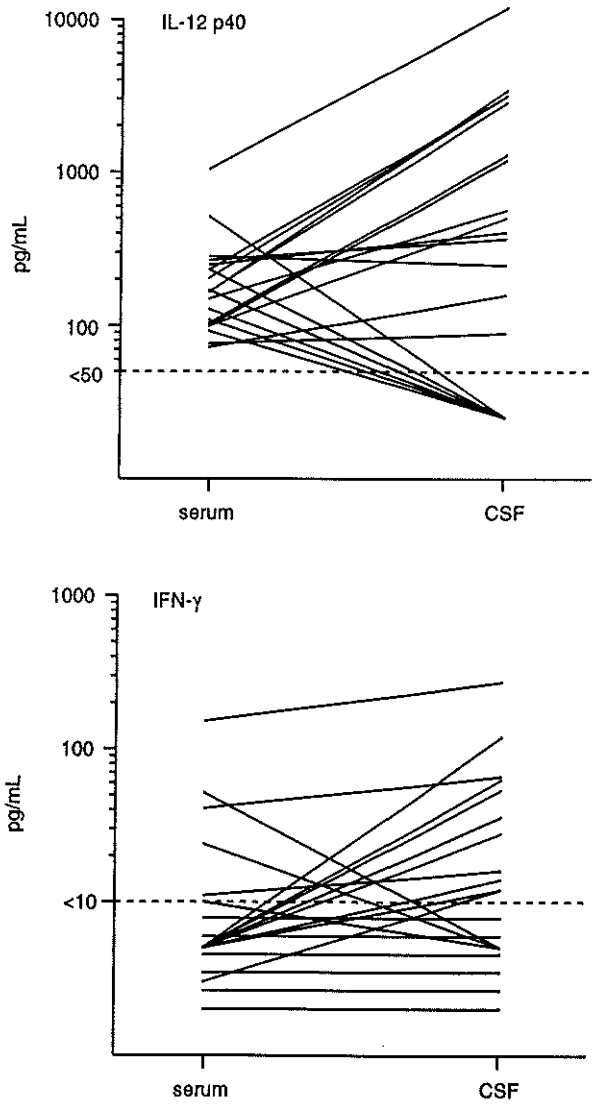

Figure 1. Serum and CSF concentrations of IL-12 p40 and IFN-γ on admission in 20 patients with bacterial meningitis. Horizontal broken line denotes detection limit.

Table 2. CSF levels of TNF-α, IL-12, IFN-γ, IL-10 and the ratio between TNF-α and IL-10 in patient with pneumococcal meningitis and those with meningitis to *H. influenzae* and *N. meningitidis*.

	H. influenzae and *N. meningitidis* (n = 28)	*S. pneumoniae* (n = 6)	*P*
TNF-α (pg/mL)	414 (<15 - 10600)	2324 (355 - 4631)	.08*
IL-12 p70 (%)	2 (7)	0 (0)	1.0 †
IL-12 p40 (pg/mL)	358 (<50 - 11936)	509 (<50 - 5390)	.55*
IFN-γ (%)	13 (46)	6 (100)	.02†
IL-10 (pg/mL)	654 (<10 - 4000)	736 (109 - 1694)	.98*
Ratio TNF-α/IL-10	0.9 (0.2 - 117.5)	2.1 (1.5 - 30.3)	.04*

Data are expressed as median (range) or as number of cases above treshold (%). *Mann-Whitney test, †Fisher Exact test. Abbreviations: CSF, cerebrospinal fluid; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; IL, interleukin.

Detectable concentrations of IL-12 p40 in CSF on admission occurred more often than in corresponding serum samples ($P = .03$, McNemar test), whereas those of IL-12 p70 and IFN-γ in serum and CSF were not significantly different (Figure 1).

Correlation between IL-12 and IFN-γ, TNF-α, IL-6, IL-8, IL-10 and other characteristics in CSF on admission

CSF levels of IL-12 p40 on admission correlated with those of TNF-α ($r = .49$, $P = .004$), IL-6 ($r = .42$, $P = .02$) and IL-10 ($r = .61$, $P < .001$) and detectable CSF levels of IFN-γ ($r = .51$, $P = .02$), but not with those of IL-8 ($r = .18$, $P = .16$). Interestingly, detectable CSF concentrations of IFN-γ on admission correlated only significantly with CSF levels of IL-12 p40 ($r = .51$, $P = .02$) and TNF-α ($r = .48$, $P = .04$) as shown in Figure 2. CSF WBC count, glucose and protein concentration were significantly correlated with CSF levels of IL-12 p40 ($r = .40$ and $P = .02$, $r = -.69$ and $P < .001$, $r = .35$ and $P = .05$ respectively). In contrast, CSF levels of IFN-γ were not associated with any of these CSF characteristics.

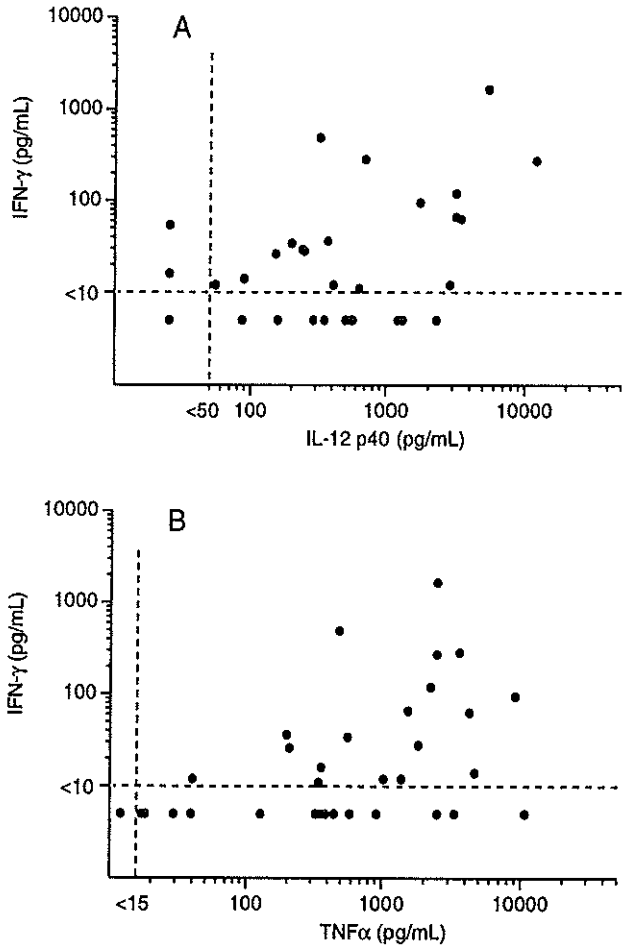

Figure 2. Correlation between admission CSF levels of IFN-γ and IL-12 p40 (A) and TNF-α (B) in children with bacterial meningitis. Horizontal and vertical lines denote the detection limits.

6.5 DISCUSSION

IL-12 is produced by phagocytic cells in response to infection and stimulates adaptive immunity by selectively inducing the Th1 cytokine pattern (IL-2 and IFN-γ) [22, 23]. Furthermore, it induces production of IFN-γ and TNF-α and activates the cytotoxic activity of NK-cells [8-10]. IFN-γ in turn enhances the function of macrophages and polymorphonuclear leukocytes by stimulating non-specific defense mechanisms such as phagocytosis and secretion of reactive oxygen intermediates [24]. Together, these responses contribute to innate host defence systems against invading microorganisms and are usually effective in reducing the load of infection. Our findings suggest that such a host defense mechanism involving IL-12 and IFN-γ is operating in the CSF compartment of patients with bacterial meningitis.

In the present study, CSF levels of IL-12 p40 in patients with bacterial meningitis were elevated while those of IL-12 p70 hardly detectable. This is in agreement with the observation that p40 is released in excess to the bioactive protein (p70) [4, 6]. Although CSF levels of IL-12 p70 were below the detection limit (< 2.5 pg/mL) in most patients, this not necessarily rules out the presence of IL-12, since biologic activity of IL-12 such as enhancement of NK-cell cytotoxicity has been reported in the presence of concentrations below 1 pM [3, 25]. Furthermore, bioactive IL-12 may have escaped detection by binding to specific receptors on cells. CSF levels of IFN-γ were also elevated in our patients with bacterial meningitis as was previously observed by others [26-29]. The levels of IL-12 p40 were significantly higher in the CSF compartment than in serum implicating intrathecal production. Our results do not allow conclusions regarding the cellular source of IL-12 in the CSF compartment.

We observed a significantly positive correlation between CSF levels of IL-12 p40 and IFN-γ, TNF-α, IL-6, and IL-10. In vitro studies suggest that these cytokines may not be responsible for the secretion of IL-12 [6]. Thus, likely these correlations reflected a common stimulus i.e. the microorganisms or their products. Experimental data indicate that IL-12 is required for IFN-γ production. This is confirmed by the observation that IL-12 circulates in the serum before the appearance of IFN-γ. Furthermore, pretreatment with anti-IL-12 antibodies inhibits production of IFN-γ in response to a challenge by endotoxin [17, 30]. IL-12 is not the only cytokine needed to induce the release of IFN-γ. In the SCID model of listeriosis, TNF-α is an important cofactor for IFN-γ production by NK cells in vivo [31] and in vitro [31, 32]. However, TNF-α by itself is unable to induce IFN-γ production. Neutralization of TNF-α with monoclonal

antibodies inhibits in vitro IFN-γ production in NK-cell and inhibits in vivo macrophage activation, resulting in increased systemic bacterial spread [31]. Furthermore, the release of IFN-γ is inhibited by IL-10 which prevents production of IL-12 and of the costimulatory cytokines IL-1ß and TNF-α by accessory cells [11]. These data together explain that CSF levels of IFN-γ in our patients were only associated with IL-12 and a possible co-stimulator TNF-α. Thus, both IL-12 and TNF-α were probably responsible for the production of IFN-γ in the subarachnoid space of patients with bacterial meningitis.

Interestingly, patients with pneumococcal meningitis had significantly higher CSF levels of IFN-γ in comparison with those with meningitis due to *H. influenzae* and *N. meningitidis* as was previously observed by others [27]. This observation can not be explained by an increased IL-12 production since CSF levels of IL-12 were not markedly higher in patients with pneumococcal meningitis. However, the high CSF ratio between the costimulator TNF-α and IL-10, which inhibits IL-12 induced IFN-γ production, in the patients with pneumococcal meningitis in comparison to those with meningitis due to other pathogens, may provide an explanation for the higher CSF levels of IFN-γ in patients with pneumococcal meningitis.

We conclude that the production of IFN-γ in the CSF compartment of patients with bacterial meningitis is at least partly induced by IL-12 with TNF-α as a costimulator. We suggest that IL-12 induced IFN-γ release may contribute to the local host defense in the subarachnoid space against bacterial meningitis.

6.6 Acknowledgements

We thank the Departments of Pediatrics of Reinier de Graaf Gasthuis (Dr. P.J.C. van der Straaten), Delft; Juliana Children's Hospital (Prof. Dr. A.J. van der Heijden), The Hague; Zuiderziekenhuis (Dr. R.N. Sukhai), Rotterdam and the Division of Pediatric Hematology / Oncology, Sophia Children's Hospital, Rotterdam for their willingness to collaborate in this study. We also thank H.M. van Vuuren, Drs. H. Koning, Ing. M.R.M. Baert and Ing. A.E.C.M. Vredendaal for their technical assistance. We are grateful to Dr. S.F. Wolf (Genetics Institute, Inc, Cambridge, MA) for providing recombinant IL-12 p40 and recombinant IL-12 p70, to Dr. M. Gately (Hofmann-La Roche, Nutley, NJ.) for mAb 20C2 and to Dr. G. Trinchieri (Wistar Institute, Philadelphia, PA) for mAb C8.6. This work was financially supported by a grant from GLAXO B.V., The Netherlands.

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Chapter 7

The Role of Nitric Oxide in Bacterial Meningitis in Children

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Journal of Infectious Diseases 1996; 174: 120-126

7.1 ABSTRACT

To investigate the role of nitric oxide (NO) in bacterial meningitis, concentrations in serum, cerebrospinal fluid (CSF), or both of the precursor (L-arginine) and degradation products of NO (nitrate, nitrite) and tumor necrosis factor (TNF)-α were measured in 35 patients and 30 controls. CSF nitrate levels were significantly elevated, mainly due to increased blood-brain barrier permeability, and are therefore not a good parameter for gauging endogenous NO production in the CSF compartment. CSF NO/nitrite levels were significantly elevated in patients. NO/nitrite levels decreased over time (26%/6 h; $P < .001$). CSF levels of NO/nitrite correlated with those of TNF-α ($r = .55$; $P = .001$) and glucose ($r = -.43$; $P = .02$). CSF levels of L-arginine were lower in patients than in controls ($P < .001$). Dexamethasone did not exert a significant effect on NO metabolism. In conclusion, enhanced NO production may contribute to anaerobic glycolysis and neurologic damage in bacterial meningitis.

7.2 INTRODUCTION

Changes in the central nervous system of patients with bacterial meningitis include metabolic alterations, elevated intracranial pressure, and a decrease in cerebral blood flow. The mechanisms leading to these changes involve bacterial components [1-3] and host factors such as cytokines, arachidonic acid metabolites [4, 5], platelet-activating factor [6-8], complement [9], granulocytes, and reactive oxygen intermediates [10, 11]. The proinflammatory cytokines tumor necrosis factor (TNF)-α and interleukin (IL)-1ß play a pivotal role in the induction of the cascade of meningeal inflammation [12-14]. However, the precise mechanisms responsible for the development of central nervous system injury are not yet completely understood.

Recently, nitric oxide (NO) has attracted attention as a potential neurotoxic factor [15]. NO is a short-lived free radical produced by a variety of cell types and involved in physiologic processes such as smooth muscle relaxation, neuronal signaling, inhibition of platelet aggregation, and regulation of cell-mediated cytotoxicity [16-19]. NO production results from the conversion of L-arginine to L-citrulline by the enzyme NO synthase (NOS). At least two classes of NO synthase are known: a constitutive, calcium-dependent form present in the brain and endothelial cells under basal conditions, and an inducible, calcium-independent form (iNOS),

which is produced after stimulation with cytokines (TNF-α, IL-1ß, interferon-γ) and lipopolysaccharides. The iNOS isoform generates large amounts of NO that may have harmful cytotoxic effects. NO ultimately decomposes to form the stable end products nitrate and nitrite. NO production is repressed by glucocorticoids, transforming growth factor ß1, IL-4, IL-10, and prostaglandin E_2 [20-25]. Cytokines stimulate the expression of iNOS in microglia and astrocytes [26-28]. This implies a possible role for NO in the central nervous system host response. Berkowitz et al. [29] reported that NO contributes to pial arteriolar dilation and impaired autoregulation of cerebral blood flow during experimental *Haemophilus influenzae* meningitis in rats. Recently, cerebrospinal fluid (CSF) levels of the degradation products of NO (nitrate and nitrite) were reported to be elevated in experimental animals and patients with bacterial meningitis [30-34].

The aim of the present study was to investigate the role of NO in the pathophysiology of bacterial meningitis. To this purpose, serum and CSF levels of the precursor (L-arginine) and the end products of NO (nitrate and nitrite) were studied in children with bacterial meningitis and in control subjects. The relationships between NO precursor and end products, CSF inflammatory characteristics, and TNF-α were studied. In addition, the effect of dexamethasone on NO synthesis was evaluated.

7.3 METHODS

Subjects

Patients. Eligible patients were children between the age of 3 months and 18 years who were diagnosed with bacterial meningitis between August 1992 and July 1994. Bacterial meningitis was defined as the presence of a positive bacterial culture from CSF or the presence of a positive blood culture in combination with clinical evidence of meningitis and a CSF white blood cell (WBC) count > 10 x 10^6/L. Patients with a history of hypersensitivity to ß-lactam antibiotics, prior antibiotic treatment or a congenital or acquired abnormality of the central nervous system were excluded.

Initial CSF and serum samples were collected from all patients. A subset of the patients was enrolled in a prospective double-blind placebo-controlled trial to study the beneficial effects of dexamethasone as adjuvant therapy in children with bacterial meningitis. These children were treated with intravenous ceftazidime (150 mg/kg/day in three doses) for at least 7

days and were given either dexamethasone sodium phosphate (0.60 mg/kg/day in four doses during 4 days) or placebo (NaCl 0.9%) in a double-blind manner. A second lumbar puncture was done in a randomized fashion at 6, 12 or 24 h after the initiation of the study medication and antibiotic treatment. The remaining patients were also treated with ceftazidime or other appropriate antibiotics. In these children, a second lumbar puncture was not done.

Controls. Paired samples of serum and CSF were obtained from pediatric cancer patients who were in remission. Lumbar punctures were done as part of diagnostic and therapeutic protocols.

Laboratory Studies

CSF samples obtained from patients and controls were examined for WBC count and levels of glucose and protein. Cultures of blood and CSF were done before the initiation of antibiotic therapy according to standard procedures [35]. CSF and serum samples were stored at -70° C until used for different assays.

Nitrate and nitrite concentrations. The concentrations of nitrate and nitrite, the stable degradation products of NO, were measured in paired samples of serum and CSF. Nitrite levels in CSF were determined by a colorimetric method [36]. The combined levels of nitrate and nitrite in CSF and in serum samples were determined by the same assay after enzymatic reduction of nitrate to nitrite using nitrate reductase (1 U/mL) from *Aspergillus* (Boehringer, Mannheim, Germany). The CSF nitrate concentration was calculated by subtracting the nitrite level from the combined level. Serum nitrite concentrations were not measured separately since they are negligible in comparison with concentrations of nitrate. The detection limit of the nitrite and nitrate assays was 0.2 μmol/L. CSF samples contaminated with blood were not analyzed.

TNF-α. CSF levels of TNF-α were assayed with amplified-sensitivity ELISA kits (Medgenix, Fleurus, Belgium) according to the manufacturer's instructions. TNF-α detected with these kits represented the total pool of immunoreactive protein, regardless of whether the cytokine was biologically active or inactive once complexed to soluble receptors or inhibitors. The detection limit for TNF-α was 10 pg/mL.

Determination of L-arginine and L-citrulline in CSF. The amino acid levels in CSF samples were analyzed after deproteinization with 5% sulfosalicylic acid. Measurements were made with an amino acid analyzer (LKB 4151 Alpha Plus; Pharmacia, Cambridge, UK) by ion-exchange chromatography. The amino acids were colored with ninhydrin reagent, and the optical density was measured at 440 and 570 nm [37].

Statistical analysis

Differences between groups in continuous variables were tested for significance with the Mann-Whitney *U* test. Differences in frequencies of findings between groups were analyzed by Fisher's exact test. The Pearson's (r) and Spearman (r_s) correlation coefficients were used to evaluate the relation between specific variables. Regression analysis for repeated measurements was used to evaluate the change in concentrations of NO degradation products over time [38]. Two-tailed $P \leq 0.05$ was considered significant.

Table 1. Clinical and laboratory characteristics of patients with bacterial meningitis and control subjects.

Characteristic	Patients			Controls (n = 30)
	Study Medication			
	Dexamethasone (n = 11)	Placebo (n = 15)	None (n = 9)	
Age (years)	1.6† (0.5-11.0)	2.5† (0.3-13.0)	1.1† (0.8- 7.7)	8.6 (3.4-17.9)
Sex (No.)				
Male	3	7	6	19
Female	8	8	3	10
Etiologic agent (No.)				
Haemophilus influenzae	6	4	6	-
Neisseria meningitidis	2	8	2	-
Streptococcus pneumoniae	2	3	1	-
Others	1	0	0	-
Cerebrospinal fluid				
WBC/mm^3	930† (96-10500)	3330† (15-34000)	3550† (180- 8700)	1 (0-5)
Glucose, mmol/L	1.8* (<0.1-4.6)	2.5 (0.1-5.3)	1.0* (0.1-3.6)	3.0 (1.9-3.7)
Protein, g/L	1.2† (0.8-3.8)	1.4† (0.2-8.0)	2.4† (0.7-3.6)	0.4 (0.2-0.6)
TNF-α, pg/mL	555† (206- 9000)	460† (<10- 3560)	914† (18-10600)	<10 -

NOTE: Except as indicated, data are expressed as median (range). Abbreviations: WBC, white blood cells; TNF-α, tumor necrosis factor α.
*$P < .05$, †$P < .001$ vs. controls.

7.4 RESULTS

Patients and controls

Clinical and laboratory characteristics of patients and control subjects are shown in Table 1. Forty-six consecutive patients were included. Thirty-five had bacterial meningitis. A causative pathogen was not found in 7 patients, whereas in 4 patients, meningitis was caused by enteroviruses. These 11 patients were excluded from further analysis.

The mean age of the 35 children was 3.2 years (range: 0.3-13.0). Sixteen patients were boys and 19 were girls. Twenty-six of the 35 patients with bacterial meningitis were enrolled in the placebo-controlled trial (11 dexamethasone and 15 placebo recipients). A second lumbar puncture was done 6 h (n = 8), 12 h (n = 4) or 24 h (n = 9) after initiation of the study medication. A second CSF sample could not be obtained in 5 patients because no fluid was obtained (n = 2), the patient withdrew from the study (n = 2), or the the patient was in critical condition (n = 1).

Control subjects included 26 patients who were in remission from acute lymphatic leukemia and 4 patients in remission from non-Hodgkin's lymphoma. All CSF samples from the controls were sterile and showed normal CSF characteristics (Table 1). Chemotherapy had been discontinued in all controls for at least 3 months. None of the control subjects underwent craniospinal irradiation. The mean (± SD) duration of remission was 2.7 ± 0.9 years. Children in the control group were significantly older than the patients. Age-related differences in NO degradation products were not observed (data not shown).

Laboratory Studies

Nitrate levels in serum and CSF. The median (range) serum and CSF levels of nitrate and the CSF-to-serum ratio of nitrate are shown in Table 2. The median CSF combined nitrate plus nitrite levels, nitrate levels, and CSF-to-serum ratios of nitrate were significantly higher in the patients. Serum nitrate levels were significantly lower in the patients.

CSF and serum nitrate levels were positively correlated in patients (n = 27; r = .41; P = .03) and in control subjects (n = 29; r = .63; P < .001). CSF nitrate levels in patients were 1.6 times higher (P = .01) than in controls at similar serum levels of nitrate as shown by analysis of covariance (Figure 1).

CSF nitrite levels. Levels of nitrite in the initial CSF samples from patients with bacterial meningitis were significantly elevated in comparison with those in control subjects (P = .05). The median (range) CSF nitrite

Table 2. Levels and ratios of nitrate and nitrite in initial cerebrospinal fluid and serum samples of patients with bacterial meningitis and control subjects.

	Patients (n = 35)	Controls (n = 30)	P
CSF nitrate+nitrite (μmol/L)	9.3 (3.4-39.6)	4.5 (1.3-13.9)	<.001
CSF nitrate (μmol/L)	5.8 (0.5-36.3)	3.9 (0.8-13.5)	.04
CSF nitrite (μmol/L)	0.9 (0.2-29.7)	0.5 (0.3- 1.4)	.05
serum nitrate (μmol/L)	22.3 (9.6-98.2)	30.6 (16.5-136.3)	.005
ratio CSF nitrate / serum nitrate	0.23 (0.02-0.51)	0.11 (0.05-0.32)	<.001
CSF L-arginine (μmol/L)	12 (4-34)	22 (18-34)	<.001

NOTE. Data are expressed as median (range). Abbreviation: CSF, cerebrospinal fluid.

levels in bacterial meningitis caused by *H. influenzae*, *Neisseria meningitidis* and *Streptococcus pneumoniae* were markedly different: 3.8 (0.2-29.7), 0.5 (0.2-3.7), and 1.2 (0.4-2.8) μmol/L respectively. CSF nitrite levels in children with *H. influenzae* meningitis were significantly elevated (P = .003) compared with those in control subjects. Serious neurologic sequelae occurred in 3 patients with *H. influenzae* meningitis. The nitrite levels in these patients were above the normal range (2.7, 3.3, and 11.5 μmol/L).

Figure 2 depicts CSF nitrite levels before initiation of the study medication and 6, 12, and 24 h afterward. Linear regression analysis for repeated measurements indicated that the mean CSF levels of nitrite decreased in time (26% [95% confidence interval, 14%-36%]/6 h; P < .001) in contrast with CSF nitrate and serum nitrate levels. Differences between CSF nitrite levels in patients treated with dexamethasone or placebo were not observed.

A higher proportion of patients with a CSF nitrite level greater than normal (> 1.2 μmol/L, mean + 2 SD of levels in control subjects) had neurologic or audiologic sequelae (or both), in contrast to the patients with normal levels (3/15 [20%] vs. 0/20; P = .07). One of the 3 patients with sequelae received dexamethasone as adjuvant therapy.

Correlations between CSF nitrite, nitrate, and TNF-α levels and CSF characteristics

Median CSF levels of TNF-α were significantly higher in patients with bacterial meningitis than in control subjects (Table 1). In addition, CSF TNF-α levels were positively correlated with CSF nitrite but not CSF nitrate levels (Figure 3). CSF nitrite levels correlated negatively with CSF glucose levels (Figure 4) but not with CSF WBC counts or CSF protein levels.

L-arginine and L-citrulline.

L-arginine levels in CSF from patients with bacterial meningitis were significantly lower than in CSF from controls (Table 2). Differences in levels of L-citrulline in both groups were not observed. CSF nitrite levels in patients were not correlated with CSF levels of L-arginine or L-citrulline.

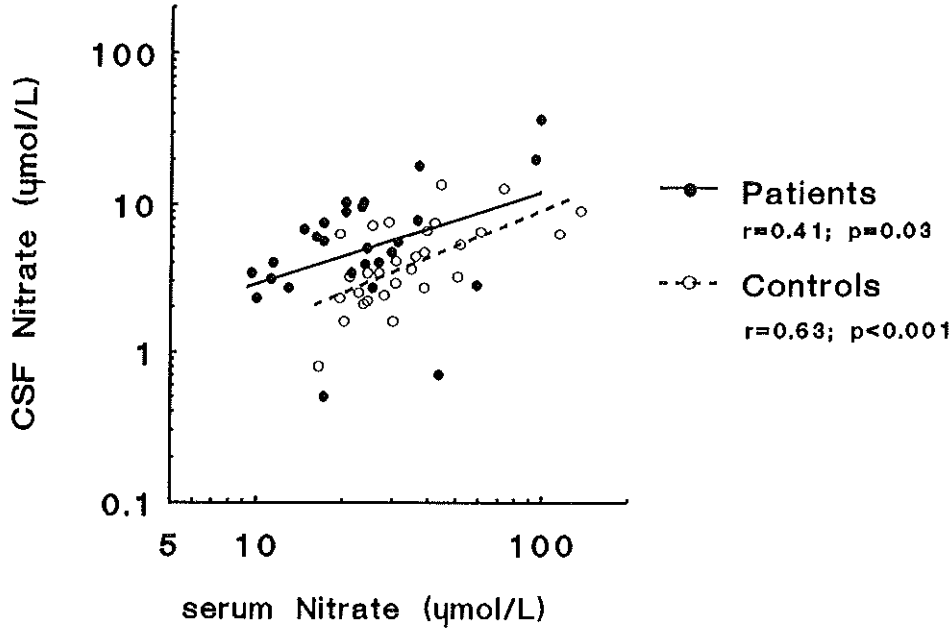

Figure 1. Relationship between cerebrospinal fluid (CSF) nitrate levels and serum nitrate levels in patients with bacterial meningitis and in control subjects.

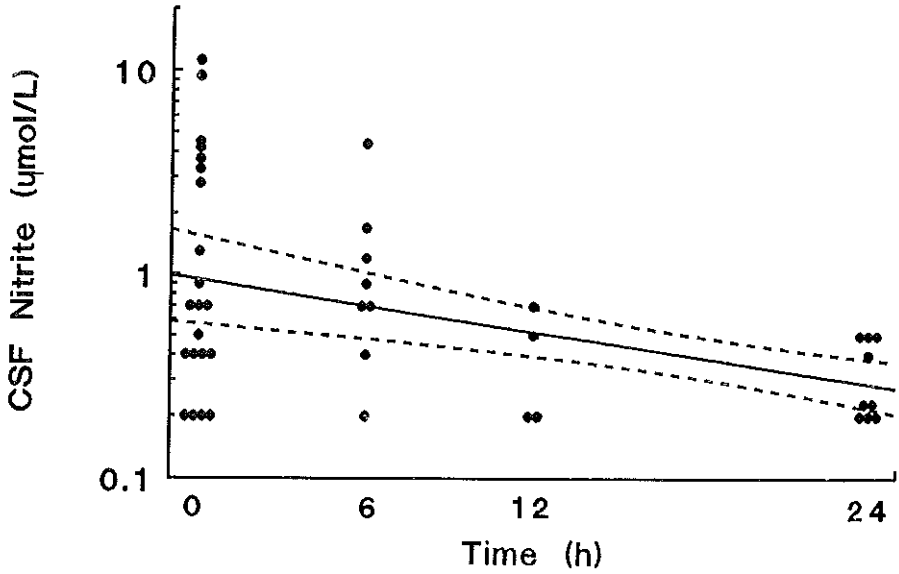

Figure 2. Mean nitrite levels versus time (t) in cerebrospinal fluid (CSF) in patients with bacterial meningitis (log[CSF nitrite] = -0.025 - 0.021 x t; $P < .001$). Dashed lines show 95% confidence limits for mean CSF nitrite level.

7.5 Discussion

NO is a short-lived free radical that can be produced in vitro by microglia and astrocytes after stimulation with cytokines and lipopolysaccharides. We questioned whether NO is involved as a mediator in the pathophysiology of bacterial meningitis in children. Visser et al. [32] recently reported that patients with meningococcal meningitis have increased CSF levels of nitrite and nitrate. Milstien et al. [31] also measured elevated levels of nitrite/nitrate in 3 patients with bacterial and 4 with viral meningitis.

In the present study, CSF concentrations of nitrate, nitrite, and combined nitrate and nitrite were significantly elevated in children with bacterial meningitis compared with levels in control subjects. CSF levels of nitrate, nitrite, or both in the control group were similar to those observed in other studies [31, 32, 39]. Serum nitrate levels were significantly higher than CSF nitrate levels in both patients and controls. In addition, serum

nitrate levels were lower in patients, probably as a result of diminished food intake (exogenous nitrites and nitrates) during the acute phase of illness [40].

The increased CSF nitrate concentration and CSF-to-serum ratio of nitrate in patients support the presence of an enhanced production of NO. However, the increased blood-brain barrier permeability in patients in the presence of relatively high serum nitrate levels may provide another explanation for the increased CSF nitrate levels. We demonstrated that CSF nitrate levels depend both on serum nitrate levels and on the presence of meningitis. Although the CSF nitrate levels were nearly two times higher in patients at similar serum levels, we were unable to distinguish whether this was caused by an enhanced NO production in the CSF compartment or by an increase in blood-brain barrier permeability. We therefore conclude that CSF nitrate levels are not a good parameter for gauging endogenous NO production in the CSF compartment of patients with bacterial meningitis.

CSF concentration of nitrite may provide a better parameter for gauging endogenous NO production in the CSF compartment. CSF is comparable to an oxygen-containing aqueous solution, a condition in which NO is oxidized primarily to nitrite with little or no formation of nitrate [41]. CSF nitrite levels were significantly higher in patients than in controls. Also, children with *H. influenzae* meningitis had significantly elevated CSF nitrite levels. *H. influenzae* is probably a stronger inducer of iNOS than is *N. meningitidis* or *S. pneumoniae.*

CSF nitrite levels in children with bacterial meningitis decreased over time (26%/6 h; $P < .001$) in contrast to CSF nitrate levels. The increased NO production seems to attenuate during recovery. CSF concentrations of L-arginine, the precursor of NO, were decreased in patients ($P < .001$). Heiblim et al. [42] also found that the CSF levels of L-arginine were decreased in patients with bacterial meningitis in comparison with those with febrile seizures. This decrease may be explained by the oxidation of L-arginine to NO by NO synthase.

CSF TNF-α levels were elevated in patients with bacterial meningitis, as previously reported by others [4, 14]. We found a strong correlation between CSF levels of TNF-α and nitrite. These data demonstrate that TNF-α may be involved in the induction of arginine-dependent NO production in the CSF compartment of patients with bacterial meningitis. These observations support the presence of an increased formation of NO/nitrite in the CSF compartment during the acute phase of bacterial meningitis.

The finding that a higher proportion of patients with CSF nitrite above the normal range had neurologic or audiologic sequelae (or both) in contrast

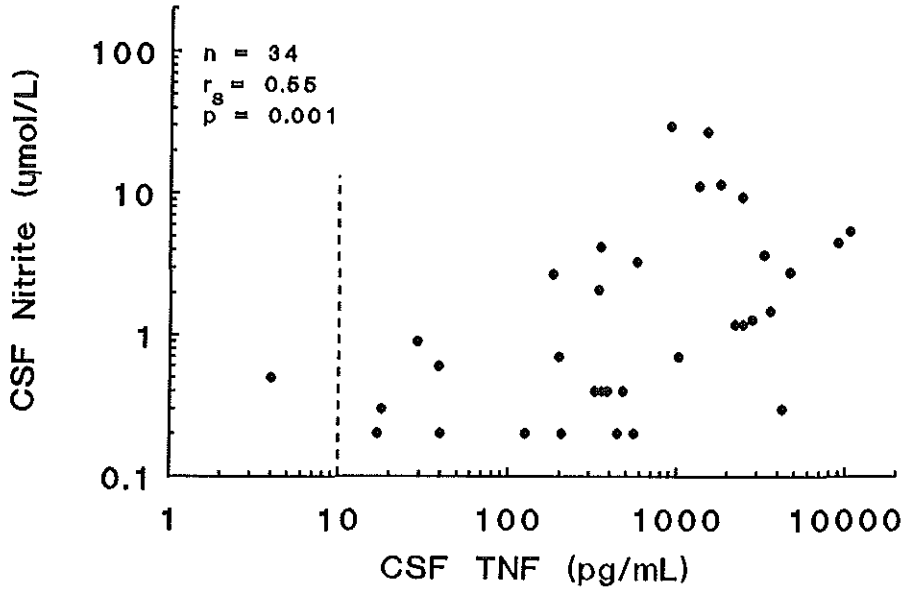

Figure 3. Cerebrospinal fluid (CSF) levels of NO/nitrite and tumor necrosis factor (TNF)-α in patients with bacterial meningitis. Dashed line represents lower detection limit of TNF-α.

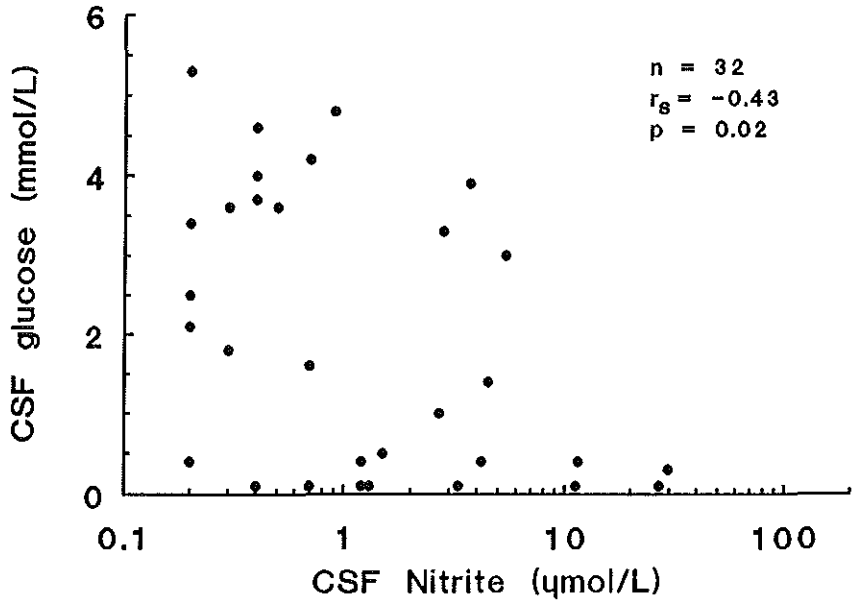

Figure 4. Cerebrospinal fluid (CSF) levels of NO/nitrite and glucose in patients with bacterial meningitis.

to the patients with normal levels suggests that NO may be responsible for neurologic damage in humans.

The cellular site of NO/nitrite production in children with bacterial meningitis is unknown. NO/nitrite can be generated by a variety of cell types, including neutrophils, microglia/macrophages, endothelial cells, astrocytes, neurons, and vascular smooth muscle cells, and by bacteria. Animal studies indicate that it is unlikely that all CSF nitrite results from formation by living bacteria (*H. influenzae, S. pneumoniae*). Intracisternal inoculation of heat-killed bacteria induces an increase in CSF nitrite comparable to that observed with living bacteria [30, 34].

We did not observe a correlation between CSF NO/nitrite levels and CSF WBC count or CSF protein levels. Therefore, WBC are probably not the major source of NO/nitrite. In contrast, CSF nitrite levels significantly correlated with CSF glucose levels. NO may inhibit the mitochondrial respiration that enhances anaerobic glycolysis. This mechanism may contribute to the decreased glucose concentration in the CSF compartment in patients with bacterial meningitis [43-45]. Alternatively, low CSF glucose levels may also be explained by inhibition of carrier-mediated transport across the blood-brain barrier [46, 47].

We did not observe differences in CSF nitrite levels at different time points after initiation of study medication (dexamethasone or placebo), although dexamethasone has been shown to inhibit NO synthase in vitro. However, by the time meningitis is diagnosed, iNOS gene expression is probably already induced. The failure of dexamethasone to inhibit iNOS mRNA and NO production after induction may explain the ineffectiveness of glucocorticoids in treatment [20]. The absence of a significant effect on NO/nitrite production in the CSF compartment may also have been due to the limited number of patients.

Adjustments for multiple testing were not made. Nevertheless, these results present a coherent picture of the role of NO in bacterial meningitis in children and are unlikely to be the result of chance alone.

We conclude that the increased CSF NO/nitrite levels, the decrease of CSF NO/nitrite over time, and the decreased CSF levels of L-arginine (the precursor of NO) indicate the presence of enhanced NO production in the CSF compartment in patients during the acute phase of bacterial meningitis. An effect of dexamethasone on NO metabolism was not observed. The increased production of NO may be partly responsible for the anaerobic glycolysis in the CSF compartment and the occurrence of neurologic damage in children with bacterial meningitis.

7.6 ACKNOWLEDGEMENTS

We thank the Department of Pediatrics of Ikazia Hospital (W.J. den Ouden), Rotterdam, and the Department of Pediatrics, Division of Hematology/Oncology, Sophia Children's Hospital, Rotterdam, for their willingness to collaborate in this study. We thank R. Jankie for expert technical assistance.

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PART II

SEPSIS

Chapter 8

Meningococcal Sepsis

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In preparation

8.1 INTRODUCTION

Meningococcal disease remains a major health problem in many countries. Clinical manifestations vary from self-limiting bacteremia to meningitis or fulminant sepsis. 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 non-specific rash are commonly observed. The diagnosis of meningococcal disease becomes obvious when petechiae are present. The mortality rate of meningococcal sepsis or septic shock varies between 20% and 50% [1-7]. Despite improvements in management and therapy, the outcome of patients with fulminant meningococcemia has not significantly changed over the past three decades. The application of molecular biological and immunological methods in animal experimental and human models of meningococcal sepsis has contributed to our understanding of the pathophysiology and opened new alleys to prevent meningococcal sepsis. This review seeks to provide a comprehensive overview on recent developments in the major areas of research on meningococcal sepsis.

8.2 EPIDEMIOLOGY

Meningococcal disease is endemic in certain geographic areas. Epidemics are reported worldwide and are predominantly caused by a particular meningococcal clone. In Europe most infections are due to meningococcal serogroup B, whereas infections in Africa are mainly caused by serogroup A [8-12]. In Europe and North America meningococcal diseases mostly occur during wintertime, while the epidemics of sub-Saharan Africa occur during the hot dry months. Group C disease may also cause outbreaks. The incidence of disease caused by serogroup C appears to increase in some areas (Italy, British Columbia) [13]. From the mid 1970s to early 1980s an increasing incidence of meningococcal disease was reported in several European countries. Before 1980 the incidence of meningococcal disease ranged between 0.7 and 2.0 cases per 100,000 inhabitants. During the 1980s the incidence of meningococcal disease gradually increased to 3.5 per 100,000 inhabitants [14].

Meningococcal disease is predominantly seen in children with a peak incidence around 2 years of age. A second peak is noted among teenagers. Serogroup C and to a lesser extent serogroup A are relatively frequent among older patients [15]. Riordan and colleagues reported a change in the

clinical presentation of meningococcal disease. The proportion of patients with sepsis increased from 7% in 1977-1985 to 36% in 1990-1993 [16]. Our group recently showed that the mortality rate in infants with meningococcal septic shock is higher than in older children [17]

The phenotype and genotype of *N. meningitidis* varies constantly in time and areas as demonstrated by Scholten et al. [12].

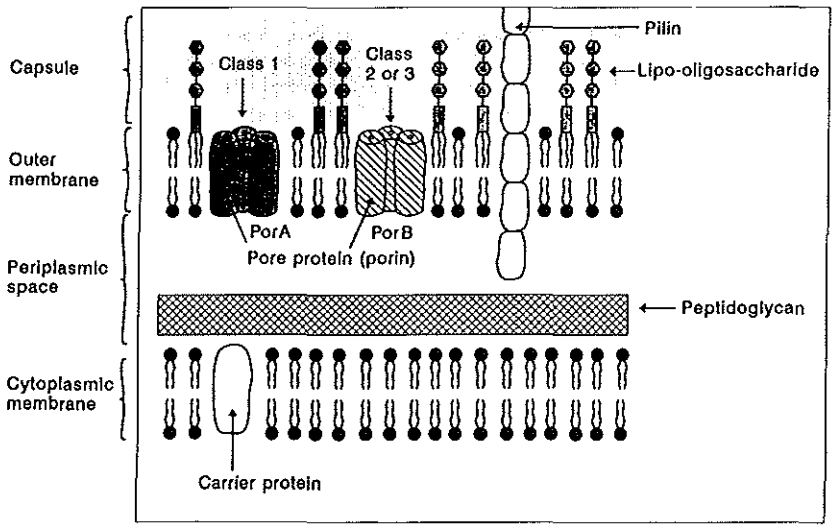

Figure 1. Diagram of the structure of the meningococcal cell wall. Reproduced from Hart et al. [37]

Table 1. Phenotypic classification system of *Neisseria meningitidis*.

System	Basis	No. of groups or types	Names
serogroups	CPS	12	A, B, C, X, Y, Z, 29E, W135, H, I, K, L
serotypes	Class 2/3 OMP	20	1, 2a, 2b, 2c,.....21*
subserotypes	Class 1 OMP	10	P1.1, P1.2, ...P1.16*
immunotypes	LPS	12	L1-L12

Abbreviations: CPS, capsular polysaccharides; OMP, outer membrane protein; LPS, lipopolysaccharides. *Not all the numbers between 1 and 21 or P1.1 and P1.16 are used.
The complete classification of a meningococcal strain is, for example: B:15:P1.16:L3. A strain can express more than one subserotype or immunotype-specific epitope, e.g. P1.7, 16 or L3,7,9. (From Verheul et al. [21])

8.3 MICROBIOLOGY

Neisseria meningitidis, is a gram-negative diplococcus that structurally resembles other gram-negative bacteria. The microorganism is characterized by surface expression of different capsular polysaccharides, by the presence of class 2 or 3 outer membrane protein (OMP), by the class 1 outer membrane protein, and by lipopolysaccharides, as described in Table 1 and depicted in Figure 1 [18]. The most important virulence factor to survive in the bloodstream is encapsulation [19]. The polysaccharide capsule inhibits neutrophil phagocytosis and prevents classical complement pathway bactericidal activity thus enhancing intravascular bacterial replication and survival. Lipopolysaccharides (LPS), also called endotoxins, are shed by meningococci partly in the form of cell-wall blebs. Endotoxin from the outer membrane has been studied extensively for its toxic and antigenic properties and biochemistry [20, 21]. The importance of endotoxin as a mediator of gram-negative sepsis was demonstrated by Brandzaeg et al. [22]. High levels of circulating lipopolysaccharide correlate with fatal outcome [22-24]. Endotoxin is a potent stimulator of both cellular and humoral immunity [25, 26]. The presence of higher-molecular-weight LPS or sialylation of cell membrane glycoconjugates correlates with increased resistance to killing by antibody and complement [27, 28]. Phase variation involving antigens that are targets of bactericidal antibodies also effects the sensitivity of a strain to serum bactericidal activity [29]. During growth, meningococci oversynthesize their outer membrane relative to the remainder of the organism. This results in the formation of bleb-like structures on the outer membrane which can be secreted [30]. 'Blebbing' and the continuous release of endotoxin in high amounts are characteristic features of meningococci. Blebs bind antibodies that would otherwise attach to whole bacteria and play a crucial role in the pathogenesis of septic shock. 'Blebbing' is also observed in other gram-negative organisms but usually to a lesser extent [31]. Bacterial properties that contribute to the pathogenicity of the organism include the presence of a capsule, pili or fimbriae [32], outer membrane proteins and IgA protease (Table 2) [26, 33, 34]. Attachment to epithelial or endothelial cells is mediated by pili and non-pilar adhesins [19, 35]. In this way and by the production of soluble products inducing ciliostasis *N. meningitidis* is able to escape from the ciliary clearance mechanisms of the nasopharyngeal mucosa. Another mechanism for survival is inactivation of IgA by IgA protease. However, this enzyme probably plays a minor role [36].

Table 2. Virulence factors of *Neisseria meningitidis*.

- Secreted compounds	IgA protease Blebs
- Surface structures	Pili Capsular polysaccharides Outer membrane proteins Regulatory iron proteins Lipopolysaccharides

The major outer membrane proteins of meningococci have been divided into five classes (class 1, 2, 3, 4 and 5) [37]. Meningococcal class 1 (PorA; serosubtype) and class 2 and 3 proteins (PorB; serotype) are porins, permitting the passage of ions across the cell membrane [38]. Gene sequencing has permitted the construction of topology models [39]. Whereas the transmembrane parts of these proteins are highly conserved, the strain-variable domains, which play a crucial role in the host immune response, are located at the tips of various surface loops in these models. The surface-exposed loops are the longest (and therefore the most exposed) in class 1 protein. This may explain why antibodies against this protein are particularly effective in bactericidal assays [39]. Class 4 OMP (Rmp) reveals homology with *Escherichia coli* OmpA [28]. The exact function of this protein is unknown, but some evidence for pore-forming properties has been obtained. Class 5 OMP's, Opa and Opc are so-called heat modifiable proteins. Opa and Opc proteins are thought to be important in bacterial adhesion and invasion in host cells [34].

8.4 Colonization and invasion

N. meningitidis may colonize the human nasopharynx. Transmission takes place by small droplets and is facilitated by crowding and lower socioeconomic status [40, 41]. The carriage rate by *N. meningitidis* has varied from a small percentage among infants to 25-35% among teenagers and young adults and declines with increasing age [42, 43]. Most people are asymptomatic carriers who may transfer the micro-organism to non-carriers. Smoking, passive exposure to cigarette smoke, viral infections, exposure to dust, teenagers and young adults, male sex, and changes in residence and virulence of meningococcal strains are all risk factors

associated with high carriage rates [13, 40, 41, 43-46]. Household contacts of a patient with meningococcal disease are at increased risk for secondary disease [47]. Most secondary cases occur within the first week after diagnosis of the index patient.

The first and most important line of defense against infection with *N. meningitidis* is integrity of the mucosal membrane [21]. Invasion across the nasopharyngeal mucosa takes place as a bacterium-directed endocytotic process by non-ciliated epithelial cells [19, 35] or by ciliated epithelial cells with decreased ciliar function [44]. This process is influenced by outer membrane proteins. Bacteria surviving in the bloodstream invariably resist antibody-independent activation of complement by several mechanisms, for example sialylation in serogroup B, C, W and Y.

8.5 Systemic Host Response

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* [21]. The complement system also 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 [48, 49].

Bacterial components, particularly endotoxin initiate a cascade of events which are responsible for the clinical presentation of meningococcal sepsis [26, 50]. The systemic inflammatory response is aimed to neutralize microorganisms and their products, but may also induce harmful tissue damage to the host. The intravascular inflammatory response can be divided into activation of cascade systems, the release of intercellular mediators from cells adjacent to circulating plasma and to the altered function of various cells in the vascular wall. In meningococcal infection coagulation, fibrinolysis, complement and kallikrein-kinin systems, as well as the production of cytokines and the activation of neutrophils and platelets, are all apparently upregulated by native LPS in a dose-dependent manner [22].

Non-specific host defence

Serum levels of endotoxin which is released systemically from gram-negative bacteria, correlate with severity of disease [51]. High density

lipoproteins, complement factors, antibodies, albumin and lipopolysaccharide binding protein (LBP) have the ability to complex with LPS. Several of these proteins appear to have a detoxifying affect [37, 52, 53]. A potent bactericidal protein produced by polymorphonuclear leukocytes is bactericidal/permeability-increasing protein (BPI). It is stored in the azurophilic granules and is also expressed on the cell surface. Bactericidal activity of BPI is caused by the strong affinity of BPI for LPS [54]. In addition to bactericidal capacity, BPI also neutralizes LPS activities in vitro and vivo [55].

Complement system activation

Fulminant meningococcal sepsis is associated with excessive complement activation [49, 51]. The complement system is an essential element in the maintenance of homeostasis. However, overstimulation or inadequate inhibition may lead to an inappropriate reaction and ultimately to tissue injury [56]. 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.

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. However, 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 [57, 58]. Normally, intravascular clearance of bacteria is mediated through the deposition of complement components: C3b for phagocytic clearance [59]; the membrane attack complex or C5b-C9 for lysis [48, 49].

The degree of complement activation in meningococcal disease is related to the amount of circulating native lipopolysaccharides. Previous studies indicate that LPS is a potent activator of the complement system [60-62]. In fulminant meningococcal septic shock, the presence of C4bc, C4bd and Bd point to activation of both the classical and alternative pathways. However, Brandtzaeg et al. suggest that complement activation is predominantly caused by alternative pathway activation.

Brandtzaeg and colleagues also showed that complement activation may persist during the first 12-24 hours of disease, when most production of other inflammatory mediators is already downregulated [51].

Pro- and counterinflammatory mediators

Lipopolysaccharides induce a release of proinflammatory mediators in gram-negative sepsis. These mediators are mainly synthesized and released by macrophages, monocytes, and endothelial cells. Experimental and clinical data have shown that tumor necrosis factor (TNF)-α and interleukin (IL)-1ß are key mediators in meningococcal sepsis. TNF-α is present in the circulation after administration of live or heat-killed bacteria or endotoxin [63, 64]. Administration of TNF-α reproduces many of the clinical symptoms of sepsis such as rigors, fever, hypotension, and tachycardia. Hematologic abnormalities include leukopenia and activation of coagulation, fibrinolytic and complement systems [65, 66]. TNF-α and IL-1ß exert their effects by different mechanisms including the induction of other cytokines, activation of neutrophils, generation of prostaglandins and production of nitric oxide.

Tumor necrosis factor-α and IL-1ß are early mediators of inflammation since they appear before the release of IL-6 and IL-8. The release of the latter mediators is triggered by LPS, TNF-α, and IL-1ß. IL-6 is a major pyrogen and is probably an important regulator of the inflammatory response. The synthesis of acute-phase proteins is stimulated by IL-6 [67]. It is not considered to be a toxic molecule. IL-8 is a potent chemoattractant and activates neutrophils [68]. Furthermore, IL-8 is thought to be involved in neutrophil-mediated vessel-wall injury [69].

Leukemia inhibitory factor (LIF) is another proinflammatory cytokine. LIF has multiple actions, many of which are shared with TNF-α, IL-1 and IL-6 [70, 71]. LIF participates in the induction of the acute-phase response and stimulates the acute-phase protein secretion and fever. Fibroblasts, monocytes, macrophages, T-lymphocytes and endothelial cells may produce LIF.

IL-12 is a recently described proinflammatory cytokine [72-74]. The precise role of IL-12 in vivo is not known, although this cytokine seems to play a key role in the differentiation of Th1 cells [75] and in the host defense against bacterial, parasitic and viral infections [73]. IL-12 also induces the production of interferon (IFN)-γ by T-cells and natural killer cells [76, 77]. IL-12 has been characterized as a major cytokine in the pathogenesis of gram-negative endotoxemia in mice [76] and in primates [78]. Hazelzet et al. showed that levels of IL-12 p40 and to a lesser extent those of IL-12 p70, are elevated in patients with meningococcal septic shock. Plasma levels of IL-12 p40 were related to outcome and severity of disease (chapter 12).

IFN-γ may contribute to mortality as well. IFN-γ activates other cytokines and is associated with increased serum levels of TNF and IL-6. A late-acting mechanism of IFN-γ is also suggested [79]. The plasma levels of IFN-γ are increased in experimental models for sepsis [79-81] as well as in human sepsis [82, 83], although not consistently [24].

The short peak of pro-inflammatory cytokines is directly followed by an increase of IL-1 receptor antagonist (IL-1RA), IL-10 and soluble TNF receptors (sTNFRs). These mediators are considered antiinflammatory because they reduce mortality in experimental endotoxemia [84-86]. 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 [4]. 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. TNF-α induces release of sTNFR-55 and sTNFR-75 from neutrophils in a time and dose dependent manner [87]. Soluble TNFR molecules are able to capture TNF-α and inhibit its biologic effects. Interleukin 10 is released in massive amounts into the systemic circulation during the initial phase of fulminant meningococcal septic shock. High serum levels of IL-10 are associated with fatality in meningococcal disease [88]. TNF-α induces IL-10 production by human monocytes. IL-10 is a potent inhibitor of cytokine production [85, 89, 90]. It also suppresses the procoagulant activity induced by LPS at the surface of human monocytes [91]. IL-10 stimulates the production of IL-1RA and induces an increased release of sTNFRs from monocytes [92].

Generally, serum levels of proinflammatory cytokines are significantly increased at the onset of disease in patients with meningococcal sepsis and are associated with severity of disease [5, 22, 24, 67, 70, 93]. These cytokines rapidly disappear in time. A strongly negative correlation between the initial levels of cytokines and the duration of petechiae and serum levels of C-reactive protein (CRP) respectively has been reported [67, 94]. This shorter duration of petechiae, as well as the lower level of CRP in non-survivors suggests a shorter disease course and associated higher levels of cytokines. The earlier admission of nonsurvivors 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 LPS or to proinflammatory cytokines [94].

Table 3. Factors involved in the pathophysiology of disseminated intravacular coagulation.

-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-Johnson [170].

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. Widespread microvascular thrombosis in various organ systems does contribute substantially to organ dysfunction and survival (Table 3). 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. Activation of the coagulation system in sepsis occurs predominantly through the extrinsic route (Figure 2). 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 [95]. 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 [96].

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]. Activated factor XII

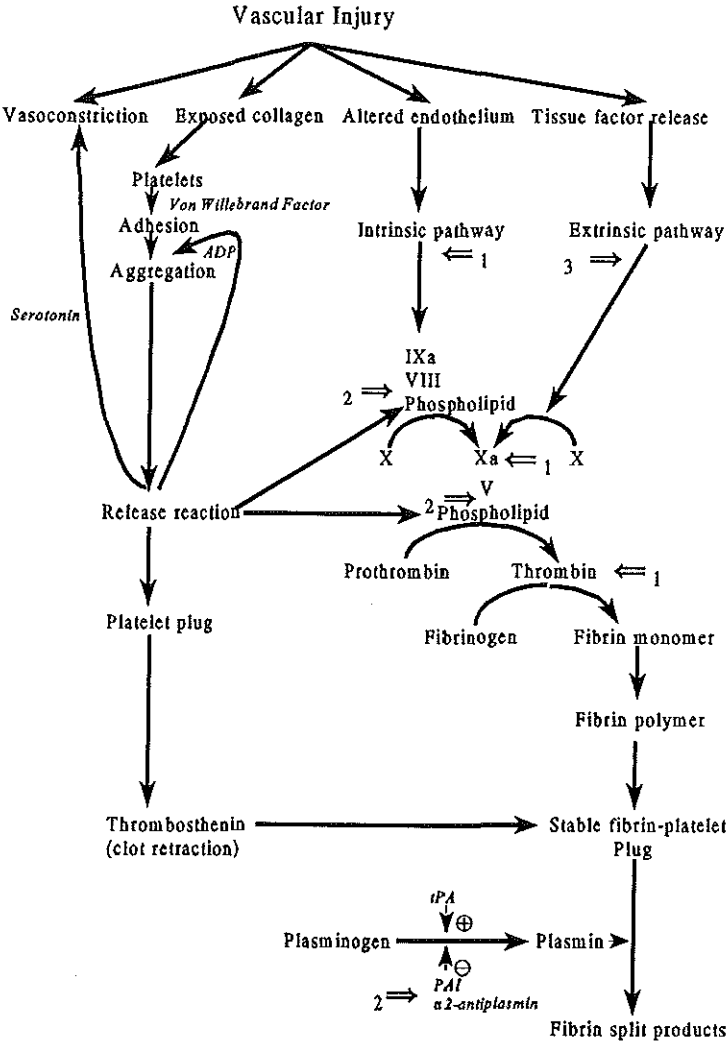

Figure 2. Hemostatic mechanism. The intrinsic pathway involves the activation of coagulation factor XII, XI and IX. The extrinsic pathway involves the activation of factor VII by tissue factor. Abbreviations: ADP, adenosine diphosphate; IXa, activated coagulation factor IX; VIII, coagulation factor VIII; X, coagulation factor X and Xa, when activated; t-PA, tissue plasminogen activator; PAI, plasminogen activator inhibitor.
Natural inhibitors (⇒) 1: antithrombin III; 2: activated Protein C; 3: tissue factor pathway inhibitor.

induces the kallikrein-kinine system. Hypotension is probably partly mediated by generation of kinins such as bradykinin. Factor XII levels are lowest in patients with septic shock [98, 99]. However, we found no significant difference between levels of factor XII in survivors and non-survivors with meningococcal septic shock (manuscript in preparation).

Levels of natural inhibitors of coagulation are markedly altered during meningococcal sepsis. Several studies confirmed that antithrombin III (ATIII) levels [100-104], protein S, and notably protein C [6, 100-102, 104-106], 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 [6, 17]. Elevated initial levels of the extrinsic pathway inhibitor (EPI), another inhibitor of coagulation, were found in patients with fulminant meningococcemia [100]. This is in contrast to the levels of ATIII and protein C. The levels of EPI were significantly higher in nonsurvivors in comparison to survivors. Furthermore, levels of EPI increased during the course of disease [100].

The fibrinolytic system becomes initially activated by tissue plasminogen activator (t-PA) during the early course of meningococcal sepsis. Subsequently, fibrinolysis is inhibited by increased levels of plasminogen activator inhibitor (PAI)-1 [17, 94, 107]. In patients with sepsis and septic shock t-PA levels are increased and related to outcome and severity of disease [108, 109]. However, we did not detect significant differences in the initial levels of t-PA in survivors and nonsurvivors with meningococcal septic shock [17, 94]. Levels of plasminogen and alfa-2-antiplasmin are low in septic shock but not related to outcome [98, 108]. However, decreased alfa-2-antiplasmin levels as well as the high ratio PAI-1/t-PA were related to worse outcome in patients with meningococcal septic shock [17, 106, 107]. These changes in fibrinolytic parameters result in an ineffective fibrinolysis. Of interest, polymorphism of the PAI-1 gene is suggested to explain higher PAI-1 levels in non-survivors at a similar TNF stimulus [94].

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 naturally occurring anticoagulants are depleted to cause purpura fulminans [6].

8.6 HEMODYNAMIC DERANGEMENTS

Shock or circulatory collapse in patients with fulminant meningococcemia is caused by a combination of an inappropriate vascular tone, myocardial dysfunction, and capillary leakage (Figure 3).

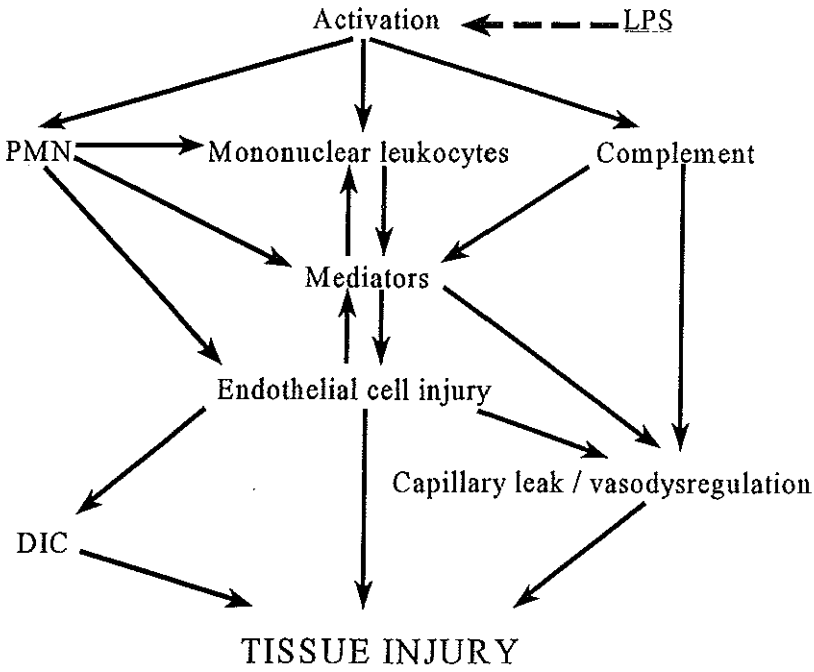

Figure 3. Pathophysiology of meningococcal sepsis. Abbreviations: LPS, lipopolysaccharides; PMN, polymorphonuclear leukocytes; DIC, disseminated intravascular coagulation.

Inappropriate vascular tone

Initially, patients with meningococcal sepsis present with intense vasoconstriction. Subsequently, the systemic vascular resistance falls due to vasodilatation in the course of the treatment which requires volume suppletion and vasopressors. A dysbalance between forces causing vasodilatation and those causing vasoconstriction of the blood vessels

results in generalized vasodilatation and hypotension. Vasoconstrictor substances that are elevated in patients with shock include catecholamines, renin, aldosterone, thromboxane A2 and endothelin [110-113]. A deficiency of PGI_2 synthesis by the endothelium is also involved in vasoconstriction in meningococcal disease [114]. On the other hand, bradykinin and nitric oxide are potent vasodilator compounds leading to hypotension [115-117].

The complement and the contact 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 [118]. During sepsis C1-INH levels were found to be normal or even decreased especially in non-survivors [119]. Hack et al. hypothesized that increased degradation of C1-INH in severe sepsis may result in an insufficient control of the complement and contact systems [118]. 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 [51].

Myocardial dysfunction

Septic shock appears to have a more rapid course in children with severe meningococcemia than in adults. Cardiac insufficiency was frequently found in the group of non-survivors from meningococcal septic shock, although it was also encountered in survivors. [120]. Heart failure has been attributed to the reduction of the coronary blood flow [121, 122], circulating myocardial depressants [123, 124], intrinsic cardiodepressant properties of endotoxin [125], acidosis [126], hypoxemia [127], and acute pulmonary hypertension caused by capillary microthrombi [128]. The mechanism of the myocardial failure in sepsis remains partly speculative. Factors such as calcium, citrate contained within fresh frozen plasma or albumin, complement, endorphins and histamine have been implied as possible myocardial depressant factors. Also, the diffuse capillary leakage may effect myocardial function. Severe myocardial edema may induce secondary modifications of myocardial compliance and/or contractility. [120]

Endothelial damage and capillary leakage

Endothelial cells form a selective permeability barrier between blood and underlying tissues. During sepsis, endotoxin and several other mediators activate vascular endothelial cells and initiate a rapid alteration of structure and function of these cells. Persistent hypovolemia in spite of large fluid infusions results from a severe capillary leak syndrome induced by endothelial damage. Capillary leakage and subsequently edema are the

result of high molecular protein leakage, separation of tight junctions between endothelial cells [129], destructive changes of endothelium (especially the glycosaminoglycan component [130]. These processes are partly induced by mediators (TNF-α, IL-1, IL-8, PAF, leukotrienes, thromboxane A_2, thrombin, vascular permeability factor, complement factors, kinins [110, 131], and the adherence of neutrophils [132-134]. Activated leukocytes also play a role in endothelial damage since these cells release of proteolytic enzymes and toxic oxygen radicals [58, 132-134]. Ultimately, increased vascular permeability leads to profound interstitial edema and diffuse parenchymal cell injury and subsequent organ dysfunction.

8.7 TREATMENT

Intensive care support

Patients with suspected meningococcal sepsis should be vigilantly observed during the first 48 hours of treatment, preferably on an intensive care unit. The most important complication requiring urgent intervention is the development of shock. Assessment of the fluid balance may be extremely difficult since patients with meningococcal shock invariably have a profound capillary leak. The aim should be to provide sufficient fluid to maintain the intravascular volume and electrolyte balance, while minimizing the accumulation of extravascular fluid. Central venous pressure or pulmonary wedge pressure monitoring should therefore be used to guide fluid administration. Pulmonary edema is a complication requiring elective ventilation. When shock continues despite aggressive correction of the volume deficit, inotropic support with or without peripheral vasodilators should be given [26].

Antibiotic treatment and profylaxis

Penicillin remains the treatment of choice for meningococcal disease. However in young children, the elderly or the immunosuppressed, infections with other organisms, including *Haemophilus influenzae* type b, *Streptococcus pneumoniae* and coliforms, may be indistinguishable from the clinical picture seen with meningococcus. For this reason a third generation cephalosporin, either cefotaxime or ceftriaxone, is the initial antibiotic treatment until the organism and its sensitivity have been determined [26].

Some countries including Spain, South Africa, Canada, the United States and the United Kingdom have documented an increasing number of

N. meningitidis isolates with a reduced susceptibility to penicillin in clinical isolates of *N. meningitidis* [135-139]. In Spain the frequency of strains with decreased susceptibility to penicillin increased from 0,4% in 1985 to 40% during the first months of 1990. These strains show varying levels of cross-resistance to other betalactam antibiotics. Multilocus enzyme electrophoresis has established a high level of genetic diversity among these isolates [140]. Resistance is due, at least in part, to a decreased affinity of penicillin-binding protein for penicillin. Continuing surveillance is important to detect penicillin-resistant meningococci which would cause serious problems in the treatment of meningococcal infections [13, 135, 137, 141].

Prophylactic antibiotics are used in order to eliminate carriage of virulent strains before they cause invasive disease or are spread to susceptible individuals. Rifampicin is most commonly used and has shown to be effective [37, 142]. Ten percent of the failures of eradication results from emergence of isolates resistant to rifampicin [143]. However cases of meningococcal disease due to rifampicin-resistant meningococci are rare [144]. When serogroup A or C strains are isolated, the close contactgroup should be offered meningococcal A or C vaccine in addition to rifampicin prophylaxis [142]. The issue if chemoprophylaxis is needed at the time of discharge after treatment for systemic meningococcal infection is still widely debated [42, 145].

Adjunctive treatment

There are a number of experimental forms of therapy designed to reduce the inflammatory process or reverse the disordered physiology. The outcome in patients with septic shock can be improved by neutralization of circulating endotoxin or inhibition of proinflammatory cytokines as observed in studies with experimental animals. However efficacy was not shown in patients with sepsis or septic shock [146, 147].

Heparin, antithrombin III concentrate, fresh frozen plasma and protein C concentrate have been studied as agents to control disseminated intravascular coagulation in patients with fulminant meningococcemia [100, 101, 148-152]. 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 specifically interferes with PAI-1 and induces a clot-selective fibrinolysis that is associated with only a little decrease of fibrinogen. Preliminary experience with rt-PA in two patients suggests that rt-PA should be considered as an investigational therapeutic

option in patients with life threatening disease and no response to conventional treatment [153, 154].

Monoclonal antibodies against the lipid A moiety of endotoxin (HA-1A) have been evaluated in adults [146, 155] and recently in children with meningococcal septic shock. The lipid A core structure of LPS is well conserved among the diverse gram-negative pathogens and antibodies against epitopes on the core glycolipid have been shown to induce cross-protection. The data of the multinational European trial in children with meningococcal septic shock show a 30% reduction in mortality, without statistical differences between the HA-1A and placebogroup. A new trial to evaluate the efficacy of protein C in the treatment of children with meningococcal septic shock is in preparation. Possible future treatment modalaties include r-BPI (phase 1 and 2 trials are initiated), C1 esterase inhibitor, and tissue factor pathway inhibitor (Table 4).

Table 4. Experimental therapeutic modalities.

Anti-endotoxin agents
monoclonal antibodies against endotoxin/lipid A
lipopolysaccharide binding protein
bactericidal permeability increasing factor
CD14 receptor antagonist
Anti-cytokine response agents
monoclonal antibodies against tumour necrosis factor
monoclonal antibodies against interleukin-1
tumour necrosis factor receptor antagonist
interleukin-1 receptor antagonist
soluble TNF receptor
Agents for the treatment of disseminated intravascular coagulation
antithrombin III
monoclonal antibodies against tissue factor
protein C and S
recombinant tissue plasminogen activator

Adapted from Cartwright et al [36].

8.8 VACCINATION

Meningococci of serogroups A, B, C, W-135 and Y may be responsible for invasive infections. Vaccines are currently available against meningococci of serogroups A, C, W-135 and Y. However, polysaccharides which are the major compounds of these vaccines, are T-cell independent immunogens and the ability to respond to them shows an age-dependent maturation [156]. Group C polysaccharide vaccines are not immunogenic in children lesser than 18 months of age. Serogroup A, Y and W135 vaccines are protective in young children, but only provide short term immunity. Persistence of vaccine-induced antibodies is also age dependent [157]. Serogroup A and serogroup C polysaccharide-protein conjugate vaccines have recently been developed and seem to be effective also in young children [158].

A vaccine against group B meningococci, the most common cause of meningococcal disease, is not yet available. Group B polysaccharides do not induce significant increases in antibodies. The poor immunogenicity of these antigens is due to immunological tolerance. Structurally and immunologically related molecules, particularly in developing brain tissue, have been identified on human cells [159]. Polysaccharide conjugate vaccines may overcome the poor antibody response. These vaccines induce an immune response against the normally non-immunogenic group B polysaccharide [158, 160]. It is conceivable that the natural tolerance to this polysaccharide structure could cross-react in vivo with host antigens and thereby could initiate and autoimmune process [159].

A promising approach to the development of an effective serogroup B meningococcal vaccine is the use of OMPs. Vaccines consisting of membrane vesicles containing a mixture of OMPs are shown to be safe and immunogenic in human volunteers [161, 162]. Efficacy studies with these vaccines demonstrated that antibodies to these antigens do provide a significant protection against serogroup B meningococcal disease [163-167]. However, these studies showed a protective effect of limited duration and an age-dependent protection in the range of 50 to 80% [165-167]. Of interest, class 1 proteins appear to be more immunogenic than class 2 or class 3 proteins and may play a central role in the development of vaccines [13]. Antibodies to the class 4 outer-membrane proteins interfere with the protective response, and should be excluded from a protein based vaccine [168]. This has led to work on a new generation of vaccines in which a nonprotective OMP is replaced by a protective OMP from another strain. In this way, a multivalent vaccine has developed with the insertion of multiple

class 1 genes into a single strain and provide protection against many of the prevalent serotypes [169]. A phase II trial with this multivalent vaccine is currently ongoing in Gloucester (England) and Rotterdam (The Netherlands).

8.9 Conclusions

Several aspects of the pathophysiology of septic shock have been clarified in recent years. Based on this improved understanding, rational therapeutic approaches that supplement antibiotic treatment can now be developed. Therapeutic interventions may be directed against harmful bacterial products, against cytokines, against WBC or against some of the consequences of disease, such as DIC. Although some experimental treatment strategies have resulted in encouraging results, prevention of meningococcal sepsis by the development and introduction of an efficacious vaccine will be the major challenge of the next decade.

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Chapter 9

Septic Shock and Purpura in Children: Clinical and Laboratory Features, Outcome, and Development of a Prognostic Score

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Submitted for publication

9.1 ABSTRACT

Clinical characteristics and outcome were studied in 87 children with septic shock and purpura. Additionally, a new prognostic scoring system was developed. The median age of the patients was 3.2 years (range 3 weeks - 17.9 years). Blood- and/or cerebrospinal fluid cultures grew *Neisseria meningitidis* in 75 children and *Haemophilus influenzae* in 2 patients. Cultures remained sterile in 10 patients. The most common phenotype of *N. meningitidis* was B:4:P1.4 (27%). A mortality rate of 25% was observed. Ten of the 66 (15%) survivors had serious sequelae. Calcium levels were significantly lower in patients with seizures. Disseminated intravascular coagulation occurred in 60% of the patients. Logistic regression analysis identified four laboratory parameters independently associated with mortality: C-reactive protein, base excess, serum potassium, and platelet count. These tests were used to develop a novel scoring system with a predictive value for death and survival of 75% and 91% respectively. The outcome was predicted correctly in 87% of the patients which is higher than in previously reported scoring systems.

9.2 INTRODUCTION

Septic shock and purpura or severe infectious purpura with shock is a life-threatening entity in previously healthy children. The syndrome is mainly caused by *Neisseria meningitidis*, although occasionally *Haemophilus influenzae* type b is involved. Meningococcal disease still remains major health problem in both developing and industrialized countries. Group B is the predominant serogroup among strains causing meningococcal disease followed by group C [1].

From 1970 to 1980 the annual incidence of meningococcal disease in the Netherlands varied between 0.7 and 2.0 cases per 100,000 population. The incidence of meningococcal disease gradually increased during the 1980s, and reached 3,5 per 100,000 inhabitants in 1990. The age-specific incidence is highest among children less than 5 years of age (~ 22.8 per 100,000) [1, 2]. In addition, the percentage of patients with meningococcal sepsis without clinical meningitis increased in the same period [3, 4].

Despite the use of antibiotics and intensive care treatment, septic shock and purpura is still associated with a high mortality and morbidity. Mortality ranges between 25% and 50% [5-7]. A relatively small percentage of the survivors has serious sequelae such as extensive skin necrosis requiring skin grafting and amputation.

The use of scoring systems combining data of prognostic significance in the assessment of patients with acute meningococcal disease or septic shock and purpura, has attracted much interest [6-18]. According to a number of studies, signs of poor prognosis on admission are the absence of meningeal inflammation, the presence of rapidly evolving hemorrhagic skin lesions, hyperpyrexia, leukocytopenia, thrombocytopenia, low plasma levels of fibrinogen, disseminated intravascular coagulation, metabolic acidosis, and rapid clinical deterioration. Combinations of clinical and laboratory parameters have been used to develop scoring systems to predict mortality. However, these systems are often partly based on subjective clinical criteria.

The purpose of the present study was to evaluate the epidemiology, clinical features, laboratory parameters, and outcome of septic shock and purpura in children admitted to the Sophia Children's Hospital between 1988 and 1995. Additionally, the prognostic significance of several clinical features and laboratory parameters was evaluated and a new prognostic score was developed.

9.3 PATIENTS AND METHODS

The records of all patients 18 years and younger admitted from October 1988 through June 1995 with a clinical diagnosis of septic shock and purpura to the Pediatric Intensive Care Unit (PICU) of the Sophia Children's Hospital were prospectively evaluated. Shock was defined as a mean arterial blood pressure more than 2 standard deviations below the normal value for age [19] and/or the presence of poor end-organ perfusion defined by at least two of the following criteria: a.) unexplained metabolic acidosis (pH $\leq$ 7.3), base excess $\leq$ -5 mmol/L or arterial plasma lactate levels > 2.0 mmol/L; b.) arterial hypoxia defined as a $PaO_2 \leq 75$ mm Hg, a PaO_2 to FiO_2 ratio < 250 or $TcO_2 \leq 96\%$ in patients without overt cardiopulmonary disease; c.) acute renal failure defined as oliguria with an urine output less than 0.5 mL/kg/hr for at least one hour despite acute volume loading or evidence of adequate intravascular volume and without preexistent renal disease; d.) sudden deterioration of the patient's mental status.

A subset of the patients was enrolled in a randomized, double-blind placebo controlled trial to study the efficacy of HA-1A human monoclonal antibody against endotoxin (Centoxin, Centocor, Malvern, PA) in meningococcal septic shock.

Medical records were analyzed for demographic parameters, clinical features, laboratory data, and outcome. The data were abstracted using a

standard form. Patients who were initially treated at other hospitals but were transferred to this hospital for intensive care treatment were also included. Decisions regarding the use of antibiotics, intravenous fluids, inotropic and vasopressor support, and the initiation of mechanical ventilation were made by the patients attending physician.

Definitions

The severity of illness on admission at the PICU was assessed using the pediatric risk of mortality (PRISM) score [20]. The duration of symptoms and petechiae was estimated as precisely as possible. Meningitis was defined as the presence of a positive bacterial culture of cerebrospinal fluid (CSF), or the presence of a positive gram-stain in the CSF, or the presence of a positive blood culture in combination with clinical evidence of meningitis and a CSF white blood cell (WBC) count above 10 cells/mm^3. Respiratory distress was defined as a condition that required mechanical ventilation because of respiratory failure. Disseminated intravascular coagulation (DIC) was defined by the combination of three of the following features: platelet count less than 150 x 10^9/L, fibrinogen less than 2 g/L, factor V less than 60%, and presence of fibrinogen degradation products (FDP) [21]. Patients were divided in different groups for statistical analyses. Survivors were compared with nonsurvivors.

Laboratory studies

Bacteriological methods: Specimens of CSF and/or blood were routinely cultured. These specimens were obtained from all patients before antibiotic therapy was initiated. Micro-organisms were identified according to standard procedures [22]. Isolates from blood and/or CSF were sent to the Netherlands Reference Laboratory for Bacterial Meningitis (Department of Medical Microbiology, University of Amsterdam, Amsterdam and National Institute for Public Health and the Environment, Bilthoven, the Netherlands). *Neisseria meningitidis* strains were classified into serogroups, serotypes and subtypes on the basis of antigenic differences in their capsular polysaccharides and in class 2/3 and class 1 outer membrane proteins (OMP's), respectively. Meningococci were serogrouped by means of Ouchterlony gel diffusion with the use of rabbit antisera (produced at the Reference Laboratory) to the capsular polysaccharides of the serogroups [23]. Serotyping and subtyping were performed by means of a whole cell ELISA [1, 24].

Clinical hematology and chemistry: Laboratory studies including a complete blood count (hemoglobin, total and differential white blood cell count, and platelet count) and serum chemistry analysis were routinely performed on admission. Blood samples for analysis of hematologic

parameters were collected in a microtainer containing EDTA(K_2). Blood samples for clinical chemistry were collected into sterilized siliconized vacutainer glass tubes (Becton Dickinson, Meylan Cedex, France) and allowed to clot at room temperature. Samples were centrifuged at 1600 g for 10 minutes at 4 °C.

Parameters of coagulation and fibrinolysis: All assays were performed with commercially available reagents and methods. Blood samples for analysis of coagulation and fibrinolysis assays was collected in trisodium citrate 0.109 M (anticoagulant to blood 1:9 vol/vol). Clotting assays were used for the determination of the activated partial thromboplastin time (APTT). Factor V (F V) was determined with a one stage assay using factor V deficient plasma and fibrinogen according to the Clauss method [25] (Behringwerke AG, Marburg, Germany). A semi-quantification of fibrin/fibrinogen degradation products (FDP) in plasma was performed by latex agglutination (Diagnostica Stago, Asnières-sur-Seine, France).

Statistical analysis

Results are expressed as means ± SD, unless stated otherwise. Comparison of various variables between groups of patients were tested with the Mann-Whitney test. Frequencies of various findings between groups were compared by the Fisher's Exact Test. Peason's (r) or spearman (r_s) correlation coefficient were used to evaluate the relation between specific variables. Multiple regression analysis was performed to evaluate factors which might affect the difference in parameters between survivors and nonsurvivors. Logistic regression analysis with backward selection was performed to develop a prognostic score for mortality based on variables obtained on admission. Two-tailed P values $\leq .05$ were considered statistically significant.

9.4 Results

Patient characteristics

Eighty-seven patients with septic shock and purpura were evaluated. Fifty-one were males and 36 were females. The children had a median age of 3.2 years (range 3 weeks - 17.9 years). Twenty-nine children (33%) were less than 2 years, 42 (48%) between 2 and 10 years, 16 (19%) were older than 10 years. Fifty-six of the children participated in the clinical trial to study the efficacy of the human monoclonal anti-endotoxin antibody HA-1A. The PRISM score at admission in the PICU ranged from 0 to 44 (median 10). Sixteen patients were directly admitted to our hospital, and 71

were referred by other hospitals. None of the patients received antibiotic treatment before or during transport to the first institution. Hospitalization occurred within 12 hours after the onset of petechiae in 88% of the patients. In 11 patients (13%) petechiae developed during hospitalization. The transferral time from the first institution to the PICU of Sophia Children's Hospital was less than 12 hours in 50 of the 71 transferred patients. The duration (mean ± SD) of symptoms and the interval between the appearance of petechiae and admission to the Sophia Children's Hospital were 18.5 ± 7.6 and 6.8 ± 5.5 hours respectively.

A lumbar puncture was performed in 55 cases at the time of admission. Meningitis was documented in 10 cases (18%). All 87 patients needed inotropic and vasopressor support. Fifty-four of the 87 patients (62%) needed mechanical ventilation.

Table 1. Distribution of serogroups, serotypes and -subtypes in 71 patients with septic shock due to *Neisseria meningitidis*.

Total no. of patients (%)	B 58 (82)	C 13 (18)
serotype		
2a	2 (3)	7 (10)
4	38 (54)	3 (4)
Other	6 (8)	1 (1)
Non-typeable	12 (17)	2 (3)
subtype		
P1.4	24 (34)	3 (4)
P1.15	5 (7)	0 (0)
Other	16 (23)	7 (10)
Non-typeable	13 (18)	3 (4)

Bacteriological findings

Cultures of blood, CSF or skin biopsies grew *Neisseria meningitidis* in 75 children and *Haemophilus influenzae* in two children. Cultures were sterile in 9 children (10%) and could not be obtained in one patient. A total of 71 strains of *N. meningitidis* were available for typing. Four other isolates were not sent to the Reference Laboratory. The distribution of the serogroups and serotypes/subtypes of *N. meningitidis* are depicted in Table 1. Fifty-eight of 71 strains (82%) were serogroup B and 13 (18%) were serogroup C. The most common phenotype of *N. meningitidis* in the present study was B:4:P1.4 (27%). The age distribution differed among the various

Table 2. Characteristics of 87 children with septic shock and purpura on admission at the PICU.

characteristic	*n*	survivors (*n* = 66)	nonsurvivors (*n* = 21)	*P*
Age (years)	87	5.7 ± 4.9	3.4 ± 3.4	NS
Sex (% male)	87	39 (59)	12 (57)	NS
Transferred	87	57 (86)	14 (64)	NS
Interval (hours) from				
- onset symptoms				
to admission PICU	83	19.3 ± 7.2	15.8 ± 8.5	.02
- appearance of petechiae				
to admission PICU	84	7.3 ± 5.7	5.2 ± 4.6	NS
PRISM score	87	10.5 ± 6.9	20.4 ± 7.6	<.001
Hematology				
Hemoglobin (mmol/L)	87	6.6 ± 0.9	6.0 ± 1.1	.05
WBC ($\times 10^9$/L)	87	14.4 ± 9.9	8.5 ± 8.5	.005
platelets ($\times 10^9$/L)	86	112 ± 50	66 ± 39	<.001
Chemistry				
sodium (mmol/L)	87	135 ± 5	138 ± 4	.02
potassium (mmol/L)	87	3.4 ± 0.6	4.2 ± 0.8	<.001
calcium (mmol/L)	81	1.92 ± 0.23	1.88 ± 0.29	NS
glucose (mmol/L)	74	6.7 ± 2.8	4.2 ± 2.6	.002
lactate (mmol /L)	81	5.0 ± 3.3	8.1 ± 4.1	<.001
ureum (mmol/L)				
creatinine (μmol/L)	81	86 ± 55	116 ± 59	.03
CRP (mg/L)	77	134 ± 67	78 ± 51	<.001
albumen (gr/L)	78	33 ± 6	32 ± 10	NS
Acid Base balance				
pH	87	7.37 ± 0.07	7.24 ± 0.14	<.001
BE (mmol/L)	86	-6.2 ± 3.8	-12.6 ± 5.0	<.001
bicarbonate (mmol/L)	87	17.3 ± 3.4	13.8 ± 3.0	<.001
Microbiology				
N. meningitidis		59 (89)	16 (76)	
H. influenzae		0 (0)	2 (10)	
No growth		7 (11)	3 (14)	

Plus-minus value are mean ± SD. Values in parentheses are percentages. Abbreviations: *n*, number of observations; PICU, Pediatric Intensive Care Unit; Hb, Hemoglobin; WBC, white blood cell; CRP, C-reactive protein; BE, base excess; NS, not significant.

serogroups. The mean age of children affected with serogroup C meningococci was significantly higher than in those with serogroup B (4.6 ± 4.6 years versus 7.7 ± 5.3 years: $P = .04$).

Outcome

Survivors vs. nonsurvivors: The mortality was 25% (95% confidence interval [CI]: 15% - 34%). Eighteen children died as a consequence of irreversible septic shock. Three patients died as a result of central nervous system complications. Nearly 50% of the deaths occurred within the first 24 hours and 90% occurred within 48 hours. The median (range) duration from the onset of symptoms until death was 34 hours (11-143 hours). The demographic and clinical characteristics of the 67 survivors and the 21 nonsurvivors at admission to the PICU are shown in Table 2. The mortality rate was higher in children younger than 4 years of age (16 of 48 [33%] vs. 5 of 39 [13%]; $P = .03$). Patients admitted primarily to the Sophia Children's Hospital had a higher mortality rate in comparison with secondary referrals (7 of 16 [44%] vs. 14 of 71 [20%]; $P = .06$]. The PRISM score of the primary referrals was significantly worse than that of the secondary referrals (16.6 ± 10.0 vs. 12.1 ± 7.6; $P = .05$). The interval between the onset of symptoms and admission to the PICU was significantly shorter in nonsurvivors.

Complications and sequelae of survivors: The median hospital stay was 13 days (range 10 - 207) among the survivors. Thirty-three of the 66 survivors were mechanically ventilated for a median duration of 7 days (range 1-24 days). Most survivors recovered without sequelae. Dermatologic or orthopedic sequelae requiring skingrafts or amputations occurred in 9 of the 66 survivors. Two survivors had serious neurological sequelae (sensorineural hearing loss, spasticity). Two patients required hemofiltration because of renal failure, one patient developed osteomyelitis. Seizures occurred in 12 patients.

Table 3. Coagulation and fibrinolysis parameters in 87 patients with septic shock and purpura.

Characteristic	reference range	*n*	survivors (*n* = 66)	nonsurvivors (*n* = 21)	*P*
Coagulation					
APTT (sec)	28-40	77	53 (29->200)	102 (53-200)	<.001
Factor V (%)*	70-140	75	41 ± 21	21 ± 12	<.001
Fibrinogen (g/L)	1.8-3.5	78	2.6 (<0.4-5.8)	1.1 (<0.4-5.4)	<.001
Fibrinolysis					
FDP (mg/L)	<5	76	48 (<5->300)	143 (35->300)	.001

Data are expressed as median (range) specified otherwise. *mean ± standard deviation. Abbreviations: *n*, number of observations; APTT, activated partial thromboplastin time; FDP, fibrin/fibrinogen degradation products.

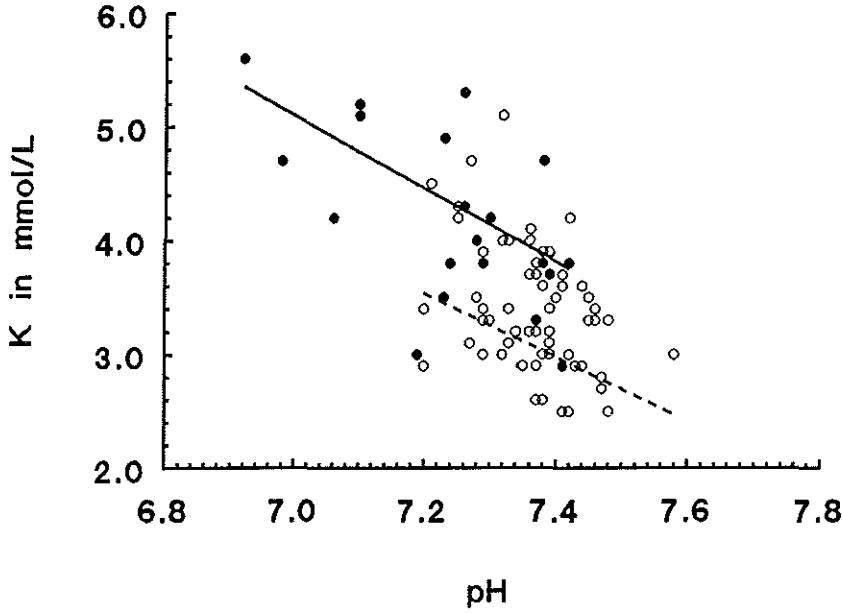

Figure 1. Relation between initial serum concentrations of potassium (K) and arterial pH in 87 children with septic shock and purpura. Solid and dashed lines indicate the regression lines through the values of each parameter for survivors and nonsurvivors respectively. Slopes between the regression lines of survivors and nonsurvivors did not significantly deviate from parallelism.

Laboratory findings

Demographic and laboratory parameters of survivors and nonsurvivors are depicted in Tables 2 and 3. Occasionally laboratory data were missing, but this never occurred in more than 12 patients for a given characteristic.

Initially, 19 patients (22%) had a peripheral white blood cell (WBC) count lower than 5 x 10^9/L. Platelet counts were below 50 x 10^9/L in 15 of 86 patients (17%). The acid-base status and the arterial serum lactate levels showed striking abnormalities that were more severe in nonsurvivors. Serum glucose levels were significantly lower in the nonsurvivors but hypoglycemia (< 2.5 mmol/L) was observed in only 5 children. Hypokalemia (< 3.5 mmol/L) was observed in 49%. Serum potassium levels were highly correlated with the arterial pH (r_s = .50, P < .001). Analysis of covariance showed that serum potassium levels were

significantly higher in nonsurvivors in comparison to survivors irrespective of the arterial pH (Figure 1). Serum calcium concentrations were measured in 81 cases (93%). Hypocalcemia (< 2.2 mmol/L) was detected in 58 patients (72%). Ionized calcium levels were only available in a limited number of patients and are therefore not shown. Of interest, serum calcium levels were lower in patients with seizures in comparison with those without seizures (1.75 ± 0.29 mmol/L versus 1.93 ± 0.23 mmol/L; $P = .05$). The serum levels of CRP were significantly lower in nonsurvivors in comparison with survivors and correlated strongly with the time interval between the onset of symptoms and petechiae and the moment of blood sampling ($r = .59$, $P < .001$ and $r = .55$, $P < .001$, respectively).

Coagulation studies were performed in all patients (Table 3). Fibrinogen levels were less than or equal to 1.5 g/L in 22 of 78 (28%) patients. DIC could be determined in 77 patients. DIC occurred significantly more often in nonsurvivors than in survivors (18 of 19 [95%] vs. 28 of 58 [48%]; $P = .003$).

Prognostic analysis

Most variables listed in Tables 2 and 3 documented at the time of admission were associated with a poor prognosis. Factors that appeared to discriminate according to the univariate analysis were considered for inclusion in a prognostic scoring system. Logistic regression analysis identified four independent variables to predict the likelihood of survival. These were the serum level of CRP and potassium, the base-excess, and the platelet count. The mathematical expression of the probability of PICU death in this study was as follows:

$$Probability\ (PICU\ death) = \frac{e^x}{1 + e^x}$$

in which X = 1.60 + 1,31 x Potassium (mmol/L) - 0.34 x Base Excess (mmol/L) - 0.023 x Platelets (10^9/L) - 4.49 x log (CRP [mg/L]). Figure 2 gives the graphical presentation of the model. This new prognostic score was compared with five other scoring systems. Each scoring system was applied to our patients. Our score had the highest predictive value for death and survival (Table 4). The newly developed Rotterdam prognostic score was highly correlated with the PRISM-score ($r = .64$, $P < .001$).

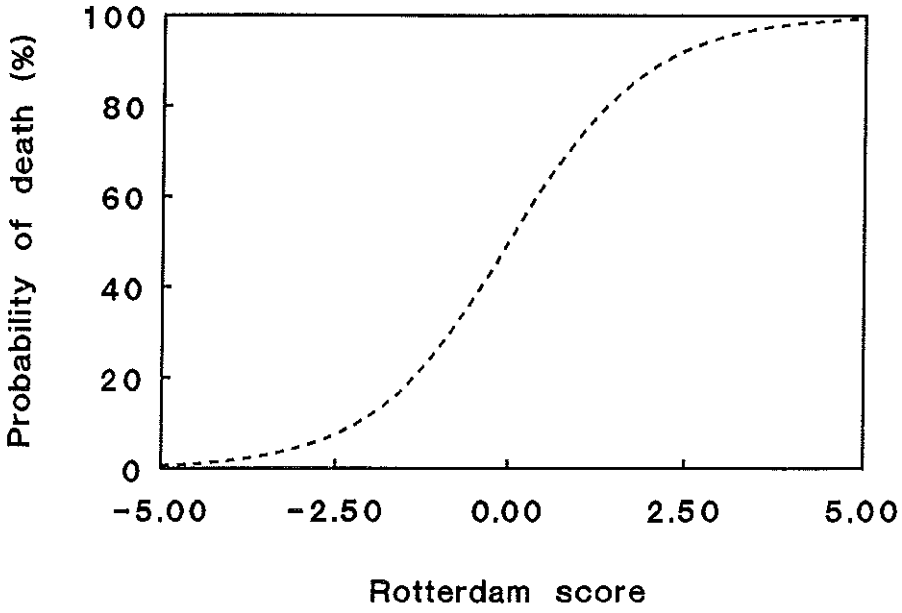

Figure 2. Probability of death according to the new prognostic scoring system.

Table 4. Outcome prediction in patients with septic shock and purpura.

Score	Patients (*n*)	Predictive value for		Accuracy
		survival	death	
Niklasson [17]	55	88%	26%	45%
Leclerc [6]	73	92%	45%	78%
CRP [11]	77	90%	42%	66%
Giraud [7]	78	88%	55%	78%
PRISM [20]	87	86%	73%	84%
Rotterdam score	76	91%	75%	87%

9.5 DISCUSSION

The clinical picture of septic shock and purpura is induced by meningococci (occasionally other bacteria) and by their products (lipopolysaccharides) and mediated by a multitude of inflammatory mediators. The inflammatory response may develop into irreversible circulatory collaps, renal failure, adult respiratory distress syndrome and death.

In the present study we show that septic shock and purpura is associated with a mortality rate of 25% and serious sequelae in 15% of the survivors. A higher mortality was observed in patients directly admitted to the Sophia Children's Hospital in comparison with secondary referrals. This was probably due to patient selection, since extremely ill patients died before referral could be organized. The clinical condition of secondary referred patients was relatively better as can be inferred from the lower PRISM-score. In contrast, Tesoro et al. observed that patients transferred from another hospital had a higher mortality [12]. In our study, the mortality rate was higher among children below 4 years of age in comparison with older children. The lower plasma levels of the naturally occurring circulating anticoagulants protein C and S in children younger than 4 years of age may contribute to the worse outcome in this group [26]. Long-term morbidity was observed in 15% of the survivors and was caused by deforming amputation or large areas of soft tissue destruction secondary to the coagulopathy and by serious neurological sequelae (sensorineural hearing loss, spasticity or paresis). A similar percentage was observed by Madden et al. [27, 28].

Meningococcal disease in the Netherlands has gradually increased from 1.1 cases per 100,000 in 1982 to 4.3 cases per 100,000 in 1993. Strain B:4:P1.4 was most frequently isolated in our patients. This strain was not found before 1980, but became the most prevalent strain in 1990 (21% of all isolates) [1].

Striking differences were observed in clinical and laboratory characteristics between survivors and nonsurvivors. The significantly shorter interval of symptoms and the lower level of CRP in nonsurvivors suggest a shorter disease course. These data indicate that nonsurvivors deteriorate more quickly because they accumulate more native lipopolysaccharides (LPS) per time span, trigger all mediator systems more intensively or because they have a higher responsiveness to LPS or proinflammatory cytokines [29].

Complex abnormalities were observed in electrolytes and acid-base status. Metabolic acidosis and increased arterial serum lactate levels are the inevitable consequence of poor end-organ perfusion leading to anaerobic glycolysis. The serum sodium level was usually normal. Interestingly, we found hypokalemia rather than hyperkalemia in patients with septic shock. Hypokalemia was more severe in survivors than in nonsurvivors even when adjusted for the degree of acidosis which would normally be expected to result in a shift of potassium from the intracellular space. Hypokalemia may be caused by the release of catecholamines leading to an increased intracellular shift of potassium into skeletal muscle [30]. The relatively higher serum potassium levels in nonsurvivors may be caused by metabolic derangements [30], the more severe renal impairment, or rhabdomyolysis. In addition, hypocalcaemia was also seen in a large number of patients as observed by others [31-33]. Interestingly, patients that had seizures during their initial disease course had lower serum calcium levels than the other children. Hypotension, acidosis and electrolyte abnormalities may play a major role in the deterioration of myocardial function and may predispose to arrhythmias and cardiac arrest.

Scoring systems for disease severity or a prognostic score have been useful in the assessment of care requirement, efficacy of therapy and prognosis. Previously, several scoring systems were developed for patients with acute meningococcal infections or septic shock and purpura. Most of these systems include the presence or absence of meningeal irritability or an elevated CSF WBC count [13, 15-18, 34]. The assessment of neck stiffness, however, is unreliable in severely ill patients. Tesoro et al. concluded that absence of meningeal involvement is not an important predictor of mortality [12]. A CSF WBC count is not always available since a lumbar puncture is usually not performed due to the unstable clinical condition on presentation. Other scoring systems require variables such as the erythrocyte sedimentation rate, and skin/rectal temperature difference determination that are not always available [10, 18]. We therefore developed a simple score for patients with septic shock and purpura which only requires objective variables available at any emergency room or PICU soon after admission. Logistic regression analysis revealed four laboratory parameters including low potassium levels, a negative base excess, a low platelet count and a low CRP level which were all significantly associated with fatal outcome. Base excess and potassium levels both reflect the degree of metabolic abnormalities. Low platelet counts are highly predictive for the presence of DIC. CRP level reflect the duration of illness since this level correlates positively with the duration of petechiae and other symptoms of patients with septic shock and purpura [29]. The mortality risk

predictor developed by us only needs simple laboratory parameters which are routinely performed. The prognostic value was higher than in previously developed scoring systems. This score will enable accurate mortality risk prediction for individuals or provide a relative scale for severity of illness. This score can also be used to evaluate the effects of future therapeutic interventions and to assess the evolution of disease in the first 24 hours.

The possible beneficial effects of the anti-endotoxin antibody HA-1A on the outcome of children in the present study with meningococcal septic shock are not yet known. However, a recent study did not find a reduction in the 14-day mortality rate in patients with gram-negative bacteremia and septic shock [35].

We conclude that septic shock and purpura in children is associated with a mortality of 25% (95% CI 15% - 34%). The mortality was even higher in children below 4 years of age. About 15% of the survivors had serious sequelae such as skin necrosis requiring skingrafts or amputation, and permanent neurologic sequelae. Logistic regression analysis identified four laboratory parameters which were used in a prognostic score to predict outcome. The predictive value for death and survival were 75% and 91% respectively. The overall outcome was predicted correctly in 87% of the cases.

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Chapter 10

The Relationship between Plasminogen Activator Inhibitor-1 and Proinflammatory and Counterinflammatory Mediators in Children with Meningococcal Septic Shock

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Journal of Infectious Diseases 1996; 173: 1148-1156

10.1 ABSTRACT

Proinflammatory cytokines (tumor necrosis factor [TNF]-α, interleukin [IL]-6 and -8), counterinflammatory compounds (IL-10 and soluble TNF receptors p55 and p75 [sTNFR-55 and -75]), and hemostatic parameters were determined in 38 patients with meningococcal septic shock. Eleven patients (29%) died. Serum levels of pro- and counterinflammatory compounds and plasma levels of plasminogen activator inhibitor (PAI)-1 were significantly higher in nonsurvivors. The interval between appearance of petechiae and blood sampling was shorter in nonsurvivors than in survivors (3.6 ± 2.4 vs. 6.1 ± 3.3 h; $P = .04$). This interval correlated strongly with the levels of TNF-α, IL-6, -8, -10, sTNFR-55 and -75, and PAI-1. However, with the exception of PAI-1, differences between concentrations of these mediators disappeared after adjustment for the interval. PAI-1 levels correlated with TNF-α concentrations ($r = .75$; $P < .001$) and were 1.9 ($P = .01$) times higher in nonsurvivors at a similar TNF-α concentration. A polymorphism in the promotor of the PAI-1 gene has been associated with differences in release of PAI-1. Thus, an increased PAI-1 response to TNF-α may be associated with fatality, probably because of polymorphism of the PAI-1 gene.

10.2 INTRODUCTION

Septic shock with purpura is a serious life-threatening disease in otherwise healthy children and young adults. The syndrome is most frequently due to *Neisseria meningitidis*, although occasionally *Haemophilus influenzae* type b is involved [1-6].

Lipopolysaccharides (endotoxin), a component of the outer membrane of gram-negative bacteria, induces the release of proinflammatory cytokines (tumor necrosis factor [TNF]-α, and interleukin [IL]-1ß, -6, -8) in patients with sepsis. Subsequently, endotoxins and cytokines stimulate the production of a wide range of additional inflammatory mediators (i.e., arachidonic acid metabolites, complement, platelet-activating factor), influence the function of leukocytes and endothelial cells, and activate hemostasis [7-11]. The production of proinflammatory cytokines and the extent of the inflammatory response is downregulated by counterinflammatory compounds, such as IL-10, and naturally occurring antagonists of TNF-α including the soluble extracellular domains of the 55- and 75-kDa membrane-bound TNF receptors (sTNFR-55 and -75).

IL-10 is produced by activated monocytes and suppresses the endotoxin-induced production of TNF-α and IL-1ß, -6, and -8 [12]. The biologic activity of TNF-α is also neutralized by sTNFR-55 and -75 [13-17]. sTNFR is shed from the cell surface of, for example, polymorphonuclear cells in response to many of the same inflammatory stimuli that induce TNF-α [18].

Endothelial cells are among the principal targets for the action of endotoxin, TNF-α and IL-1ß. These cells change to a procoagulant state and can modulate the fibrinolytic system by secretion of tissue-type plasminogen activator (t-PA) and plasminogen activator inhibitor (PAI)-1, which respectively activate and inhibit fibrinolysis. The activation of coagulation together with the inhibition of the fibrinolysis are responsible for the development of a hypercoagulable state, fibrin deposition, and microthrombi [19]. Fibrin deposition and complement activation cause extensive endothelial damage and are associated with multiple organ failure [20-22].

Inflammatory mediators and coagulation disorders are involved in the pathophysiology of septic shock and should be associated with disease severity. Thus, we investigated the balance between proinflammatory cytokines (TNF-α, IL-6, IL-8) and counterinflammatory compounds (IL-10, sTNFR-55, sTNFR-75) in admission serum samples of 38 consecutive children with meningococcal septic shock (MSS) and studied their relationship with indicators of hemostasis.

10.3 Methods

Patients

We prospectively recruited patients between ages 3 months and 18 years with septic shock and petechiae or purpura. Primary or secondary referrals were admitted to the pediatric intensive care unit (PICU), Sophia Children's Hospital, between October 1991 and September 1994. Patients were eligible for inclusion if they met the following criteria: Presence of petechiae or purpura for < 12 h and presence of shock (systolic blood pressure < 75, < 80, < 85, or < 100 mm Hg in children ages 3 months to 1 year or 1-5, 6-12, and > 12 years old, respectively). Children were also included when poor end-organ perfusion was present (defined as occurrence of at least two of the following criteria): (1) unexplained metabolic acidosis (pH ≤ 7.3), base excess -5 mmol/L or lower, or arterial plasma lactate levels > 2.0 mmol/L; (2) arterial hypoxia (PO_2 ≤ 75 mm Hg, PO_2-to-FiO_2

ratio < 250, or TcO_2 ≤ 96% in patients without overt cardiopulmonary disease); (3) acute renal failure (oliguria with urine output < 0.5 mL/kg/h for > 1 h despite acute volume loading or evidence of adequate intravascular volume and without preexistent renal disease); or (4) sudden deterioration of the patient's mental status. Most of the patients were enrolled in a randomized, placebo-controlled trial to study the efficacy of the human monoclonal anti-endotoxin antibody HA-1A (Centoxin; Centocor, Malvern, PA) in MSS. Initial blood samples were obtained before administration of HA-1A or placebo. There was no selection in the HA-1A trial to bias the present study.

On PICU admission, the severity of illness was assessed using the pediatric risk for mortality (PRISM) score, a severity-of-illness index [23]. Parents were asked to indicate the onset of symptoms as precisely as possible. The time of onset of petechiae was defined as the mean time between observation of the child with and without petechiae. The times of initiation of antibiotic treatment, PICU admission, and blood sampling were carefully registered by the investigators. Decisions regarding the use of antibiotics, intravenous fluids, inotropic and vasopressor support, and initiation of mechanical ventilation were made by the patient's attending physician.

Laboratory studies

Cerebrospinal fluid and blood specimens were routinely cultured. Blood samples for the determination of biochemical parameters, TNF-α, IL-6, -8, and -10, and sTNFR-55 and -75 were obtained from an arterial catheter and collected into sterilized siliconized glass tubes (Vacutainer; Becton Dickinson, Meylan, France) and allowed to clot at room temperature. The samples were centrifuged at 1600 *g* for 10 min at 4° C. Aliquots were stored at -80° C until assayed. Blood for the platelet and leukocyte counts was collected in a microtainer containing $EDTA(K_2)$. Blood for the coagulation and fibrinolysis assays was collected in 0.109 *M* trisodium citrate (anticoagulant to blood 1:9 vol/vol) and in a 0.109 *M* trisodium citrate mixture (theophylline, adenosine, dipyridamole; Diatube H, Diagnostica Stago, Asnières-sur-Seine, France). These samples were immediately chilled on ice and centrifuged at 2800 *g* for 15 min and then at 45,000 *g* for 30 min at 4° C. Platelet and white blood cell counts were determined by flow cytometer (H1 system; Technicon Instruments, Tarrytown, NY); C-reactive protein (CRP) was measured by immunonephelometric assay [24].

Cytokines and inhibitors. Levels of TNF-α, IL-6, -8, and -10, and sTNFR-55 and -75 in serum were determined by ELISA (Medgenix, Fleurus, Belgium). Mediators were determined according to the manufacturer's instructions with the following detection limits (lowest positive standard): TNF-α, 15 pg/mL; IL-6, -8, and -10, respectively, 30, 7, and 11 pg/mL; and sTNF-55 and -75, respectively, 0,4 and 1.0 ng/mL.

Parameters of coagulation and fibrinolysis. All assays were done with commercially available reagents and methods. Clotting assays were used for the determination of the activated partial thromboplastin time (APTT). Factor V was determined with a one-stage assay using factor V-deficient plasma and fibrinogen according to the Clauss method [25] (Behringwerke, Marburg, Germany). Antithrombin III (ATIII) activity and protein C activity were determined by chromogenic substrate assays (Behringwerke). Total protein S was measured by ELISA (Diagnostica Stago). Plasminogen was determined spectrophotometrically using a chromogenic synthetic substrate (Behringwerke). Plasma t-PA antigen concentration was measured by ELISA as described [26] as was PAI-1 (Diagnostica Stago). A semiquantification of fibrin-fibrinogen degradation products (FDP) in plasma was done by latex agglutination (Diagnostica Stago).

Disseminated intravascular coagulation (DIC) was defined by the combination of three of the following features: platelet count < 150 x 10^9/L, fibrinogen < 2 g/L, factor V < 60%, and the presence of FDP [27].

Statistical analysis

Results are expressed as mean ± SD unless stated otherwise. Differences between groups of variables were tested by the Mann-Whitney test. Frequencies of various findings between groups were compared by Fisher's exact test. Pearson's correlation coefficient was used to evaluate the relation between specific variables. Multiple regression analysis was done to evaluate factors that might affect the difference in levels of mediators between survivors and nonsurvivors. Two-tailed *P* values ≤ .05 were considered statistically significant.

10.4 Results

During the 3-year study period, 43 patients with septic shock and purpura were admitted to the PICU. Five patients did not fulfill the entry criteria: 3 had purpura > 12 h before admission, 1 was < 3 months old, and informed consent was not obtained for 1 child.

Table 1. Characteristics of patients with meningococcal septic shock.

Characteristic	Survivors (n = 27)	Nonsurvivors (n = 11)	P
Age (years)	7.3 ± 5.7	5.1 ± 3.9	.43
Sex (% male)	16 (59)	7 (64)	1.0
Transferrals*	22 (82)	7 (64)	.40
Interval (h) from			
- Onset symptoms to admission PICU	18.5 ± 7.7	14.4 ± 4.8	.09
- Appearance of petechiae to admission PICU	6.1 ± 3.3	3.6 ± 2.4	.04
Duration of antibiotic treatment (h)	4.7 ± 1.8	2.8 ± 1.8	.10
PRISM score	8.6 ± 5.4	18.6 ± 5.5	<.001
Clinical hematology			
White blood cells (x 10^9/L)	15.1 ± 10.3	5.4 ± 3.2	.004
Clinical chemistry			
Creatinine (μmol/L)	102 ± 68	135 ± 65	.08
C-reactive protein (mg/L)	131 ± 60	81 ± 43	.02
Microbiology			
N. meningitidis	25 (93)	9 (82)	.56
No growth	2 (7)	2 (18)	

Note. Date are mean ± SD or no. (%). Abbreviations: PICU, pediatric intensive care unit; PRISM, pediatric risk for mortality score. *Patients transferred from first institution to PICU, Sophia Children's Hospital.

Patient characteristics

Thirty-eight patients (23 boys, 15 girls) entered the study. Of these, 36 participated in the clinical trial to study the efficacy of HA-1A human monoclonal antibody. The median age was 4.1 years (range, 0.7-17.9 years). Twenty-nine patients were referred from another hospital. The PRISM scores at PICU admission ranged from 1 to 25. Cultures of blood, cerebrospinal fluid, or skin biopsies from 34 children grew *N. meningitidis*. In 4 cases with sterile cultures, the diagnosis was made on the basis of typical clinical findings. None of the patients received antibiotic treatment before or during transport to the hospital. The duration (mean ± SD) of symptoms and the interval between the appearance of petechiae and admission to Sophia Children's Hospital were 17.4 ± 7.2 and 5.4 ± 3.3 hours respectively. All patients needed inotropic and vasopressor support. Twenty-five of the 38 patients required mechanical ventilation. The overall fatality rate was 29%. Table 1 shows the demographic and clinical characteristics of the 27 survivors and 11 fatalities. Survivors and nonsurvivors were evenly distributed in regard to time of onset of petechiae

and time of hospitalization. The interval between onset of petechiae and PICU admission was significantly shorter in nonsurvivors. Serum CRP levels were also significantly lower in nonsurvivors than in survivors and were highly correlated with the interval between the onset of symptoms and petechiae and the moment of blood sampling (r = .56, P < .001 and r = .45, P = .005, respectively).

Table 2. Coagulation and fibrinolysis data in patients with meningococcal septic shock

Characteristic	Reference range	Survivors (n = 27)	Nonsurvivors (n = 11)	P
Coagulation				
Platelets (x 10^9/L)	150-450	120 ± 45	65 ± 37	.002
APTT* (sec)	28-40	52 (33->200)	92 (58-200)	<.001
Factor V (%)	70-140	43 ± 23	22 ± 12	.007
ATIII act. (%)	80-120	66 ± 14	51 ± 12	.01
Protein C activity (%)	70-140	21 ± 11	17 ± 8	.08
Protein S total (%)	65-108	57 ± 18	41 ± 10	.006
Fibrinogen* (g/L)	1.8-3.5	2.6 (<0.4-5.3)	1.2 (<0.4-2.5)	.005
Fibrinolysis				
Plasminogen (%)	75-140	62 ± 13	53 ± 14	.10
t-PA antigen (ng/mL)	<10	25 ± 14	35 ± 19	.13
PAI-1 antigen (ng/mL)	4-40	971 ± 848	2500 ± 1390	<.001
FDP* (mg/L)	<5	70 (20->300)	120 (100-220)	.02

Note. Data are mean ± SD unless specified otherwise. Abbreviations: APTT, activated partial thromboplastin time; ATIII, Antithrombin III, t-PA, tissue-type plasminogen activator; PAI, plasminogen activator inhibitor; FDP, fibrin/fibrinogen degradation products.
*Median (range).

The parameters of coagulation and fibrinolysis are summarized in Table 2. DIC was observed in all nonsurvivors and in 13 (48%) of the 27 survivors (P = .003). The APTT was significantly more prolonged in those who did not survive. The inhibitors of coagulation (ATIII, protein C, and protein S) were generally decreased, but more so in the nonsurvivors. Plasminogen levels were similar in survivors and nonsurvivors. The t-PA, PAI-1 antigen, and FDP levels were higher in nonsurvivors than in survivors.

Table 3. Serum levels of cytokines and soluble (s) tumor necrosis factor (TNF) receptors (R) in patients with meningococcal septic shock.

	Survivors (n = 27)	Nonsurvivors (n = 11)	P
TNF-α (pg/mL)	144 (35 - 3130)	450 (74 - 2680)	.03
IL-6 (pg/mL)	107,600 (2990 - 4,515,000)	1,081,000 (25310 - 5,758,000)	.005
IL-8 (pg/mL)	746 (31 - 113,100)	30,760 (599 - 118,500)	.005
IL-10 (pg/mL)	1479 (68 - 20,440)	14,780 (636 - 28,070)	.01
sTNFR-55 (ng/mL)	15.5 (6.2 - 32.3)	27.2 (8.5 - 36.6)	.05
sTNFR-75 (ng/mL)	51.2 (22.5 - 149.4)	79.6 (10.0 - 119.7)	.04

Note. Data are median (range). IL, interleukin.

Proinflammatory cytokines and counterinflammatory compound levels at admission

At admission, serum levels of proinflammatory cytokines and counterinflammatory compounds were significantly higher in the patients who subsequently died (Table 3). Highly significant positive correlations were observed between all of these mediators (Table 4). In addition, serum cytokine levels were negatively correlated with the interval between the appearance of petechiae and blood sampling ($P < .001$ for all; TNF-α: $r = -.55$; IL-6: $r = -.57$; IL-8: $r = -.58$; IL-10: $r = -.59$; Figure 1). Multiple regression analysis for the relation between serum cytokine levels and survival and duration of skin lesions showed that the time-adjusted concentrations of the cytokines TNF-α and IL-6, -8, and -10 were not significantly higher in children who died versus survivors. sTNFR-55 and -75 were significantly higher in nonsurvivors and also correlated with the interval between the onset of petechiae and initial serum measurements (sTNFR-55: $r = -.36$, $P = .03$; sTNFR-75: $r = -.61$, $P < .001$). Both sTNFRs remained markedly elevated during the first 24 h after hospitalization (data not shown).

Serum cytokine levels were also associated with the duration of antibiotic treatment (TNF-α: $r = -.40$, $P = .02$; IL-6: $r = -.37$, $P = .03$; IL-8: $r = -.33$, $P = .05$; IL-10: $r = -.39$, $P = .02$). However, these associations were weaker than those with the duration of petechiae. The duration of

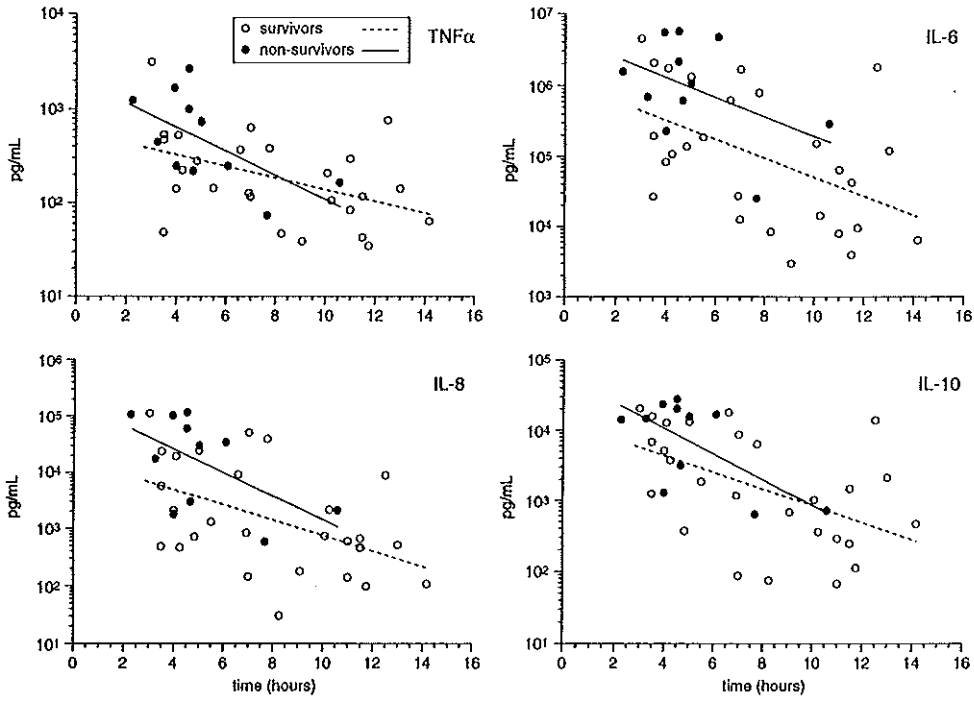

Figure 1. Relation between initial serum concentrations of inflammatory compounds (tumor necrosis factor [TNF]-α, interleukin [IL]-6, -8, -10) and interval between onset of petechiae and blood sampling in 38 children with meningococcal septic shock. Solid and dotted lines indicate regression through values for each parameter. Slopes between regression lines of survivors and nonsurvivors did not significantly deviate from parallelism for each parameter.

petechiae and the duration of antibiotic treatment were significantly correlated (r = .50, P = .002). When these time intervals were simultaneously related by multiple regression analysis to the levels of mediators, the duration of petechiae was most predictive (P < .005 for TNF-α, IL-6, IL-8, IL-10) while an additional significant predictive value was not observed for the duration of antibiotic treatment.

Correlation between inflammatory cytokines and clinical features

The relationship between cytokines and certain hematologic parameters was assessed. The peripheral white blood cell count and the CRP level were negatively correlated with levels of TNF-α (r = .59, P < .001 and r = .46, P = .004), IL-6 (r = .67, P < .001 and r = .62, P < .001), and IL-8 (r = .68, P < .001 and r = .62, P < .001) and with the interval between the onset

Table 4. Correlation between serum levels of cytokines and soluble (s) receptors (R) for tumor necrosis factor (TNF) in patients with meningococcal septic shock.

	TNF-α	IL-6	IL-8	IL-10	sTNFR-55
IL-6	.90	-			
IL-8	.90	.92	-		
IL-10	.79	.85	.89	-	
sTNFR-55	.82	.84	.80	.82	-
sTNFR-75	.87	.82	.78	.75	.77

Note. Probabilities for all correlations were < .001. Abbreviation: IL, interleukin.

of petechiae and blood sampling (r = .54, P < .001 and r = .45, P = .005). Initial serum TNF-α levels correlated significantly with the APTT (r = .47, P = .003) and the concentrations of factor V (r = -.51, P < .001), t-PA (r = .63, P < .001) and PAI-1 (r = .75, P < .001). PAI-1 levels were significantly higher in nonsurvivors than in survivors (2500 ± 1390 vs. 971 ± 848 ng/mL; P < .001) even when adjusted for duration of skin lesions before blood sampling (P = .02) (Figure 2). Of interest, PAI-1 concentrations were 1.9 times higher in nonsurvivors (P = .01) than in survivors at similar TNF-α serum levels as shown by analysis of covariance (Figure 3). This relationship between the levels of PAI-1 and TNF-α was not affected by their association with the time interval (partial r = .60, P < .001).

10.5 Discussion

Systemic meningococcal disease has a wide spectrum of severity, ranging from benign meningococcemia to fulminant septic shock with multiple organ failure and death. TNF-α and IL-1ß are thought to play a central role in the pathophysiology of this disease. These cytokines are involved in the induction of other proinflammatory cytokines, such as IL-6 and -8, and are involved in the activation of the coagulation and fibrinolysis. Our study confirms the findings of other investigators that disease severity and outcome are related to concentrations of TNF-α, IL-6, -8, and -10, and sTNFR-55 and -75 [4, 28-33].

A wide interindividual variability in TNF-α release after stimulation by endotoxin has been demonstrated in vitro in whole blood samples and

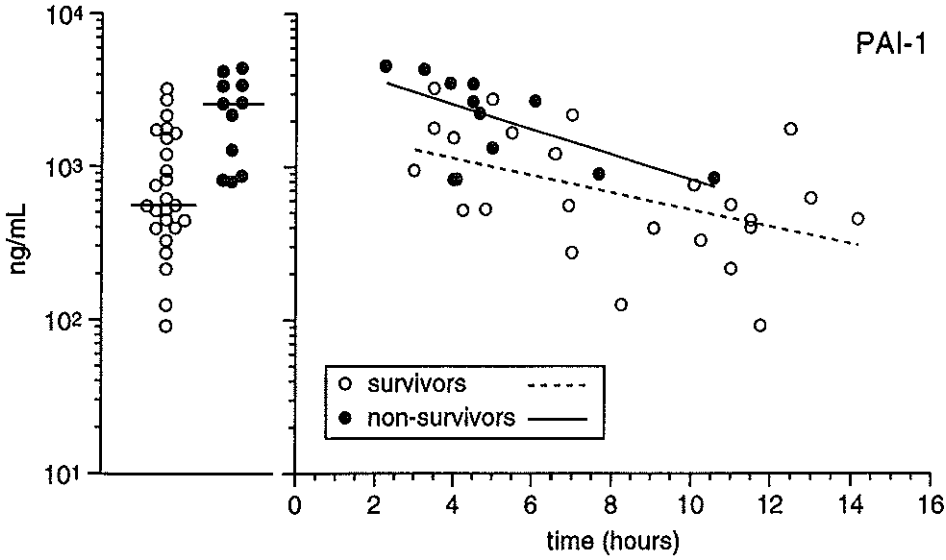

Figure 2. Initial plasma levels of plasminogen activator inhibitor (PAI-1; $n = 36$). Left, relation between PAI-1 levels and survival. Right, initial PAI-1 plasma levels in relation to interval between onset of petechiae and blood sampling. Slope between survivors and nonsurvivors did not significantly deviate from parallelism. Time-adjusted PAI-1 levels were 2.1 times higher in nonsurvivors ($P = .02$).

peripheral blood mononuclear cells isolated from healthy volunteers [34, 35]. The high initial TNF-α levels in those who do not survive MSS have been interpreted as the result of an exaggerated response to circulating endotoxin [36]. This production of inappropriately large quantities of TNF-α may be due to the presence of a genetic variant in the promotor region of the TNF gene (TNF2 allele) as previously observed in patients with cerebral malaria [37]. The TNF2 allele has been associated with higher constitutive expression and greater secretion of TNF-α after induction [38].

The initial concentrations of TNF-α in this and previous studies were significantly higher in MSS nonsurvivors than in survivors. However, the magnitude of TNF-α serum levels and of other proinflammatory cytokines (e.g., IL-6 and -8) is also determined by the duration of disease when blood samples are obtained, perhaps because these cytokines rapidly disappear during the acute phase of septic shock. [29, 39-41]. Accordingly, we found a strong negative correlation between initial cytokine levels and the interval between onset of purpuric skin lesions and blood sampling. This association is in contrast to previous reports [4, 32, 39]. In addition, in the present

study, CRP levels, which indirectly reflect the duration of illness [42], were significantly correlated with the interval between the onset of petechiae and blood sampling. This significantly shorter interval and the lower level of CRP in nonsurvivors suggest a shorter disease course and may therefore explain the higher levels of cytokines. The earlier PICU admission of nonsurvivors may indicate that persons who do not survive accumulate more native lipopolysaccharide in a shorter time, trigger all mediator systems more intensively, and are recognized as more severely ill earlier in the course of disease. Alternatively, nonsurvivors may have been admitted earlier because of a more rapid deterioration due to greater responsiveness to lipopolysaccharides or proinflammatory cytokines.

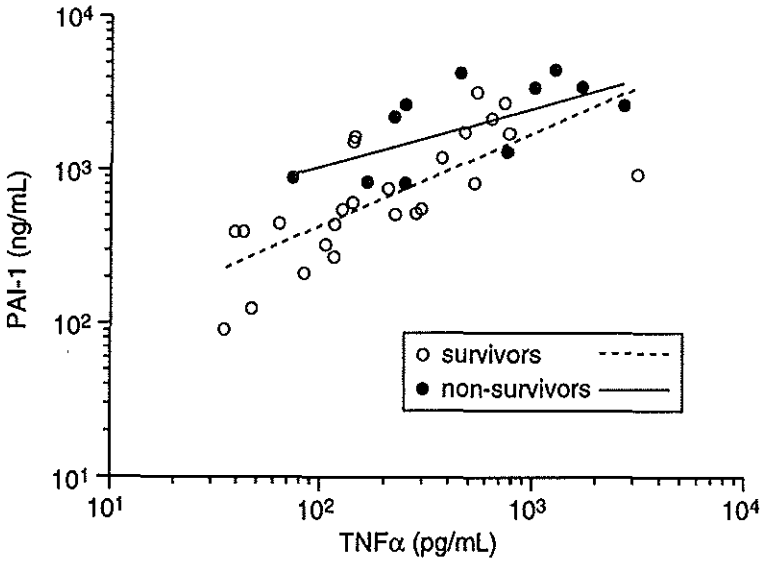

Figure 3. Relation between initial levels of tumor necrosis factor (TNF)-α and plasminogen activator inhibitor-1 (PAI-1) in 36 patients with meningococcal septic shock. Slope between regression lines of survivors and nonsurvivors did not significantly deviate from parallelism (P =

The clinical features of patients with MSS show similarities to those observed in experimental endotoxemia in humans. A challenge with endotoxin in healthy volunteers results in a transient occurrence of a sepsis-like syndrome. The peak levels of cytokines correspond with the transient leukopenia that occurs shortly after the endotoxin challenge [10, 43]. In experiments in baboons infused continuously with *Escherichia. coli*, peak levels of TNF-α also occured very early in the course of disease [44, 45].

Similarly, high levels of TNF-α, IL-6 and -8, low white blood cell counts, and low CRP levels were observed in patients from whom blood was obtained shortly after the onset of petechiae. We therefore assume that the onset of petechiae is a useful setpoint during the course of invasive meningococcal disease. This assumption is supported by multiple regression analysis, which indicated that serum levels of cytokines are dependent on the duration of petechiae and not on the duration of antibiotic treatment.

Of interest, the differences in the concentrations of mediators between survivors and nonsurvivors disappeared after adjustment for the time between onset of petechiae and blood sampling. These data suggest that the survivors and nonsurvivors in the present study may have had similar releases of inflammatory cytokines (TNF-α, IL-6, IL-8).

The proinflammatory cytokines are counteracted by counter-inflammatory compounds. We observed significantly higher concentrations of IL-10 in nonsurvivors with MSS. Lehmann et al. [29] recently reported that high IL-10 levels in patients with meningococcal disease are associated with fatality. In contrast, Derkx et al. [39] did not confirm this observation in patients with MSS, which may be explained by the relatively small number of patients evaluated. However, the time-adjusted IL-10 levels in the present study were similar between survivors and nonsurvivors. IL-10 acts as a potent inhibitor of the release of the proinflammatory cytokines TNF-α, IL-1ß, IL-6, and IL-8 from T-cells [46], polymorphonuclear leukocytes [47], and monocytes and macrophages [12, 48, 49]. In animal models of sepsis, IL-10, given before or soon after challenge with gram-negative bacterial endotoxin or staphylococcal enterotoxin B, reduced TNF-α production and mortality [50-52]. Chernoff et al. [53] showed that a single intravenous injection of IL-10 in humans reduces mitogen-induced T cell proliferation and suppresses TNF-α and IL-1ß production from whole blood stimulated ex vivo with endotoxin. In the present study, the strong correlation between IL-10 and the proinflammatory cytokines TNF-α, IL-6, and IL-8 in survivors and nonsurvivors suggests the presence of an adequate IL-10 response to down-regulate the production of proinflammatory cytokines.

In addition, sTNFR-55 and -75 can neutralize the biologic activity of TNF-α. Girardin et al. [31] found that high levels of the sTNFRs are associated with an increased likelihood of fatality, and Froon et al. [54] suggest that increased serum levels of sTNFRs in patients with sepsis syndrome are merely the result of renal failure. Normally, the majority of sTNFRs is removed from the circulation by the kidneys, although the liver and lungs are probably also involved [55]. The higher serum creatinine

levels of nonsurvivors (P = .08) in the present study may at least partly explain the differences in sTNFR concentrations.

Abnormalities of coagulation and fibrinolysis play an important role in the pathophysiology of MSS. It has been known for some time that endotoxin, TNF-α and IL-1ß contribute to the activation of the coagulation and fibrinolysis [56, 57]. Normally the endothelial cell provides a blood vessel lining that reduces the coagulability of blood. TNF-α causes endothelial cells to have procoagulant activity by enhancing the expression of tissue factor and by suppressing cofactor activity for the anticoagulant protein C [57-61]. The prolonged APTT in patients in the present study indicates a massive consumption of coagulation clotting factors, leading to a bleeding tendency. In addition, the plasma levels of the natural inhibitor of coagulation, ATIII, and of protein C and protein S were markedly depressed, resulting in a procoagulant state. The hypercoagualability that occurs during DIC results in the generation and deposition of fibrin, leading to the formation of microvascular thrombosis in various organs and perhaps to multiple organ failure and ultimately death. In the present study, DIC occurred in 13 of the 27 survivors and in all of the nonsurvivors (P = .003).

Endotoxin, TNF-α, and IL-1ß modulate the fibrinolytic system to secrete both t-PA and PAI-1, which respectively activate and inhibit fibrinolysis [62-68]. Moreover, fibrin and thrombin formed during coagulation are also potent inducers for the release of t-PA and PAI-1 [69, 70]. Fibrinolysis can be initiated by the release of t-PA from vascular endothelium that converts plasminogen into the active enzyme plasmin that degrades fibrin in the thrombi. The activity of t-PA in patients is counter-regulated by PAI-1 that binds to and thereby inhibits t-PA. Protein C can inhibit PAI-1 activity. In the present study, the levels of PAI-1 antigen were significantly higher in nonsurvivors. This finding together with decreased protein C activity result in insufficient fibrinolytic activity during a markedly procoagulant state that is associated with vital organ microembilization [19-22]. Administration of recombinant t-PA may therefore be considered an adjuvant therapeutic option in patients with fulminant meningococcemia as suggested by Zenz et al. [71].

In previous studies, PAI-1 levels rapidly decreased after hospitalization [72, 73], and PAI-1 and TNF-α levels were strongly associated [74, 75]. Further analysis of our findings showed that PAI-1 levels were significantly dependent on the interval between onset of petechiae and blood sampling and survival. Of interest, PAI-1 concentrations in this study were significantly higher in nonsurvivors at a similar TNF-α concentration. We questioned whether interindividual differences in responsiveness to TNF-α, for example, may contribute to outcome in patients with MSS. The

presence of a single base pair insertion/deletion (allele frequency 0.53/0.47) polymorphism in the promotor of the PAI-1 gene has been associated with differences in release of PAI-1 in postoperative patients [76] and in patients with an increased risk of recurrent myocardial infarction [77].

The promotor containing the deletion allele produced six times more mRNA than the insertion allele in response to IL-1ß [78]. The insertion/deletion polymorphism in the PAI-1 promotor is of functional importance in the regulation of the expression of the PAI-1 gene [78]. These data support the hypothesis that individuals homozygous for the del allele may respond with increased PAI-1 expression in the acute phase of MSS. The possible presence of PAI-1 gene polymorphism in patients with MSS is strengthened by the relatively low PAI-1 response (945 ng/mL) in 1 patient with an extremely high TNF-α level (3130 pg/mL) who survived MSS.

The possible beneficial effects of the HA-1A human monoclonal antibody against endotoxin on the outcome of children with MSS is not yet known. However, a recent study did not find a reduction in the 14-day mortality rate in patients with gram-negative bacteremia and septic shock [79].

We conclude that high levels of proinflammatory cytokines and counterinflammatory compounds are associated with fatality. After the levels of inflammatory mediators are adjusted for time after the onset of petechiae, the differences between survivors and nonsurvivors disappeared. We therefore propose that the outcome in patients with MSS is probably not related to TNF gene polymorphism. However, the increased PAI-1 response to, for example, TNF-α in the fatal cases suggests the presence of polymorphism in the expression of the PAI-1 gene that may contribute to the outcome of MSS.

10.6 Acknowledgements

We thank Miranda R.M. Baert, Atie Bruggeling, C. Erik Hack, Hedda Koning, Astrid E.C.M. Vredendaal, Henriëtte M. van Vuuren, Alma M.A. van Zantwijk for advice and technical support.

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Chapter 11

The 4G/5G Polymorphism in the Promoter of the Plasminogen Activator Inhibitor-1 Gene is associated with the Development of Meningococcal Sepsis

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In preparation

11.1 ABSTRACT

Meningococcal sepsis is a life-threatening disease characterized by diffuse intravascular coagulation. Endotoxin and cytokine induced production of plasminogen activator inhibitor (PAI)-1 is one of the major causes of deleterious thrombosis in children with meningococcal sepsis. Previously high plasma levels of PAI-1 were associated with an increased mortality. A 4G/5G genetic polymorphism in the promoter of the PAI-1 gene has been associated with differences in plasma PAI-1 activity. The 4G allele is associated with higher basal production of PAI-1 and an increased responsiveness to stimulation with cytokines and endotoxin. We therefore questioned whether the high production of PAI-1 in children with meningococcal sepsis was associated with this polymorphism. To this purpose DNA samples of 37 Dutch children who suffered from meningococcal sepsis were analyzed by PCR and compared with DNA samples from control subjects. The genotype was determined by allele specific oligo-melting. The results of this study show that the 5G/5G genotype is significantly less present in children with meningococcal sepsis in comparison with control subjects. We propose that children with meningococcemia and a 4G/4G or 4G/5G genotype have a significantly increased production of PAI-1 leading to more severe disease than in children with a 5G/5G genotype.

11.2 INTRODUCTION

Meningococcal sepsis is a life-threatening entity in which the systemic inflammatory response on micro-organisms or their products - in particular endotoxins - results in the release of inflammatory mediators, complement activation, abnormalities of coagulation and fibrinolysis, endothelial cell damage, capillary leakage, myocardial dysfunction, and finally multiorgan failure frequently followed by death [1]. Endothelial cell secretion of tissue-type plasminogen activator (t-PA) and plasminogen activator inhibitor (PAI)-1, which respectively activate and inhibit fibrinolysis, are principal determinants of the procoagulant state in children with meningococcal septic shock. t-PA is directly involved in the conversion of plasminogen to plasmin, an active protease which degrades and removes fibrin deposits [2]. PAI-1 is a 50-kDa glycoprotein, which inhibits t-PA [3]. PAI-1 is in vitro produced by several cell types including endothelial cells, hepatocytes, platelets, and smooth muscle cells.

Previously, we and others found significantly increased plasma levels of PAI-1 antigen in children with meningococcal septic shock [4, 5]. High initial plasma levels of PAI-1 are associated with an increased mortality [4]. Serum levels of tumor necrosis factor (TNF)-α, which is in vitro one of the stimulators of PAI-1 production [6] are also significantly higher in non-survivors versus survivors [4]. We simultaneously detected an association between the duration of skin lesions and serum levels of both PAI-1 and TNF-α. Differences in TNF-α levels between survivors and nonsurvivors disappeared after adjustment for the duration of skin lesions. However, these differences were after adjustment still statistically significant for PAI-1. Furthermore, PAI-1 concentrations were significantly higher in nonsurvivors at a similar TNF-α concentration [4]. This suggests that differences in the innate PAI-1 response to TNF-α may play a role in the outcome of disease.

Recently, a common polymorphism in the promoter of the PAI-1 gene has been associated with an increased risk for myocardial infarction and coronary artery disease in adults with non-insulin-dependent diabetes mellitus [7-10]. This increased risk is probably caused by an allele-specific increase in the transcription of the PAI-1 gene, resulting in a significantly higher production of PAI-1 antigen especially after stimulation with cytokines such e.g. TGFß, IL-1, and TNF-α [7, 11].

We questioned on the basis of our clinical and laboratory observations whether the 4G/5G polymorphism in the PAI-1 gene promoter could be associated with an increased risk for meningococcal sepsis. To this purpose, DNA samples from 37 Dutch children who suffered from meningococcal sepsis were analyzed.

11.3 MATERIALS AND METHODS

Patients and controls.

A total of 37 children with meningococcal sepsis admitted to the pediatric intensive care unit (PICU) of the Sophia's Children Hospital were included in this study. The control population was a group of 137 infants between 3 months and 1 year who participated in a study to evaluate the immunogenicity of a combined vaccine against diphtheria, pertussis, tetanus, poliomyelitis and *Haemophilus influenzae*. Children between 3 months and 18 years with septic shock and petechiae or purpura were prospectively recruited between January 1992 and December 1995. Meningococcal infection was documented by a positive culture of blood, CSF or skin biopsy specimen or by serological tests. Patients were eligible for inclusion if they met the following criteria: The

presence of shock (systolic blood pressure < 75, < 80, < 85, or < 100 mm Hg in children ages 3 months to 1 year or 1-5, 6-12, and > 12 years old, respectively). Children were also included when poor end-organ perfusion was present (defined as occurrence of at least two of the following criteria): (1) unexplained metabolic acidosis (pH ≤ 7.3), base excess -5 mmol/L or lower, or arterial plasma lactate levels > 2.0 mmol/L; (2) arterial hypoxia (PO_2 ≤ 75 mm Hg, PO_2-to-FiO_2 ratio < 250, or TcO_2 ≤ 96% in patients without overt cardiopulmonary disease); (3) acute renal failure (oliguria with urine output < 0.5 mL/kg/h for > 1 h despite acute volume loading or evidence of adequate intravascular volume and without preexistent renal disease); or (4) sudden deterioration of the patient's mental status.

On admission to the pediatric intensive care unit, the severity of illness was assessed using the pediatric risk for mortality (PRISM) score, a severity-of-illness index [12].

Genetic analysis of the PAI-1 promoter polymorphism.

DNA was prepared from serum or blood using a standard small-scale isolation technique. An 890-bp region of the PAI-1 promoter from each subject was amplified using the polymerase chain reaction (PCR), and the genotype was determined by allele-specific oligo (ASO) melting, as described by Dawson and coworkers [11]. Controls for ASO melting consisted of DNA samples of known genotype determined by DNA sequence analysis.

Statistical analysis.

A X^2 test was used to compare the observed numbers of each PAI-1 genotype with those expected for a population in Hardy-Weinberg equilibrium. Allele frequency was estimated by gene counting and X^2 analysis.

11.4 Results

DNA samples of 37 patients with meningococcal septic shock (21 boys, 16 girls) were analyzed. The median age of the patients was 4.1 years (range, 0.5-17.9 years). The PRISM scores at PICU admission ranged from 1 to 25. The mortality rate of this group of patients was 30%. The results of the genetic analysis of patients and control subjects are presented in the Table. A section of the ASO melting autoradiograph is shown in Figure 1. The PAI-1 4G/5G polymorphism was in Hardy-Weinberg equilibrium in the healthy controls. The genotype distribution was significantly different between patients and controls (P = .03). However, differences in genotypic distribution between

survivors and nonsurvivors were not found. The risk of meningococcal sepsis was significantly higher in patients homozygous or heterozygous for the 4G allele in comparison with those homozygous for the 5G allele.

Table. Distribution of 4G/5G genotypes in children with meningococcal sepsis and controls.

	Patients (n = 37)		Controls (n = 137)
No. (%) with 5G/5G genotype	2 (5)		35 (26)
No. (%) with 4G/5G genotype	24 (65)		70 (51)
No. (%) with 4G/4G genotype	11 (30)		32 (23)
Relative risk		6.0 (1.4 - 26.3)	
Frequency of 5G allele	0.38		0.49
Frequency of 4G allele	0.62		0.51

The relative risk of meningococcal sepsis between patients groups and controls with the 5G/5G genotype and subjects with the 4G/5G and 5G/5G genotype was calculated. The 95% confidence interval is shown in parentheses. The allele frequency was determined by gene counting. A comparison between patients with meningococcal sepsis and controls revealed a difference in genotypic distribution (P = .03) and in allele frequency (P = .04).

11.5 Discussion

PAI-1 is a 50-kDa plasma protein involved in the inhibition of fibrinolysis. The production of PAI-1 is stimulated by TNF-α, IL-1, TGFß and endotoxin [6]. These proinflammatory molecules are released at high levels in patients with meningococcal sepsis leading to activation of coagulation, inhibition of fibrinolysis, the development of a hypercoagulable state, fibrin deposition, and microthrombi [13]. These form the basis for the extensive endothelial damage and multiple organ failure and ultimately death in children and adults with meningococcal sepsis [14]. One could infer from these findings that a higher production of PAI-1 in patients with meningococcemia might contribute towards more serious disease. The results of the current study clearly indicate that polymorphism in the promoter of the PAI-1 gene is associated with the development of meningococcal sepsis. We found that the distribution of PAI-1 polymorphisms was in Hardy-Weinberg equilibrium in control subjects. In contrast, patients with meningococcal sepsis had a

significantly decreased 5G allele frequency, whereas the 5G/5G genotype was significantly underrepresented. Previously Dawson et al. have shown by in vitro experiments that the presence of a 4G/5G polymorphism is associated with a significantly higher mRNA production of PAI-1 in response to interleukin (IL)-1 [11]. They suggested that individuals homozygous for the 4G allele might have a different response to acute phase proteins [11]. Several investigators subsequently reported that the 4G/5G genetic polymorphism in the promoter of the PAI-1 gene is associated with differences in PAI-1 activity in adults with non-insulin-dependent diabetes mellitus, coronary artery disease and myocardial infarction [7-10]. Ye et al. reported an association between homozygosity for the 4G allele and elevated PAI-1 plasma levels and impaired fibrinolysis, although they did not find an increased risk for myocardial infarction in this study [15]. The increased PAI-1 levels in patients with one or two 4G alleles versus those homozygous for the 5G allele are attributed to the absence of a DNA binding site for a repressor protein on the 4G allele [7]. This results in an increased basal expression of PAI-1 transcription.

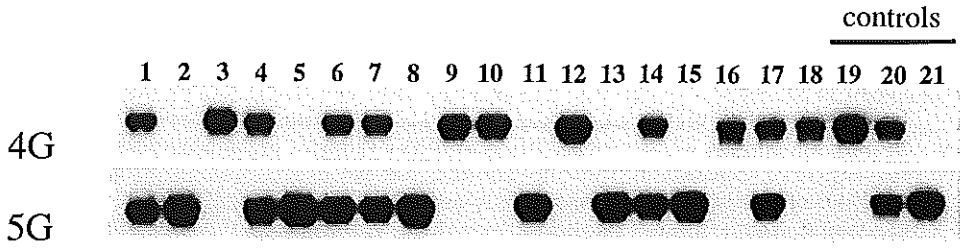

Figure. ASO melting autoradiograph demonstrating the 4G/5G polymorphism in 18 healthy controls (lanes 1 to 18). 4G: the 4G allele; 5G: the 5G allele. The control DNAs homozygous 4G (lane 19), heterozygous 4G/5G (lane 20) and homozygous 5G (lane 21) are also included

We propose that patients with meningococcemia and a 4G/4G or 4G/5G genotype have a significantly increased production of PAI-1 leading to more severe disease than in children with a 5G/5G genotype. Alternatively, children with a 5G/5G genotype may develop milder disease or acquire meningitis. We previously showed that PAI-1 plasma levels were significantly higher in nonsurvivors in comparison with survivors of meningococcal sepsis [4]. This raises the question whether 4G/5G polymorphisms of the PAI-1 gene is also associated with mortality. However, analysis of differences between survivors and nonsurvivors yielded negative results.

Previously Bredius et al. reported that a genetic polymorphism in the Fcγ receptor IIa on phagocytes contributes to the susceptibility for meningococcal sepsis [16]. Westendorp et al. recently suggested that interindividual differences in the release of tumor necrosis factor may also influence to the outcome of meningococcal disease [17]. Our data and those of previous investigators clearly underscore the need for further studies to unravel the complex genetic factors involved in the host response against and the outcome of patients with meningococcal disease.

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Chapter 12

Interleukin-12 Levels during the Initial Phase of Septic Shock with Purpura in Children: Relation to Severity of Disease

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Submitted for publication

12.1 ABSTRACT

Plasma levels of interleukin (IL)-12, a recently described cytokine consisting of two different polypeptide subunits (p40 and p35), were measured together with interferon (IFN)-γ and other cytokines in 46 children with septic shock and purpura. The median (range) plasma IL-12 p40 level on admission was 457 (244-2677) pg/mL in non-survivors versus 189 (< 40-521) pg/mL in survivors (P < .001). IL-12 p70 levels were elevated in only 9 patients. IL-12 p40 plasma levels were positively correlated with tumor necrosis factor (TNF)-α, IL-6, IL-8, IL-10 and PRISM-score, whereas they were negatively correlated with CRP, WBC and serum glucose levels. Twelve (29%) of the patients had detectable levels of IFN-γ. Thus, circulating levels of IL-12 p40 and to a lesser extent those of IL-12 p70, are elevated in children with septic shock and purpura, and correlate with severity of disease and outcome.

12.2 INTRODUCTION

Septic shock with purpura is a clinical syndrome predominantly caused by *N. meningitidis* and characterized by a sudden onset and rapid progression of disease. Children younger than 10 years are most frequently affected. Lipopolysaccharides (LPS) released from gram-negative bacteria such as meningococci initiate the production of pro-inflammatory cytokines by cells of the mononuclear-macrophage lineage and endothelial cells. Circulating levels of these cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-1 [1], IL-6 [1, 2], IL-8 [3], and IL-10 [4, 5], are increased in children with septic shock and purpura. Severity of disease is related to the initial plasma levels of LPS [6] and of these cytokines.

Interleukin-12 is a recently described cytokine [7, 8] initially called natural killer cell stimulatory factor or cytotoxic lymphocyte maturation factor. Among cytokines, IL-12 is unique in that it is a heterodimeric protein composed of two different polypeptide subunits, p40 and p35 (for review: [8-14]). The precise role of IL-12 in vivo is not known, although this cytokine seems to play a key role in the differentiation of Th1 cells [10, 15], and in the host defense against bacterial, parasitic and viral infections [8]. IL-12 also induces the production of interferon (IFN)-γ by T-cells and natural killer (NK)-cells [16, 17]. The plasma levels of IFN-γ are increased in experimental models for sepsis [18-20] as well as in human sepsis [21, 22], although not consistently [1]. Recently, IL-12 was

characterized as a major cytokine in the pathogenesis of gram-negative endotoxemia in mice [16] and in primates [23]. We therefore questioned whether IL-12 and IFN-γ play a role in the pathogenesis of septic shock and purpura in humans. To this purpose initial plasma levels of IL-12 and IFN-γ were measured in children with this disease and their relation with outcome and severity of disease were studied. In addition, plasma levels of TNF-α, IL-6, IL-8, and IL-10 were determined and the possible correlation between these cytokines and IL-12 and IFN-γ, respectively, was studied.

12.3 PATIENTS AND METHODS

Study protocol

Children above 3 months and below 18 years of age with septic shock and petecchiae/purpura were enrolled in this study. Primary or secondary referrals were admitted to the pediatric intensive care unit (PICU) of the Sophia Children's Hospital between April 1991 and October 1994 Patients were eligible for inclusion when they met the following criteria: 1. presence of petecchiae/purpura for less than 12 hours; 2. presence of shock defined as sustained 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) requiring intensive care treatment, 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 mmol/L); b. arterial hypoxia (PaO_2 ≤ 75 mm Hg, a PaO_2/FiO_2 ratio < 250, or a transcutaneous SaO_2 ≤ 0.96) in patients without overt cardiopulmonary disease; c. acute renal failure (diuresis < 0.5 mL/kg/hr for at least one hour despite acute volume-loading or evidence of adequate intravascular volume) without preexisting renal disease; or d. sudden deterioration of the baseline mental status. The pediatric risk of mortality (PRISM) score [24] was calculated using the most abnormal value of each variable recorded during the first 4 hours after admission at the PICU. All patients received maximal supportive therapy: antibiotics, volume suppletion, inotropic support, and mechanical ventilation. Informed consent was obtained from the parents or legal representatives. The Medical Ethics Committee of the University Hospital Rotterdam approved the study protocol.

Collection of blood

On admission arterial blood was collected within two hours. Blood for cytokine analysis was collected in vials containing 3.8% trisodium citrate, immediately chilled on ice, and centrifuged at 2800 g for 15 minutes and then at 45,000 g for 30 minutes at +4°C. Plasma was stored at -70°C until tests were performed.

Assays

White blood cell (WBC) count, as well as lactate, glucose and C-reactive protein (CRP) levels were determined routinely. WBC counts were determined using a flow cytometer (Technicon H1-system, Technicon Instruments, N.Y.). Lactate was measured by enzymatic endpoint determination. CRP by a nephelometric assay [25].

Plasma levels of TNF-α, IL-6, IL-8, IL-10, and IFN-γ were measured with enzyme-linked immunosorbent assays obtained from the Department of Immune Reagents (Central Laboratory of the Bloodtransfusion service CLB, Amsterdam) and were performed according to manufacturers' instructions. Normal levels (detection limit, taking the dilution of samples into account) for these assays are: < 5 (1 pg/mL) for TNF-α; < 10 (1 pg/mL) for IL-6; < 20 (4 pg/mL) for IL-8; < 30 (30 pg/mL) for IL-10; < 10 (2 pg/mL) for IFN-γ.

Assays of IL-12

IL-12 p40 antigen was measured with an ELISA [26]. Briefly, mAbC11.79 and biotinylated mAbC8.6, both directed against the IL-12 p40 subunit [27], were used as coating and detecting antibodies, respectively. Streptavidin polymerized horseradish peroxidase (poly-HRP; CLB, Amsterdam, The Netherlands) was used to quantify bound antigen. Recombinant human p40 was used as a standard. Taking the dilution of tested samples into account, the lower limit of detection was 20 pg/mL. Normal values in 21 healthy adults were ≤ 160 pg/mL. We measured the IL-12 p40 levels in 5 normal children age: 43 months (36-48), the median value was: 28 pg/mL. As levels of IL-12 p40 were similar in children and adults (and healthy children of young age are difficult to obtain blood from), we used both groups together as control for the septic children.

IL-12 p70 antigen was measured using a newly developed ELISA [23]. Shortly, mAb20C2, which has relative specificity for the IL-12 p70 heterodimer [27], and mAb C8.6 were used as a capture and detecting antibodies, respectively. The ELISA did not measure recombinant human p40 unless concentrations > 20 ng/mL were tested. In contrast, recombinant human p70, which was used as a standard, could be detected at

concentrations as low as 0.25 pg/mL. To avoid cross reaction with the p40 chain, plasma samples were analyzed at least at a tenfold dilution. Therefore, the actual detection limit was 2.5 pg/mL. Normal values are below this detection limit.

Statistical analysis

Results are expressed as medians (range) unless otherwise specified. Differences between groups were tested with the Mann-Whitney test or Fisher's Exact test in case of percentages. Correlation coefficients given are Spearman's. Two-tailed P values $\leq .05$ were considered statistically significant.

12.4 RESULTS

Patients

Forty-six patients admitted to the PICU were enrolled in the study: 29 males (63%) and 17 females (37%). The median age was 3.4 years (range 0.5-17.9). Cultures of blood, cerebrospinal fluid or skin biopsies revealed *Neisseria meningitidis* in 40 patients and *Haemophilus influenzae* in 1 patient. Cultures were sterile in 5 patients. Thirty-one (67%) patients needed mechanical ventilation. Forty-four of the children participated in a randomized, placebo controlled trial to study the efficacy of a human monoclonal antibody against endotoxin, HA-1A (Centoxin®, Centocor, Malvern, PA, USA), in meningococcal septic shock. HA-1A or placebo were administered after blood was collected for the determination of cytokines and other laboratory parameters. Twenty four of these patients were treated with a placebo, 17 patients survived (71%) 7 patients died (29%). The results of only these 24 patients were used for outcome analysis.

Clinical and laboratory parameters

Clinical and laboratory parameters obtained on admission (PRISM-score, arterial lactate, WBC count, serum levels of glucose and CRP) for the total group and separately for survivors and non-survivors of the placebo group, are indicated in Table 1. As expected, all parameters were significantly associated with outcome.

Table 1. Clinical and laboratory parameters on admission of children with septic shock with purpura and their relation with outcome.

	Total group (n = 46)	Survivors* (n = 17)	Nonsurvivors* (n = 7)	P
Age (years)	3.8 (0.5-17.9)	4.9 (0.5-17.9)	2.2 (1.4-12.3)	.259
Male / female	29/17	9/8	3/4	.653
PRISM (score)	13 (1-38)	9 (1-20)	21 (17-25)	<.001
Lactate (mmol/L)	5.0 (1.1-20.0)	4.2 (1.1-15.5)	7.2 (4.0-20.0)	.047
WBC (x10^9/L)	9.2 (1.3-44.4)	17.0 (6.1-44.4)	4.9 (1.3-8.2)	<.001
Glucose (mmol/L)	5.6 (1.0-14.2)	8.4 (1.9-14.2)	2.8 (1.0-10.1)	NS
CRP (mg/L)	110 (34-250)	167 (39-250)	70 (38-162)	.002

All data shown are median (range); *data shown were obtained in patients that did not receive mAb; P-value for the difference between survivors and non-suvivors (Mann-Whitney test). Abbreviations: PRISM, pediatric risk of mortality; WBC, white blood cell; CRP, C-reactive protein; NS, not significant.

IL-12 p40 and p70 levels on admission

Levels of IL-12 p40 in surviving (and also in non-surviving) patients were significantly higher than in the controls (P < .001).The median (range) plasma IL-12 p40 level on admission (Figure 1) was 457 (244-2677) pg/mL in non-survivors versus 189 (40-521) pg/mL in survivors (P < .001). In contrast, IL-12 p70 was elevated in only 9 patients. The median level of IL-12 p40 for those patients with detectable IL-12 p70 levels (n = 9) was significantly higher (P = .007) in comparison with those without detectable levels of IL-12 p70 (n = 32): 457 (76-2677) and 207 (40-1007), respectively. The ratio (p40/p70) in the 9 patients with detectable IL-12 p70 levels was 117 (26-203) (Figure 2).

Table 2. Correlation between IL-12 p40 and TNF-α, IL-6, IL-8, or IL-10 on admission of children with septic shock and purpura.

	IL-12 p40 (n = 41)	
	r*	P
TNF-α	.45	.003
IL-6	.56	<.001
IL-8	.60	<.001
IL-10	.51	.001

*Spearman's rank coefficient of correlation. Abbreviations: TNF, tumor necrosis factor; IL, interleukin.

Table 3. Correlation between IL-12 p40 and several clinical or laboratory parameters on admission of children with septic shock and purpura.

	IL-12 p40 (n = 44)	
	r*	P
PRISM	.42	.005
Lactate	.16	.286
WBC	-.56	<.001
Glucose	-.39	.01
CRP	-.33	.028

*Spearman's rank coefficient of correlation. Abbreviations: PRISM, pediatric risk of mortality; WBC, white blood cell; CRP, C-reactive protein.

Relation between IL-12 and other cytokines on admission

IL-12 p40 plasma levels on admission were positively correlated with TNF-α, IL-6, IL-8, and IL-10 (Table 2). The association between IL-12 p70 and the other cytokines was different in comparison with that between IL-12 p40 and the other cytokines. Patients with detectable IL-12 p70 levels had significantly higher levels of IL-8 (P = .042) and IL-12 p40 (P = .007) levels than patients with undetectable levels of IL-12 p70.

Relation of IL-12 to clinical and laboratory parameters

A negative correlation was found between CRP levels or WBC versus plasma IL-12 p40 levels (Table 3). Plasma IL-12 p40 levels correlated positively with the PRISM-score and negatively with serum glucose levels. Patients with detectable levels of IL-12 p70 had significantly lower serum glucose levels (P = .019).

Interferon-γ

Twelve of the 41 (29%) patients had detectable levels of IFN-γ. In those 12 patients, levels of TNF-α, IL-6, IL-8, and IL-10, but not IL-12 p40 were significantly (P < .005) increased in comparison with patients with undetectable levels of IFN-γ. In addition, those 12 patients had significantly (P < .005) lower WBC and a significantly (P < .05) higher serum lactate. From the 9 patients with a detectable level of IL-12 p70, 5 had a detectable level of IFN-γ (56%), while from the 30 patients without detectable levels of IL-12 p70, only 6 had a detectable level of IFN-γ (20%). Due to small numbers this difference just did not reach statistical significance (P = .08).

12.5 DISCUSSION

This is the first report showing that levels of IL-12 p40, and to a lesser extent those of IL-12 p70, are elevated in meningococcal sepsis. Plasma levels of IL-12 p40 were related to outcome and to severity of disease.
IL-12 or NK cell stimulatory factor is a recently described [7] heterodimeric cytokine, which appears to play an important role as a functional bridge between natural resistance and adaptive immune response [12]. During endotoxemia in mice, IL-12, both p40 and p70, was detected shortly after injection of LPS. Bioactive IL-12 circulated in serum before the appearance of IFN-γ. Pretreatment with anti-IL-12 antibodies blocked the production of IFN-γ [16], thus protecting against lethality [17]. Similar findings were reported in a model of a generalized Shwartzmann reaction in mice [28]. In baboons challenged with *Escherichia coli* [23], the systemic release of IL-12 p40 and p70 was also reported. Our study confirms that circulating levels of IL-12 are also increased in human sepsis.
IL-12 p70 was detected in only 9 of the patients and these levels were only slightly increased. In these 9 children IL-8, IL-12 p40 and IFN-γ levels were significantly higher than in patients with undetectable IL-12 p70. Levels of p40 were approximately 100 times higher than those of p70. Such an excess-production of IL-12 p40 was also found in in vitro experiments with human peripheral mononuclear [10, 27] and polymorphonuclear cells [29] as well as in septic baboons [23]. The physiological significance of this excess-production of the free p40 subunit in comparison with the biologically active p70 heterodimer is not clear. It has been suggested that the p40 subunit has a biological activity distinct from that of p70 heterodimer. Mattner et al. have suggested that IL-12 bioactivity is inhibited by free p40 molecules [30]. Studies by Ling et al. [31] revealed that human p40, as described in mice, exists in a monomeric and dimeric form. Again as in mice, the dimeric form was at least 20-fold more effective than the monomer to inhibit the activity of IL-12 or its binding to human IL-12 receptor (IL-12R). However, in contrast to the mouse homodimer, which binds to the mouse IL-12R with similar affinity as heterodimeric mouse IL-12 itself, the receptor binding and bioactivity of the human homodimer were only 10 % of the receptor binding and bioactivity of the human heterodimer. Perhaps the excess production of p40 in relation to the p70 has a regulatory role [32, 1995]. Nevertheless, in vitro an in vivo studies have clearly shown that the production of p40 is linked to that of IL-12 [8, 23], and hence elevated levels of p40 subunit in our patients probably reflected the production of bioactive IL-12. Consistent herewith

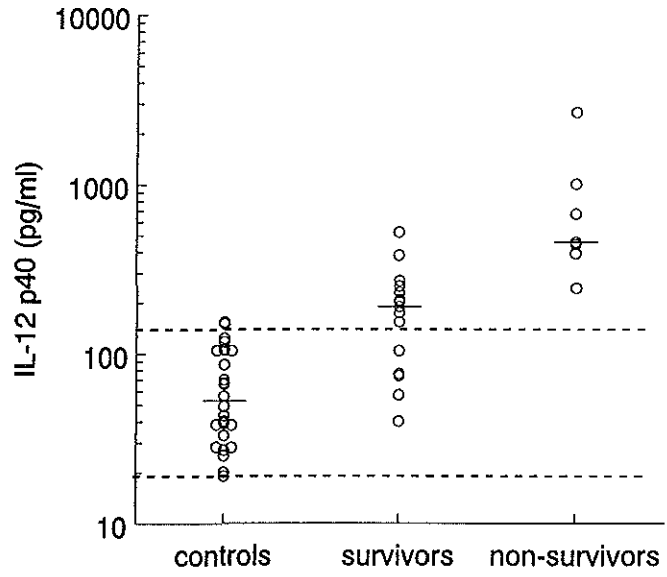

Figure 1. Plasma levels of IL-12 p40 on admission of children with septic shock with purpura (placebo treated, n = 24) and of healthy controls (n = 26). Solid lines represent median values. Dotted lines indicate the range of normal values.

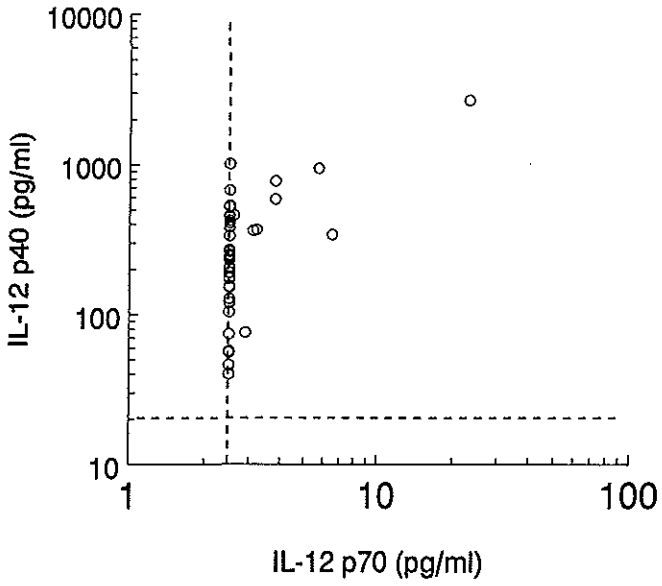

Figure 2. Scattergram of IL-12 p40 versus IL-12 p70. Data represent the levels on admission. Dotted lines represent the lower detection limits.

was the observation that patients with detectable IL-12 p70 levels had higher IL-12 p40 levels than those without detectable IL-12 p70 levels. Apparently the threshold of the IL-12 p70 assay is too high.
The positive correlation between plasma levels of IL-12 p40 or p70, and other proinflammatory cytokines, was not surprising since this probably reflects stimulation of cells by endotoxins. However, plasma levels of IL-10, a counter-inflammatory cytokine, also correlated positively with IL-12. In vitro, IL-10 is a potent inhibitor of LPS-dependent IL-12 production [29, 33, 34]. Moreover, a negative correlation between IL-10 and IL-12 was found in baboons with sepsis suggesting that IL-10 downregulates the release of IL-12 in this sepsis model [23]. Thus, the positive correlation between IL-12 and IL-10 in our patients was in contrast to the findings in baboons. We propose that the synthesis of pro- and anti-inflammatory cytokines is so strongly and continuously stimulated [5, 35] in patients with meningococcal sepsis, that counterregulatory mechanisms are insufficient to suppress excessive production.
IL-12 can induce IFN-γ production by T- and NK-cells in the presence of cofactors as TNF-α or IL-1 β [7]. The role of IFN-γ during in vitro LPS-challenge [33], in vivo endotoxemia in mice [16-20], or the generalized Shwartzmann reaction in mice [28, 36, 37], has well been established. Disseminated intravascular coagulation and shock associated with meningococcal sepsis are considered to be the clinical counterparts of the "classical" generalized Shwartzmann reaction. However, it is not known whether IFN-γ similarly contributes to mortality in human sepsis. IFN-γ levels and outcome were not correlated in adult patients with septic shock [22] and in children with meningococcal septic shock [1]. In contrast, Girardin et al. reported high levels of IFN-γ in children with severe meningococcal septic shock. Their plasma concentrations of IFN-γ were related to severity of the disease and correlated with serum levels of TNF-α [21]. In our study only 12 patients had plasma IFN-γ levels above the detection limit. Those children also had significantly higher levels of other cytokines. The proportion of children that had elevated IL-12 p70 levels was higher, although just not significantly, in the group with detectable IFN-γ, compared to the group with undetectable IFN-γ. A possible explanation for the absence of a relation between IFN-γ and IL-12 p40 is that these cytokines were not released simultaneously, as was observed in animal models for sepsis [23].
The clinical and laboratory parameters in this study are commonly used to assess the severity of disease in patients with meningococcal septic shock. PRISM score is a scoring system to calculate the risk of mortality in pediatric intensive care patients [24]. Serum lactate is related to the degree

of circulatory failure [2]. WBC and CRP are negatively correlated with the fulminant evolution of meningococcal septic shock [38, 39]. Low serum glucose levels are reported by some authors [40], although this finding is not well understood. IL-12 p40 levels in our patients, correlated with all these parameters reflecting severity of the disease, except for serum lactate. IL-12 p70 was only related to low serum glucose. IFN-γ was negatively related to the WBC.

In conclusion, this study is the first to report a systemic release of IL-12 and its relation with outcome, severity of disease and other cytokines, in children with septic shock and purpura. We suggest that new immunomodulatory agents in sepsis should also be studied for their effects on IL-12 production.

12.6 Acknowledgments

The mAbs c11-79 and c8.6 were kindly provided by Dr. G. Trinchieri; mAb20c2 was a generous gift from Dr. M. Gately. Recombinant human IL-12 p40 and p70 were kind gifts from Dr. S.F. Wolf.

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Chapter 13

Age-related Differences in Outcome and Severity of DIC in Children with Septic Shock and Purpura

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Thrombosis and Haemostasis 1996. In press

13.1 ABSTRACT

We studied the influence of age on mortality and severity of clotting abnormalities in 79 children (median age: 3.1 years) with meningococcal sepsis. Parameters of coagulation and fibrinolysis and plasma levels of cytokines were prospectively measured on admission. The mortality rate was 27%. The age of survivors was significantly different from that of non-survivors (P = .013). With the exception of FVII, vWF and t-PA, parameters of coagulation and fibrinolysis, as well as plasma cytokine levels were related to outcome. Patients were divided in two groups: younger and older than median age. The mortality in children ≤ 3.1 years was 40% versus 13% in children > 3.1 years (P = .006). In contrast to cytokine levels, which were not different between the two age groups, fibrinogen, prothrombin, factors V, VII, VIII, vWF, protein C, antithrombin, FDP, and the ratio PAI-1/t-PA were related to age, indicating a more severe coagulopathy in children ≤ 3.1 years despite a similar degree of inflammatory response. A relative deficiency of coagulation factors due to an immature state of the clotting system, as well as an inadequate fibrinolytic response, both related to age may have caused this more severe coagulative response in younger children, and may have contributed to the higher mortality rate.

13.2 INTRODUCTION

Septic shock with purpura is an overwhelming, rapidly progressive and often fatal syndrome. This syndrome is frequently diagnosed in children and adolescents and predominantly caused by *Neisseria meningitidis* and. A key event in the pathophysiology is disseminated intravascular coagulation (DIC), leading to microvascular thrombosis and end-organ damage during the early stage and occasionally to permanent tissue destruction and amputation. Septic purpura associated DIC is characterized by a marked activation of coagulation, consumption of platelets and coagulation proteins, and inhibition of the activated fibrinolytic system [1-5]. Natural anti-coagulants, such as antithrombin (ATIII) and protein C and S play an important role in this process [6-9]. Levels of protein C and S are decreased in septic shock and purpura, which may explain the severe procoagulant state [8, 10-17]. Although consumption of protein C certainly contributes to the decreased levels, immaturity of the protein C system may also play a role. The latter could explain the rapid evolution and the high incidence of

purpura fulminans in young children [16]. We observed the highest mortality in our population in younger patients. We therefore questioned whether higher mortality in young children is associated with more severe clotting abnormalities. To this purpose coagulation and fibrinolysis parameters and plasma levels of tumor necrosis factor (TNF)-α, interleukin (IL)-6, 8, 10 were prospectively measured on admission of children with septic shock and purpura. The relation between these parameters and the outcome and severity of disease was studied.

13.3 PATIENTS AND METHODS

Study protocol

Children above 3 months and below 18 years of age with septic shock and petechiae/purpura were enrolled in this study after informed consent was obtained from their parents or legal representatives. They were admitted to the pediatric intensive care unit (PICU) of the Sophia Children's Hospital between August 1988 and December 1994. Our PICU is the only center for pediatric intensive care in the south-western part of The Netherlands. All children younger than 14 years who need pediatric intensive care are referred to the PICU. Children between 14 and 18 years are occasionally referred to adult intensive care units irrespective of the severity of disease. Patients were eligible for inclusion when they met the following criteria: 1. presence of petechiae/purpura for less than 12 hours; 2. presence of shock defined as sustained 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) requiring intensive care treatment, or evidence of poor end-organ perfusion. The latter was defined when at least two of the following criteria were present: a. unexplained metabolic acidosis (pH ≤ 7.3 or base excess ≤ -5 mmol/L or plasma lactate levels > 2 mmol/L); b. arterial hypoxia (PaO_2 ≤ 75 mm Hg, a PaO_2/FiO_2 ratio < 250, or a transcutaneous SaO_2 ≤ 0.96) in patients without overt cardiopulmonary disease; c. acute renal failure (diuresis < 0.5 mL/kg/hr for at least one hour despite acute volume repletion or evidence of adequate intravascular volume defined by the presence of a palpable liver, a cardiothoracic ratio on chest radiography > 0.4, and a central venous pressure > 5 mmHg) without pre-existing renal disease; or d. sudden deterioration of the baseline mental status. The pediatric risk of mortality (PRISM) score [18] was calculated using the most abnormal value of each variable recorded during the first 4 hours after

admission at the PICU. All patients received maximal supportive therapy: antibiotics, volume-administration, inotropic support, and mechanical ventilation. Disseminated intravascular coagulation (DIC) was defined by the combination of three of the following features: platelet count less than $150x10^9$/L, fibrinogen levels below 2 g/L, factor V less than 60%, or increased levels of fibrin and fibrinogen degradation products (FDP). The study protocol was approved by the Medical Ethics Committee of the University Hospital Rotterdam.

Collection of blood

Within two hours after admission arterial blood samples were collected. Blood for analysis of cytokines and coagulation parameters was collected in vials containing 3.8% trisodium citrate and in CTAD tubes (containing citrate-theophylline-adenosine-dipyridamole). The vials were immediately chilled on ice, and centrifuged at 4000 g for 10 minutes and then at 20,000 g for 30 minutes at +4°C. Plasma was stored at -80°C until tests were performed.

Assays

White blood cell and platelet counts were determined using an automated platelet counter (Technicon H1-system, Technicon Instruments, N.Y.). Lactate was measured by enzymatic end-point determination and CRP by nephelometric assay [19]. Plasma levels of TNF-α, IL-6, IL-8, and IL-10 were measured with enzyme-linked immunosorbent assays obtained from the Department of Immune Reagents (CLB, Amsterdam) and were performed according to manufacturers' instructions. Normal levels (detection limit, taking the dilution of samples in the assays into account) for these assays are: < 5 (1 pg/mL) for TNF-α; < 10 (1 pg/mL) for IL-6; <20 (4 pg/mL) for IL-8; < 30 (30 pg/mL) for IL-10. The clotting assays as well as the chromogenic assays were performed with commercially available reagents and methods from Behring Diagnostica (Behring-Werke A.G., Marburg, Germany). The enzyme-linked-immuno-sorbent (ELISA) determinations were done with reagents from Diagnostica Stago (Asnières sur Seine, France). Results are expressed in % of normal adult values unless indicated otherwise. The activated partial thromboplastin time or coagulation time, surface induced (APTT) was measured with the Neotromtin reagent, in which vegetable phospholipids and ellagic acid as a particulate activator are used. (N 28-40 seconds). Fibrinogen was determined with a modified Clauss method [20] (N > 2 g/L). The activities of coagulation prothrombin, factors VII, V and X were measured by one stage clotting assays, based on the coagulation time, tissue factor-induced,

Table 1. Clinical and laboratory parameters on admission in children with septic shock and purpura in relation with outcome and age.

Parameter	Total group (n = 79)	Survivors (n = 58)	Nonsurvivors (n = 21)	≤ 3.1 years (n = 40)	> 3.1 years (n = 39)
Age (years)	3.1 (0.3-17.9)	4.4 (0.3-17.9)	2.1 (0.4-12.3)*	1.3 (0.3-3.1)	9.0 (3.2-17.9)
Sex (male/female)	47/32	35/23	12/9	26/14	21/18
Need for inotropics	74 (94%)	53 (91%)	21(100%)§	38 (95%)	36 (92%)
Mechanical ventilation	51 (65%)	30 (52%)	21 (100%)§	33 (83%)	18 (46%)§
PRISM	14 (1-44)	11 (1-38)	21 (8-44)§	17 (8-44)	8 (1-25)§
DIC	62/77 (81%)	42/57 (74%)	20/20 (100%)†	33/39 (85%)	29/38 (76%)
Lactate (mmol/L)	5.0 (1.1-20.0)	4.2 (1.1-15.5)	7.3 (2.9-20.0)§	5.1 (1.8-16.7)	4.4 (1.1-20.0)
WBC (x10^9/L)	8.1 (1.3-44.4)	12.5 (1.4-44.4)	5.3 (1.3-12.9)§	6.4 (1.3-26.5)	12.9 (1.4-44.4)‡
Glucose (mmol/L)	5.8 (1.0-14.2)	6.8 (1.0-14.2)	3.7 (1.0-10.1)‡	4.9 (1.0-11.4)	6.9 (1.3-14.2)‡
CRP (mg/L)	98 (14-250)	132 (34-250)	65 (14-162)§	80 (14-224)	128 (38-258)*

Values are medians (range) or numbers (%); significance of the difference between groups (Mann-Whitney U-test or Fisher's exact test): *$P \leq .05$, †$P \leq .01$, ‡$P \leq .005$, §$P \leq .001$. Abbreviations: PRISM, pediatric risk of mortality; DIC, disseminated intravascular coagulation; WBC, white blood cells; CRP, C-reactive protein.

using the commercial deficient plasmas. (N 70-140%). Factors VIII and IX were done with one stage assays based on the APTT. The quantitative determination of the Von Willebrand Factor (vWF) was performed with a sandwich type ELISA. (N 80-120%). The same procedure was performed for the amounts of protein C and S antigen. (N 70-120%). The functional protein-C assay was measured by a chromogenic assay with a specific snake venom as the particulate activator (N 70-140%). The antithrombin, the plasminogen as well as the plasmin inhibitor (alpha-2 antiplasmin) activities in plasma were determined in quantitative kinetic tests by a synthetic chromogenic substrate method (N 70-140%). Fibrin + fibrinogen degradation products (FDP) were determined in a semiquantitative assay using latex particles coated with antibodies to FDP (N <5 µg/mL). C4BP amounts were measured in quantitative determinations by electro-immunodiffusion (Laurell rocket technique). (N 68-140%, ±250 µg/mL). Plasminogen activator inhibitor (PAI)-1 antigen was determined using a

sandwich-type radioimmunoassay previously described [21] and expressed as ng/mL. Thrombin-antithrombin complexes (TAT), plasmin-plasmin inhibitor complexes (plasmin-α2-antiplasmin complexes) (PAP), and plasminogen activator, tissue type (tissue-type plasminogen activator) (t-PA) were measured with ELISA's (TAT, t-PA) or radioimmunoassay (PAP) previously described [22]. Results were expressed as nmol/L (PAP), µg/L (TAT) or ng/mL (t-PA).

Statistical analysis

Results are expressed as medians (range) unless otherwise specified. Differences between groups were tested with the Mann-Whitney test or Fisher's Exact test in case of percentages. Correlation coefficients given are Spearman's. Two-tailed *P*-values ≤ .05 were considered as statistically significant.

13.4 RESULTS

Demographics.

Seventy-nine patients fulfilling the entry criteria and admitted to the PICU were enrolled in the study. Forty-seven were males (60%), 32 females (40%) (Table 1). The median age was 3.1 years (range 0.3-17.9). The patients were divided into two groups according to the median age, i.e. 3.1 years or younger, or above 3.1 years. Fifty-eight of the 79 patients (73%) survived and 21 (27%) died. The mortality in children below 3.1 years was 40 %, which was significantly (P = .006) higher than in patients above 3.1 years (13%). Sixteen of the 21 patients who died were younger than 3.1 years; five were older than 3.1 years. In each group one patient died due to brain edema leading to herniation and brain death. In the other 19 patients the principal cause of death was circulatory collapse. Cultures of blood, cerebrospinal fluid or skin biopsies revealed *Neisseria meningitidis* in 67 (85%) patients and *Haemophilus influenzae* in 2 patients. Cultures were sterile in 10 patients. Fifty-one (65%) patients needed mechanical ventilation. For practical reasons, i.e lack of sufficient plasma sample size, coagulation parameters were not determined on admission in 3 patients. Fifty-two of the 79 children participated in a randomized, placebo controlled trial to study the efficacy of a human monoclonal antibody, HA-1A (Centoxin®, Centocor, Malvern, PA, USA), in meningococcal septic shock. HA-1-A or placebo were administered after blood was collected for the determination of cytokines or coagulation parameters.

Severity parameters

Clinical and laboratory parameters obtained on admission (PRISM-score, presence of DIC, need for mechanical ventilation, need for inotropic support for more than 24 hours, arterial lactate, WBC, serum levels of glucose and CRP) are indicated in Table 1 after stratification according to outcome (survivors versus non-survivors) and age (younger or older than 3.1 years). The ages of survivors and non-survivors were significantly (P = .013) different: the median age was about 2 years higher for the survivors. All clinical and laboratory parameters, with the exception of lactate and need for inotropic support, were significantly associated with outcome and age. Inotropic support longer than 24 hours was necessary in 74 of the 79 patients. In only two of the 40 children < 3.1 years (5%), and 3 of the 39 children > 3.1 years (7.7%) inotropic support was not started or could be discontinued within 24 hours. These five children with a relatively rapid recovery still needed 36 mL/kg (range: 32-50 mL/kg) plasma to normalize their blood pressure. Four of the five children had an increased arterial lactate level (4.2 mmol/L; range: 1.1-5.5). There was no significant age related difference in duration of inotropic support in the survivors.

Cytokines

Cytokine levels were measured in 49 patients. The median plasma levels of TNF-α, IL-6, IL-8, and IL-10 were significantly higher in non-survivors as compared to survivors (Table 2). These parameters did not significantly correlate with age.

Table 2. Cytokines on admission in children with septic shock and purpura in relation with outcome and age.

Cytokine	Survivors (n = 35)	Nonsurvivors (n = 14)	≤ 3.1 years (n = 22)	> 3.1 years (n = 27)
TNF-α (pg/mL)	7 (2-165)	32 (1-272)*	14 (1-79)	8 (2-272)
IL-6 (pg/mL)	15,200 (36-1.5x10^6)	280,000 (87-2.0x10^6)†	66,000 (36-2.0x10^6)	15,000 (87-1.5x10^6)
IL-8 (pg/mL)	1100 (13-0.38x10^6)	88,000 (23-0.37x10^6)‡	6500 (13-0.37x10^6)	1100 (23-0.38x10^6)
IL-10 (pg/mL)	2260 (41-0.11x10^6)	19,000 (30-0.10x10^6)*	7200 (41-0.10x10^6)	2260 (30-0.11x10^6)

Values are medians (range); significance of the difference between groups (Mann-Whitney U-test): *P ≤ .05, †P ≤ .005, ‡P ≤ .001. Abbreviations: TNF, tumor necrosis factor; IL, interleukin.

Coagulation parameters

The relation between coagulation parameters and outcome or age is indicated in Table 3. The median number of platelets was significantly decreased in non-survivors compared with survivors. The APTT was markedly increased in non-survivors versus survivors, and in patients below versus above 3.1 years. Fibrinogen, prothrombin and plasma coagulation factors (V, VII, VIII, IX, and X) were all decreased. Factors VII and X levels were not significantly different between children above and below 3.1 years and between survivors and non-survivors, respectively. Levels of factor IX and TAT-complexes were only different between survivors and non-survivors. The plasma level of Von Willebrand factor (vWF) was significantly lower in patients below 3.1 years, but in both groups very high compares to normal values.

The results of anti-coagulant and fibrinolytic tests are summarized in Table 4. Protein C was markedly decreased and serum levels were significantly related to outcome and age. Plasma levels of antithrombin, plasmin inhibitor, and FDP were also related to outcome and age. In addition PAI-1 levels tended to be higher in children below 3.1 years than in children above 3.1 years. The ratio between PAI-1 and t-PA was significantly different between survivors and non-survivors, but also between younger and older children. When survivors were analyzed separately, this ratio was still significantly (P = .011) different between children above and below 3.1 years.

Relationship of cytokines to coagulation parameters

APTT, fibrinogen, FVIII, TAT, PAP, t-PA, PAI-1, as well as the PAI-1/t-PA ratio correlated well with the levels of most of the cytokines. A weak, positive correlation was found between FVII and the cytokine plasma levels and an inverse relation between cytokine levels and C4BP. Correlation coefficients were estimated separately for the patients younger and older than 3.1 years. Differences between the correlation patterns for both groups were: the strong negative correlations between fibrinogen, FVIII, or protein C levels and cytokine levels in the younger age groups, which correlations were weaker or absent in the older age group; the strong positive correlation in the younger age group between APTT and plasma cytokine levels, which was not found in the older age group; finally, PAI-1 in both groups correlated with cytokines, yet the production of t-PA in the younger age group was not related to plasma cytokine levels, whereas in the older age group a strong, positive correlation between these parameters was found.

Table 3. Coagulation parameters in children with septic shock and purpura in relation to outcome and age.

Coagulation parameter	Survivors (n = 51)	Nonsurvivors (n = 16)	≤ 3.1 Years (n = 30)	> 3.1 Years (n = 37)	Normal values
platelets (x10^9/L)	106 (15-214)	53 (14-141)‡	85 (15-214)	101 (14-189)	150-350
APTT (s)	52 (29-200)	103 (56-220)‡	68 (33-220)	51 (29-200)‡	28-40
Fibrinogen (g/L)	2.6 (0.3-5.8)	1.1 (0.2-2.5)‡	1.7 (0.2-5.3)	2.7 (0.2-5.8)‡	1.8-3.5
Prothrombin (%)	51 (24-82)	41 (11-57)‡	43 (12-79)	55 (11-82)*	70-120
FV (%)	40 (5-116)	16 (2-48)‡	25 (2-116)	38 (3-77)*	70-120
FVII (%)	19 (3-41)	21 (5-41)	25 (5-41)	18 (3-34)*	70-120
FVIII (%)	84 (3-286)	22 (2-62)‡	42 (4-133)	87 (2-286)‡	70-150
vWF (%)	422 (200-711)	394 (227-629)	376 (227-570)	454 (200-711)*	80-120
vWF/FVIII	5.0 (1.9-147)	17.5 (5.7-264)‡	8.6 (2.4-30.6)	5.2 (1.9-264)*	1
FIX (%)	50 (16-118)	40 (22-86)‡	48 (16-86)	47 (23-118)	70-120
FX (%)	50 (27-98)	44 (17-60)*	49 (21-90)	52 (17-98)	70-120
TAT (ng/mL)	14 (3-455)	175 (12-811)‡	40 (3-467)	14 (3-811)	< 4

All data shown are medians (range); significance of the difference between groups (Mann-Whitney U-test): *$P \leq .05$, ‡$P \leq .005$, ‡$P \leq .001$. Abbreviations: APTT, activated partial thromboplastin time; F, factor; vWF, Von Willebrand Factor; TAT, thrombin-antithrombin complexes.

13.5 Discussion

This study in 79 children confirms that disturbances in the coagulation system are associated with outcome and severity of the disease in children with severe septic shock and purpura. The coagulation profile in these children is characterized by excessive activation resulting in massive consumption of platelets and coagulation factors. These abnormalities in

Table 4. Anti-coagulation and fibrinolysis parameters in children with septic shock and purpura in relation to outcome and age

Anti-Coag/Fibr parameter	Survivors (n = 51)	Nonsurvivors (n = 16)	≤ 3.1 Years (n = 30)	> 3.1 Years (n = 37)	Normal values
Protein C act (%)	23 (5-58)	14 (5-32)&	19 (5-47)	23 (6-58)*	70-120
Protein C ag (%)	22 (3-63)	13 (5-36)†	16 (3-49)	22 (12-63)	70-120
Protein S ag (%)	63 (21-112)	44 (27-80)§	48 (21-112)	62 (30-92)	70-120
C4BP (%)	72 (33-108)	53 (24-89)*	61 (24-100)	73 (32-108)	70-140
Antithrombin (%)	65 (29-93)	56 (25-68)§	58 (29-93)	68 (25-88)*	70-140
Plasminogen (%)	62 (25-94)	52 (27-72)*	56 (25-94)	62 (37-93)	70-140
Plasmin inhibitor (%)	64 (30-108)	51 (12-75)§	55 (29-103)	65 (12-108)*	70-140
FDP (µg/mL)	63 (5-325)	125 (70-325)§	110 (5-325)	50 (5-275)&	<5
PAP (nmol/L)	14 (3-56)	23 (4-54)†	17 (3-52)	14 (3-56)	<7
t-PA (ng/mL)	24 (5-79)	27 (7-77)	19 (5-58)	26 (8-79)	<10
PAI (ng/mL)	670 (92-3268)	2679 (828-4578)§	1409 (92-4351)	617 (126-4578)	<30
PAI/t-PA	26 (6-206)	90 (28-298)§	82 (6-298)	26 (6-205) §	<3

All data shown are medians (range); significance of the difference between groups (Mann-Whitney test): *$P \leq .05$, †$P \leq .01$, ‡$P \leq .005$, §$P \leq .001$. Abbreviations: act, activity; ag, antigen; FDP, fibrin/fibrinogen degradation products; PAP, plasmin-α2-antiplasmin complexes; t-PA, tissue-type plasminogen activator; PAI, plasminogen activator inhibitor.

coagulation result from activation of endothelial and other cells by endotoxins and cytokines, and are the cause for microvascular thrombosis with hemorrhagic necrosis. However, our observation of age-related differences in coagulation parameters suggests that part of the observed abnormalities results from age dependent relative liver insufficiency.

Previously we and others reported that protein C (PC) levels in patients with meningococcal sepsis had decreased to a level at which they are at risk to develop thrombosis [10, 14, 16, 17]. There were no

differences between PC antigen and PC activity. Increased levels of PC inhibitor did, therefore, not explain the decreased PC levels [23]. We propose that decreased PC levels reflect massive consumption. In agreement herewith, administration of activated protein C to baboons with lethal septic shock not only prevented coagulopathy, but also improved outcome [24]. In addition, inhibition of PC activation using a neutralizing antibody resulted in a more severe response even against a sublethal dose of *E. coli*, implying that decreased PC levels in patients with meningococcal sepsis predispose for a more severe disease. Furthermore, PC not only regulates coagulation, but also enhances fibrinolysis by inhibition of PAI [25]. Together, these data suggest that administration of protein C concentrates may have beneficial effects in children with septic shock and purpura.

Activation of coagulation is accompanied by activation of fibrinolysis. In animal experiments [26], studies in volunteers [4], and human septic shock patients [27], elevated levels of tissue plasminogen activator (t-PA) antigen have been demonstrated. In one study these were related to the severity of the disease as reflected by the APACHE score [28]. In our study t-PA levels, though increased, were not associated with outcome and only correlated weakly with serum lactate (r = .45, P =.001) and WBC (r = .31, P < .05). The levels of t-PA did correlate weakly with the TAT complexes (r = .34, P < .05), indicating that the fibrinolytic and coagulative responses may have been linked either because one (fibrinolysis) occurred in response to the other, or because both were induced by the same stimuli. Plasmin inhibitor levels were significantly lower in non-survivors, as was previously reported [2, 29]. This presumably reflects consumption of this anti-fibrinolytic protein, since nearly all patients had increased circulating PAP-complexes. Decreased plasmin inhibitor levels facilitate enhanced fibrinolytic activity. Yet, we believe that overall fibrinolysis was relatively inhibited in our patients due to the release of high amounts of PAI-1 into the circulation. Increased levels of PAI-1 indeed have been shown in experimental sepsis [26], and in human sepsis [2, 27, 29-31] and are associated with a decrease in levels of PAP-complexes [32]. We speculate that the levels of PAP complexes, though increased, presumably reflect an insufficient fibrinolytic response. Increased levels of PAI-1 were strongly related to outcome and, as was previously reported by our group, an increased PAI-1 response to TNF-α may be associated with fatality, probably because of polymorphism of the PAI-1 gene [33].

Mortality of patients with septic shock and purpura is probably related to the severity and extent of coagulation disturbances which lead to micro-thrombi and hemorrhagic necrosis. Mortality rates differ from 10% to 40% depending on patient selection criteria. Additionally 10% of all patients

with meningococcal disease suffer from serious sequelae like amputation, secondary to the coagulopathy. This study shows that mortality was three times higher in patients below three years. Although the maturation of the coagulation system is only partly unraveled, most coagulation factors do not reach adult levels until after the first 6 months of life [34]. Protein C is somewhat unusual since it does not reach adult levels until the fourth year of life [35]. Powars et al. have suggested that infants are more susceptible to severe depletion of protein C. These authors showed a relation between protein C and both age and outcome in children with meningococcal sepsis [16]. We studied the relation between coagulation parameters and age, and did not only find differences in protein C, but also in other parameters of coagulation and fibrinolysis. So, either both patient groups had the same degree of inflammatory stimulus, and the younger patients a more disturbed coagulation, or, the younger patients were more severely ill and for this reason had a more severely disturbed coagulation. Yet, cytokine levels were comparable in both groups indicating that younger and older children had at least comparable inflammatory responses. Thus, apparently a similar inflammatory response yields a more severe coagulative response in the younger children. We suggest that this more severe coagulative response was due to the immature state of their clotting system, in particular of the anticoagulant pathways, resulting in extensive consumption of coagulation factors. Striking was the increased levels of vWF caused by endothelial cell and platelet activation. Because of this activation factor VIII is also increased during sepsis just as vWF [2]. In our patients consumption and extravasation were probably so extreme, that factor VIII levels were much lower in non-suvivors, and in younger patients indicating a higher consumption in these patients. But since we only analyzed some of the cytokine levels and no other fluid phase cascade systems, there remains uncertainty about the degree of intravascular inflammation in the two groups. Plasma levels of PAI-1 of both age groups correlated strongly with cytokine levels, as well as with TAT-complexes (r =.73, $P < .001$; r = .80, $P < .001$). Furthermore, in contrast with the older age group, t-PA levels in the younger age group did not correlate with those of cytokines. Moreover, the correlation between t-PA levels and TAT-complexes, was different for both groups: whereas no correlation was found in the younger age group (r = -.05, P = .81), there was a very strong correlation found in the older age group (r = .80, $P < .001$). This may indicate, that in addition to the relative deficiency of coagulation factors, an inadequate fibrinolytic response may have contributed to the activation of coagulation. This seems to be contradictory to the finding that FDP's were significantly higher in patients $\leq$ 3.1 years vs > 3.1 years and in non-survivors vs survivors.

However, FDP's are a combination of fibrinogen and fibrin degradation products. Fibrinogen was much lower in non-survivors than in survivors. So, fibrinogen consumption was more pronounced in non-survivors. Consistent herewith TAT complexes were much higher in non-survivors, making it likely that more fibrin was formed in this group. Although there was detectable activation of fibrinolysis as estimated by increased FDP's and PAP-complexes, we speculate that this was insufficient to compensate for coagulation activation.

The parameters reflecting severity of disease, PRISM, lactate (higher in non-survivors), and WBC, glucose, and CRP (lower in non-survivors), were also different between the two age groups, the patients younger than 3 years being more severely ill than the older patients. We speculate that this resulted from the increased tendency to develop a more severe coagulopathy. Additionally, lack of immune competence i.e. lack of bactericidal and opsonophagocytic antibodies combined with certain Fc-γ receptor allotypes on specific cells may contribute towards the bad prognosis in infants and young children.

The question remains whether coagulopathy and micro thrombosis contributes to circulatory collapse and mortality. The coagulopathy could be causally related to the circulatory collapse but could also be two separate manifestations of massive endotoxemia. The latter has been shown in baboon experiments. Blocking FXII did ameliorate the hypotensive response but that activation of the coagulation was not influenced [36]. However, the same group established that protein C treatment prevented the coagulopathic as well as the lethal effects [24]. The same combined effect was described for tissue factor pathway inhibitor [37]. Therefore one may argue that FXII is more related to hypotension and that blocking of this system possibly leads to improvement of hypotension without changing the coagulopathy. However, this does not prove that circulatory collapse and coagulopathy are separate manifestations.

So, despite a similar degree of inflammatory response, a more severe coagulopathy was found in children below the age of 3.1 years, consisting of a relative deficiency of coagulation factors, as well as an inadequate fibrinolytic response, both related to age, which may have contributed to the higher mortality observed in younger children with meningococcal sepsis.

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Chapter 14

General Discussion

14.1 INTRODUCTION

The morbidity and mortality in children with bacterial meningitis and sepsis did not decrease during the last decade despite the availability of excellent antibiotics, improvements in intensive care therapy, and application of new immunotherapeutic agents. Recently research in this field has been directed towards efforts to enhance our understanding of the host response to the major pathogens involved in bacterial meningitis and sepsis: *Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Neisseria meningitidis*. These studies which were carried out in animal experimental models and in humans have elucidated the major role of the host inflammatory response in the clinical symptomatology and outcome. Genetically encoded variability in the immune system also exerts major effects on the clinical course and outcome of infectious diseases [1]. Examples are the increased risk for meningococcal infections in patients with complement deficiency, the increased risk in certain children for invasive disease by *H. influenzae* or *S. pneumoniae* due to an impaired antibody response and the association between certain Fcγ receptor allotypes on leukocytes and insufficient phagocytosis of encapsulated microorganisms. The growing knowledge on the virulence factors of bacteria discussed in this thesis also points to an important role of strain specific characteristics in the pathogenesis of disease. It is generally believed that a greater understanding of the host response against sepsis and meningitis and a better characterization of the molecular microbiological characteristics of bacteria involved in these diseases are essential elements for the development of new therapeutic strategies. The aims of this thesis were 1.) to study laboratory parameters, clinical characteristics and outcome of patients with bacterial meningitis and sepsis and 2.) to enhance understanding of the host response against the major bacteria involved in these disease entities.

14.2 THE OUTCOME OF CHILDHOOD BACTERIAL MENINGITIS AND MENINGOCOCCAL SEPSIS

Bacterial meningitis and sepsis are clinical entities with a high morbidity and mortality. Baraff et al. [2] reported a mortality rate of 3.8% in children with meningitis due to *H. influenzae*. The mortality rate in pneumococcal meningitis in children was 15.3% [2]. Meningococcal meningitis, the most common cause of bacterial meningitis in the Western

World, especially in countries with immunization programs against *H. influenzae* type b, is associated with a mortality rate of 7.5% [2]. Unfortunately, laymen and even health professionals sometimes do not clearly understand the difference between meningococcal meningitis and meningococcal sepsis [3]. This confusion is based on the historical idea, that meningococcal meningitis and sepsis are the same illness (cerebrospinal fever). Another issue, which has contributed to this misconception is that bacteremia is present in the majority of patients with meningitis and that cerebrospinal fluid (CSF) cultures yield positive results in a subset of patients with meningococcal septic shock. The real mortality rate for patients with meningococcal meningitis is probably less than 5%. Meningococcal sepsis, in contrast, is associated with a mortality rate up to 50% in patients with severe shock [4-6]. The outcome of these diseases also depends on factors such as age range of the study population, type of microorganism, underlying diseases, primary or secondary referral, and duration of the follow-up period.

We investigated the outcome of children with pneumococcal meningitis and meningococcal septic shock. The overall mortality rate in our population of patients with pneumococcal meningitis, which included a larger number of children than in any other previous report, was 17%. This rate is relatively high in comparison with that in previous studies [2, 7-9]. We attribute this difference to the large number of patients referred by other hospitals. In chapter 3, we report the presence of significant differences in mortality between children with primary and secondary referral for pneumococcal meningitis (7% vs. 34%). The higher mortality rate among secondary referrals could be expected since these patients were more severely ill. A similar observation was made by Bohr et al. [8]. The mortality rate among adult patients is substantially higher than that among children. This is most likely due to the frequent presence of underlying disorders in the elderly. In a recent retrospective study of pneumococcal meningitis in Dutch adults a mortality rate of 33% was reported [10]. The fatal outcome of pneumococcal meningitis in adults is mainly due to the development of cardiorespiratory failure. In contrast, children often die as a result of neurological complications. Clonal relatedness between the pneumococcal isolates was not observed (chapter 4).

In chapter 9 the clinical outcome is described of children with meningococcal septic shock. We found a mortality rate of 25%. In contrast to patients with pneumococcal meningitis, primarily referrals with meningococcal septic shock had a higher mortality than secondary referrals: 44% vs. 20%. The pediatric risk of mortality (PRISM) score of the primary referrals was significantly worse than that of the secondary referrals. The

mortality rate was also higher in the group of children younger than 4 years (16 of 48 [33%] vs. 5 of 39 [13%]; $P = .03$). These findings underscore that epidemiologic data on mortality rate should be interpreted with care since confounding factors such as age and clinical condition at referal may influence them.

Survivors of these serious invasive infections do frequently suffer from sequelae. A meta-analysis of children with bacterial meningitis demonstrated at least one major adverse outcome (spasticity, paresis, deafness, seizures or severe intellectual disability) in 16.2% of these patients. Approximately 30% of children with pneumococcal meningitis has sequelae [2]. Many of these neurological abnormalities do however resolve within one year [11].

Neuropsychological evaluation is not routinely performed during follow-up of children with bacterial meningitis. Only few studies have addressed long-term consequences of bacterial meningitis and whether additional abnormalities of visuomotor coordination, auditory perception, and higher cognitive functions compromise the future learning abilities and development of these children [12-16]. In addition, many of these studies are mainly pertinent to *H. influenzae* type b meningitis and the results cannot be generalized to meningitis due to *N. meningitidis* and *S. pneumoniae*. Because most motor or cognitive skills are immature at the time of meningitis, deficits may not be detected until the child is older and has started school. Therefore, future long-term follow-up studies on neuropsychological, social, and schoolfunctioning are urgently needed.

The use of scoring systems combining prognostic parameters to assess patients with meningococcal disease, has attracted much interest. The application of a prognostic score forces clinicians to make observations in areas of prognostic significance and enables them to estimate the severity of disease. Scoring systems can be readily applied to management protocols thus contributing to the establishment of clinical indications for invasive monitoring and aggressive therapy. Scoring systems may also be used to stratify subsets of patients for inclusion in new therapeutic trials. We developed a prognostic scoring system for patients with meningococcal septic shock (chapter 9). This system is exclusively based on the use of laboratory parameters which is a major advantage above previous scoring systems which use subjective characteristics or laboratory parameters that are difficult to obtain.

The pathophysiological effects of meningococcal sepsis and other related syndromes result from tissue damage by the uncontrolled production of inflammatory mediators. Recently novel immunotherapeutic regimens have been developed to inhibit the induction of the inflammatory cascade

by endotoxin. Other experimental therapies aim to inhibit the activation of various mediators or to modulate this response by administration of antiinflammatory mediators such as monoclonal antibodies and other drugs directed against bacterial products or cytokines.

Natural or recombinant tumor necrosis factor (TNF)-α or interleukin (IL)-1ß do induce in animal models clinical and laboratory features similar to sepsis. Inhibitors of these cytokines can reverse the features of septic shock in these models. In experimental gram-negative sepsis, neutralization of endotoxin by monoclonal antibodies also reduces mortality [17]. Although beneficial effects of these new drugs have been documented in experimental animals, studies in man thusfar did not lead to improvements in outcome [18]. A recent study even documented an increase in mortality of patients treated with tumor necrosis factor:Fc fusion protein [19]. Inhibition of the proinflammatory cytokine IL-1 by means of IL-1 receptor antagonist also did not result in a reduction of mortality [20, 21]. These data are in accordance with animal models of infection by salmonella species or by *Listeria monocytogenes* in which inhibition of TNF-α or interleukin-1 production may worsen the outcome of disease [22-24]. Treatment with anti-TNF-α antibody was also either ineffective or detrimental in mice with localized peritonitis [25, 26]. TNF-α or IL-1 may actually protect against endotoxin-hyporesponsiveness in C3H/HeJ mice during systemic infection [27]. Despite great expectations, clinical trials in human sepsis have not been as encouraging as animal studies. Further research is necessary to elucidate the pathophysiological mechanisms in patients with sepsis before new therapeutic strategies can be succesfully applied.

The use of vaccines against *H. influenzae*, *N. meningitidis* and *S. pneumoniae* will circumvent the problems described above. Recently, conjugate vaccines against *H. influenzae* type b (Hib) have been developed [28-30]. Hib vaccination has been introduced in childhood vaccination programs in an increasing number of countries. Subsequently a 90% reduction in *H. influenzae* meningitis has been observed [31]. However, a substantial number of children have meningitis or sepsis by *N. meningitidis* or *S. pneumoniae*. Meningococcal disease is caused by several serogroups of which *N. meningitidis* serogroup B is the most common cause in Europe, North-America, and several countries in Latin America. A meningococcal non-conjugate tetravalent A, C, Y, and W135 polysaccharide vaccine is available, safe and immunogenic in adults, but not in young infants. The serogroup B polysaccharide, however, is poorly immunogenic in humans and cannot be used as a major vaccine component [32]. Therefore, vaccines based on outer membrane proteins are currently evaluated for their efficacy

[33, 34]. Recent field trials with such vaccines have demonstrated partial protection against group B infection [35, 36]. Our group currently performs a trial to study the immunogenicity of a hexavalent meningococcal vaccine strain developed by Dr. Jan Poolman (RIVM, Bilthoven). This vaccine uses class 1 outer membrane protein representing the serosubtypes P1.5,2; P1.7,16; P1.19,15; P1.12,13; P1.7h,4; P1.5c,10. These antigens represent 75-80% of *N. meningitidis* serogroup B.

Several pneumococcal conjugate vaccines combining the most relevant serotypes coupled to different protein antigens are already available and are currently studied for their effects on prevention of otitis media and invasive infections by *S. pneumoniae* [37].

14.3 MEDIATORS INVOLVED IN THE HOST DEFENCE

Patients with bacterial meningitis and sepsis undergo profound pathophysiological changes induced by a variety of pro- and antiinflammatory mediators. Proinflammatory cytokines including TNF-α, IL-1, IL-6, IL-8, IL-12 and interferon (IFN)-γ upregulate the host inflammatory response in these disease entities. Endogenous antiinflammatory mediators such as soluble TNF receptors (sTNFR) and IL-10 downmodulate the host response. We and others recently showed that the highly reactive molecule nitric oxide is an important mediator in the CSF compartment of patients with bacterial meningitis. Abnormalities in coagulation and fibrinolysis also play an important role in the pathophysiology of sepsis as illustrated in this thesis.

Cytokines

Cytokines are important molecules in the pathogenesis of bacterial meningitis (chapters 5 and 6) and meningococcal sepsis (chapters 10 and 11). Proinflammatory cytokines (TNF-α, IL-1ß, IL-6 and IL-8) are present in high concentrations in CSF specimens of patients with bacterial meningitis [38-40]. Higher cytokine levels have been associated with poor neurological outcome [38]. Proinflammatory cytokines have also been implicated as mediators of inflammatory changes and blood-brain barrier injury in the central nervous system of patients with bacterial meningitis [41, 42]. These compounds appear to be locally produced by the brain in response to bacterial cell wall components, and are thought to cause an influx of granulocytes from cerebral capillaries to the subarachnoid space by upregulation of leukocyte selectins on cerebral capillary endothelium

[43]. We investigated in this thesis the kinetics of CSF levels of proinflammatory mediators (chapter 5). CSF levels of TNF-α, IL-6, and IL-8 decreased rapidly. This observation is similar to that in consecutive serum samples of experimental models of bacteremia or endotoxemia and in patients with sepsis [44]. Production of these proinflammatory cytokines has been associated with the development of central nervous system injury. The precise mechanism has not been elucidated. It has been suggested that cytokines are produced by glial cells and brain capillary endothelial cells [45]. Intracisternal inoculation of endotoxin is followed by detection of TNF-α and IL-1ß activity. Subsequently, leukocytosis and changes in protein, glucose and lactate concentrations are observed within hours. The inflammatory response in the CSF of experimental animals after intracisternal inoculation of bacterial products such as endotoxin or live *H. influenzae* may be inhibited by dexamethasone. This is only effective when dexamethasone is initiated before or at the same time as antibiotics [46-48]. Clinical trials showed an improvement in the outcome of bacterial meningitis when dexamethasone was used as adjuvant therapy [49, 50].

We measured serum levels of cytokines on admission of patients with meningococcal sepsis as reported in chapter 10. Levels of proinflammatory mediators were highly elevated. Nonsurvivors had higher levels on admission than survivors in agreement with studies by other investigators [5, 51, 52]. Our data indicate that the height of serum levels of TNF-α and other proinflammatory cytokines (e.g., IL-6 and -8) is also determined by the duration of disease i.e. duration of petechiae. This conclusion is supported by the presence of a strongly negative correlation between initial cytokine levels and the interval between onset of purpuric skin lesions and blood sampling. In addition, C-reactive protein (CRP) levels, which indirectly reflect the duration of illness [53], were significantly correlated with the interval between the onset of petechiae and blood sampling. The significantly shorter period of disease and the lower level of CRP in nonsurvivors suggested a shorter disease course and may explain the presence of higher levels of cytokines. We speculate that serum levels of proinflammatory cytokines were already over or near their maximum when the patients were admitted. This is also illustrated by the rapidly disappearance of cytokines during the acute phase of septic shock [54-57].

The earlier admission on a pediatric intensive care unit of nonsurvivors with meningococcal septic shock may indicate that these patients accumulate more native lipopolysaccharide in a shorter time, trigger all mediator systems more intensively, and are recognized as more severely ill earlier in the course of disease. Alternatively, nonsurvivors may have been

admitted earlier because of a more rapid deterioration due to greater responsiveness to lipopolysaccharides or proinflammatory cytokines.

Interestingly, differences in the concentrations of mediators between survivors and nonsurvivors disappeared after adjustment for the time interval between the onset of petechiae and blood sampling. We speculate all that patients with meningococcal septic shock may have a similar pattern of release of proinflammatory cytokines, irrespective of the outcome.

We also studied the role of interleukin-12, a relatively novel proinflammatory cytokine, in the pathophysiology of both bacterial meningitis (chapter 6) and meningococcal sepsis (chapter 11). Interestingly, findings in patients with bacterial sepsis and meningitis were similar in many ways. The results do not allow definitive conclusions on the role in both clinical entities. Bioactive IL-12 is a heterodimeric protein of 70 kDa (p70) which consists of 40 kDa (p40) and 35 kDa (p35) subunits. IL-12 is produced by phagocytic cells in response to infection and stimulates the natural immunity. The production of IFN-γ is induced and in turn enhances the function of macrophages and polymorphonuclear leukocytes by stimulation on non-specific defence mechanisms such as phagocytosis and reactive oxygen intermediates. Several studies have shown that IL-12 contributes to the control of the host defence against infections with intracellular microorganisms such as *Mycobacterium tuberculosis*, *Listeria monocytogenes*, and *Leishmania donovani*. IL-12 mediated protection may be invoked by through its ability to stimulate IFN-γ, which inhibits the parasite. Concentrations of IFN-γ are significantly increased in CSF samples from children with tuberculous meningitis [58]. We propose that analysis of IL-12 in these children would be interesting and would probably reveal to a major role of this cytokine in this type of meningitis. The role of IL-12 in bacterial meningitis and sepsis is less clear. However, CSF concentrations of IL-12 p40 and IFN-γ were markedly elevated in patients with bacterial meningitis. In addition, CSF levels of IFN-γ correlated positively with IL-12 and the costimulator TNF-α. Interestingly, patients with pneumococcal meningitis had significantly higher CSF levels of IFN-γ in comparison with those with meningitis due to *H. influenzae* and *N. meningitidis* as was previously observed by others [17]. This observation can not be explained by an increased IL-12 production since CSF levels of IL-12 were not markedly higher in patients with pneumococcal meningitis. However, the high CSF ratio between the costimulator TNF-α and IL-10, which inhibits IL-12 induced IFN-γ production, in the patients with pneumococcal meningitis in comparison to those with meningitis due to

other pathogens, may provide an explanation for the higher CSF levels of IFN-γ in patients with pneumococcal meningitis.

In patients with meningococcal septic shock, plasma levels of IL-12 p40 were elevated and related to the outcome and severity of disease. Bioactive IL-12 could not be detected in the large majority of children with meningitis and sepsis. Previously, other investigators studies already reported that levels of p40 were much higher than those of p70 [59, 60]. The physiologic significance of the excess production of the free p40 subunit is not clear. Mattner et al [61] suggested that IL-12 bioactivity is inhibited by free p40 molecules. Nevertheless, in vitro and in vivo studies have clearly shown that the production of p40 is linked to that of bioactive IL-12 p70 [60, 62]. Further studies will be necessary to elucidate the exact role of IL-12 in bacterial meningitis and sepsis.

Counterinflammatory mediators

The production of proinflammatory cytokines and the extent of the inflammatory response is downregulated by counterinflammatory compounds, such as IL-10, and naturally occurring antagonists of TNF-α including the soluble extracellular domains of the 55- and 75-kDa membrane-bound TNF receptors (sTNFR-55 and -75). IL-10 is produced by monocytes and macrophages, the Th2 subset of T helper lymphocytes, and B lymphocytes and suppresses the synthesis of proinflammatory cytokines by T cells, polymorphonuclear leukocytes, and monocytes and macrophages. We and others found significantly elevated CSF levels of IL-10 in patients with bacterial meningitis, suggesting a role for IL-10 in the control of the inflammatory response in the CSF compartment (chapter 5). In vitro studies have shown that TNF-α is cytotoxic for neuronal cells, can disrupt myelin, and damage oligodendrocytes [63, 64]. High levels of IL-10 in CSF may have a protective role in bacterial meningitis, by reduction of the TNF production, thus preventing the occurrence of neurological sequelae. In an experimental bacterial meningitis model, intracisternal or intravenous injection of IL-10, in combination with antibiotics, also led to a significant reduction of TNF levels in CSF [65]. When dexamethasone was given together with IL-10, TNF-α levels further decreased. IL-10 may also inhibit the inflammatory reaction in the CSF compartment, induce a decrease in intracranial pressure, and water content of the brain and a reduction of the number of polymorphonuclear leukocytes in the CSF, as demonstrated in experimental pneumococcal meningitis.

We observed that nonsurvivors with meningococcal septic shock had significantly higher concentrations of IL-10 than survivors. Lehman et al. recently reported that high IL-10 levels in patients with meningococcal

disease were associated with fatality [54]. However, we found in our study, that after adjustment for the time between the onset of petechiae and blood sampling (as measure for the duration of the disease course) IL-10 levels were similar in survivors and non-survivors.

IL-10 protects against TNF-mediated lethality in murine models of endotoxemia [66]. In animal models of sepsis, IL-10, administered before or soon after challenge with gram-negative bacterial endotoxin or staphylococcal enterotoxin B, reduced TNF-α production and mortality [66-68]. Chernoff et al. [69] showed that a single intravenous injection of IL-10 in humans reduces mitogen-induced T cell proliferation and suppresses TNF-α and IL-1ß production from whole blood stimulated ex vivo with endotoxin. In the study presented in chapter 10, the strong correlation between IL-10 and the proinflammatory cytokines TNF-α, IL-6, and IL-8 in survivors and nonsurvivors suggests the presence of an adequate IL-10 response to down-regulate the production of proinflammatory cytokines.

The biologic activity of TNF-α is also neutralized by sTNFR-55 and -75 [70]. sTNFR is shed from the cell surface of, for example, polymorphonuclear cells in response to many of the same inflammatory stimuli that induce TNF-α [71]. In addition, sTNFR-55 and -75 can neutralize the biologic activity of TNF-α. We and others measured significantly elevated levels of sTNFR-55 and sTNFR-75 in CSF and serum of patients with bacterial meningitis [51]. Of interest, levels of sTNFRs in our study were exclusively associated with concentrations of IL-10 but not with concentrations of TNF-α, IL-6, or IL-8. This is in agreement with the recent observation that IL-10 induces an increase in the synthesis and cell-surface turnover of sTNFRs from monocytes [72, 73]. Other studies also indicate that IL-10 stimulates the release of IL-1 receptor antagonist, another antiinflammatory compound. IL-10 thus may suppress TNF activity in the CSF compartment by an inhibition of TNF-α secretion, stimulation of the release of sTNFRs, and down-regulation of the expression of surface TNFRs [72, 73]. In our study, serum and CSF levels of sTNFR-55 and sTNFR-75 were similar in patients with bacterial meningitis. The relatively low CSF levels of sTNFRs may be partly explained by a release of sTNFRs from polymorphonuclear leukocytes before entry of these cells into the CSF compartment. Alternatively, shedding of sTNFRs in the CSF compartment may be lower because of the approximately 10-fold lower white blood cell (WBC) count in CSF than in peripheral blood in these patients. The significantly higher stimulus by TNF-α and IL-10 for release of sTNFRs in the CSF compartment apparently does not compensate for this inequality. The elevated CSF levels of sTNFR-55 and sTNFR-75 in the present study persisted for a remarkably long time (at least 24 h), as was previously

observed in bacteremia [74]. This may be due to slow clearance from the CSF compartment or to continuing release. The elevated levels of sTNFR may stabilize the activity of TNF-α, and provide a reservoir of TNF-α that is available for gradual release [75]

In patients with meningococcal septic shock, we and others [76] reported that high levels of the sTNFRs are associated with an increased likelihood of fatality. Froon et al. [77] suggest that increased serum levels of sTNFRs in patients with sepsis syndrome are merely the result of renal failure. Normally, the majority of sTNFRs is removed from the circulation by the kidneys, although the liver and lungs are probably also involved [78]. The higher serum creatinine levels of nonsurvivors in the present study may at least partly explain the differences between survivors and nonsurvivors in sTNFR concentrations due to a reduced renal clearance.

Nitric oxide

Nitric oxide (NO) is one of the most versatile molecules produced by mammalian cells. NO is a short-lived free radical produced by a variety of cell types and involved in smooth muscle relaxation, neuronal signaling, inhibition of platelet aggregation, and regulation of cell-mediated cytotoxicity [79-81]. NO production results from the conversion of L-arginine to L-citrulline by the enzyme NO synthase (NOS). Three different NOS isoforms have been cloned and characterized: neuronal [81, 82], endothelial [83, 84], and cytokine inducible. The last group includes macrophage [85, 86], and vascular smooth muscle NOS [87]. The role of NO in inflammatory processes has been the subject of intense investigation since its discovery in 1987. NO was shown by us to be involved in the pathophysiology of both bacterial meningitis and sepsis.

The involvement of NO in the pathophysiology of bacterial meningitis was previously investigated in several animal studies. In experimental meningitis induced by intracisternal injection of TNF-α, an increase of the NO level in CSF, a reduced oxygen uptake, a decreased CSF blood-glucose ratio, an increased lactate concentration in CSF, and an increased intracranial pressure were found [88]. In our study (chapter 7), we detected a strong correlation between CSF levels of TNF-α and nitrite in children with bacterial meningitis. In addition, CSF levels of the precursor L-arginine were significantly decreased. These data demonstrate that TNF-α may be involved in the induction of arginine-dependent NO production in the CSF compartment of patients with bacterial meningitis. Rats inoculated intracisternally with live gram-positive and gram-negative bacteria, or LPS showed elevated NO/nitrite levels in CSF that increased with an increased blood-brain barrier permeability [89]. In the study presented in chapter 7,

the decreased CSF levels of L-arginine (the precursor of NO), the increased CSF NO/nitrite levels, and the decrease of CSF NO/nitrite over time, and indicate the presence of enhanced NO production in the CSF compartment in patients during the acute phase of bacterial meningitis.

The mechanism by which NO may contribute to the pathophysiology of bacterial meningitis is not yet completely understood. NO disrupts various enzyme systems associated with mitochondrial respiration, DNA replication, and the citric acid cycle [90-94]. NO also initiates the production of other strong oxidants which may contribute to cellular destruction and alterations in central nervous system homeostasis. One such strong oxidant is peroxynitrite, which is produced through a reaction of NO with superoxide anion [95-101]. Peroxynitrite may well be more damaging than NO itself.

The cellular site of NO/nitrite production in children with bacterial meningitis is unknown. NO/nitrite can be generated by at least three separate mammalian cells. Experimental studies present increasing evidence pointing to an induction of the calcium-independent isoform of nitric oxide synthase (iNOS) in astrocytes and glial cells by gram-positive cell wall fragments and endotoxin. The second source of CSF nitrite may be the microvascular endothelium which constitutes the blood-brain barrier. Finally, a third source of CSF nitrite could be neutrophils that cross into the CSF during bacterial meningitis. Both human and rat neutrophils synthesize relatively high levels of nitrite when activated by various stimuli. We did not observe a correlation between CSF NO/nitrite levels and CSF WBC count or CSF protein levels in patients with bacterial meningitis. Therefore, we propose that WBC are probably not the major source of NO/nitrite, although we intend to investigate this question by applying immunocytochemical techniques on CSF neutrophils.

Drugs as well as endogenous mediators are able to reduce the NO production. Although the present data may suggest, that a therapeutic downregulation of NO production may be a useful approach one has to realize that NO has both beneficial and detrimental effects. Positive effects of elevated NO levels in CSF may be sealing of the vascular layer and subsequently reduced leakage of plasma proteins through postcapillary venules, and inhibition of endothelial cell activation. De Caterina et al. showed that NO can limit the degree of activation of human endothelial saphenous vein endothelial cells. The ability of NO to inhibit the expression of endothelial-leukocyte adhesion molecules and certain proinflammatory cytokines may reduce the inflammatory response [102]. In contrast, Buster et al. demonstrated that NO contributes to the general inflammatory response during experimental bacterial meningitis, thus promoting

production of chemotactic factors and leukocyte-endothelial cell adhesion molecules which facilitate entry of leukocytes into the subarachnoid space [89].

Since high levels of NO are reported to be cytotoxic for neuronal cells and thus may contribute to the development of neurological sequelae during bacterial meningitis, inhibition or reduction of the NO production may be useful [103, 104]. However, the relevance of NO as cause of neuronal injury in humans is not yet known. Rat astrocytes and microglia produce NO in neurotoxic concentrations in response to bacterial products. Human microglia do not seem to synthesize such concentrations of NO [105, 106]. Human astrocytes, however, are able to generate NO [105]. Since astrocytes are the most numerous cell type in the brain, their activation by bacterial products could play a role in the development of brain injury in bacterial meningitis in humans.

NO production is suppressed by glucocorticoids, transforming growth factor ß1, IL-4, IL-10, and prostaglandin E_2 [107-111]. Dexamethasone may improve neurologic outcome in pediatric bacterial meningitis and is frequently used as adjunctive therapy [49, 50]. This beneficial effect is thought to result from the reduction of inflammation, although the precise mechanism is unknown. Dexamethasone suppresses production of NO in astrocytes and markedly reduces the associated neurotoxicity after challenge with LPS [112, 113]. In the study presented in chapter 7, we did not observe differences in CSF nitrite levels in time between those patients with and without dexamethasone as adjuvant therapy. However, by the time meningitis is diagnosed, iNOS mRNA expression is already induced. The failure of dexamethasone to inhibit iNOS mRNA and NO production after induction may explain the ineffectiveness of glucocorticoids [107]. The absence of a significant effect on NO/nitrite production in the CSF compartment may also be due to the relatively small sample of patients or the initiation of antibiotic therapy prior to administration of dexamethasone.

Inhibition of NOS with N-nitro-L-arginine methyl ester (L-NAME) in animals with meningitis results in a reduction of CSF nitrite of CSF nitrite levels and a corresponding reduction in WBC counts and a decrease in blood-brain barrier permeability [89]. Administration of L-NAME was also accompanied by an increased mortality [114, 115]. In addition, Tauber et al. showed that NO synthase inhibition during meningitis by aminoguanidine leads to an increase in cerebral ischemia. This increased cerebral ischemia is a likely cause of the worsened neuropathological outcome in animals with group B streptococcal meningitis treated with aminoguanidine [116].

NO also appears to play a role in the hemodynamic instability associated with septic shock. Cytokines and endotoxin stimulate synthesis

of inducible NOS. NO which is subsequently produced in large amounts over an extended period, may be the key mediator in the pathogenesis of septic shock. Previous investigators measured elevated levels of nitrate in patients with sepsis. We also found elevated serum levels of nitrate in patients with meningococcal septic shock (unpublished data). However, differences in nitrate levels between survivors and non-survivors were not found, although patients with persistent hypotension had significantly higher levels of nitrate. Comparison between animal studies shows a great variability, which may be attributable to differences in dosing regimens or in models of septic shock. Data obtained from human studies are more consistent, but are limited to a few case series. The results indicate that NOS inhibitors increase blood pressure and systemic vascular resistance and decrease cardiac output. The effects of NOS inhibitors on morbidity and mortality could not be assessed because of the lack of an appropriate sample size. Further studies are rquired to determine appropriate dosing regimens of NOS-inhibitors and to study the effects of these agents on morbidity and mortality in patients with septic shock.

Coagulation and fibrinolysis

Meningococcal septic shock is associated with severe disturbances of coagulation and fibrinolysis. Its most profound manifestation is disseminated intravascular coagulation (DIC) characterized by micro-vascular thrombosis, consumption of platelets and coagulation proteins and by stimulation of the fibrinolytic system. Meningococcal infections may evolve into the most severe clinical form of DIC: purpura fulminans with hemorrhagic necrosis and peripheral gangrene. We questioned whether the higher mortality in young children is associated with the presence of more severe clotting abnormalities (chapter 12).

The coagulation is activated through the extrinsic pathway (tissue factor). In vitro studies demonstrated that endotoxin, IL-1ß, and TNF-α enhance tissue factor expression on endothelial cells [117, 118]. Tissue factor binds to factor VII. The complex of activated factor VII (factor VIIa) and tissue factor binds to factors IX and X and this sequence of proteolytic activation results in the formation of thrombin from prothrombin. Thrombin cleaves fibrinogen yielding monomeric fibrin which then polymerizes to form the fibrin clot [119]. The massive consumption coagulopathy is characterized by low levels of coagulation factor VII, X, V, prothrombin, fibrinogen, and platelets.

The natural inhibitors of coagulation, antithrombin III (ATIII) and the protein C-protein S system, contribute to the regulation of coagulation. The levels of these inhibitors are markedly altered in patients with

meningococcal septic shock. The ATIII levels are significantly decreased. The major mechanism by which ATIII activity declines in sepsis is acute consumption during the process of coagulation.

Procoagulant effects of TNF-α are also amplified by the impaired function of the protein C-protein S inhibitory pathway due to downregulation of thrombomodulin and increased serum levels of the protein S binding factor C4bBP. Normally, thrombomodulin binds thrombin and thereby reduces its coagulative potential and activate protein C [118-122].

In the study presented in chapter 12 markedly decreased plasma levels of protein C and protein S were found, resulting in a procoagulant state. Although consumption of protein C certainly contributes to the decreased levels, immaturity of the protein C system may also play a role. Most coagulation factors reach adult levels at 6 months of age [123]. Protein C, however, does not reach adult levels until the fourth year of life [124]. Powars et al. have suggested that infants are more susceptible to severe depletion of protein C. These authors showed a relation between protein C and both age and outcome in children with meningococcal disease [125]. Therefore, the lowest level of the endogenous anticoagulans protein C in young children may at least partly explain the significantly lower levels of most coagulation factors in young children with meningococcal septic shock.

Activation of coagulation is accompanied by activation of fibrinolysis. Endotoxin, TNF-α, and IL-1ß modulate the fibrinolytic system to secrete both tissue plasminogen activator (t-PA) and plasminogen activator inhibitor (PAI)-1, which activate and inhibit fibrinolysis, respectively [126-131]. Fibrinolysis is initiated by the release of t-PA from vascular endothelium which converts plasminogen into the active enzyme plasmin resulting in degradation of fibrin in the thrombi. Administration of low intravenous doses of endotoxin or TNF-α in healthy volunteers initially leads to activation of coagulation and fibrinolysis. However, a few hours later, an increasing inhibition of the fibrinolytic system occurs due to a strong elevation of PAI-1, aggravating the procoagulant state [44, 126, 129, 135]. These data are supported by in vitro studies on human endothelial cells showing that endotoxin enhances the release of PAI-1 coinciding with suppression of fibrinolytic activity [128-130]. Moreover, fibrin and thrombin formed during coagulation are also potent inducers for the release of t-PA and PAI-1 [133, 134]. The activity of t-PA in patients is counter-regulated by PAI-1 that binds to and thereby inhibits t-PA.

We showed in chapter 10 by multiple regression analysis that levels of PAI-1 correlated with the duration of petechiae and were higher in

nonsurvivors. The inhibition of fibrinolytic activity represented by the ratio PAI-1 to t-PA was significantly higher in nonsurvivors compared to survivors. Comparison between children below and above 3 years of age showed a significantly lower fibrinolytic activity in the youngest age group. This may be indirectly caused by the higher plasma levels of thrombin and fibrin in the youngest age group which also stimulate the release of PAI-1. Of interest, the more severe coagulopathy in children below 3 years of age occurred in the presence of similar serum levels of proinflammatory cytokines in younger and older children. This indicates that the severity of clotting abnormalities may of greater importance than the degree of the inflammatory response. Therefore activation of coagulation together with the inhibition of the fibrinolysis are responsible for the development of a hypercoagulable state, fibrin deposition, microthrombi and DIC, especially in young children [135-138]. Fibrin deposition and complement activation cause extensive endothelial damage and are associated with multiple organ failure [136-138].

Heparin, antithrombin III concentrate, fresh frozen plasma, and protein C have been tried as therapeutic agents to control DIC in patients with fulminant meningococcemia [139-143]. However with exception of fresh frozen plasma, and protein C, which have some fibrinolytic properties, these interventions do not contribute to the dissolution of already formed clots [144]. The administration of protein C may have a beneficial effect on patients with meningococcal septic shock and DIC, since protein C not only regulates coagulation, but also enhances fibrinolysis by inhibition of PAI [144]. A multicenter, blinded study to assess the efficacy and safety of protein C concentrate in the treatment of patients with septic shock and purpura will therefore be initiated this year. Administration of recombinant t-PA to enhance fibrinolysis may be an alternative adjuvans therapy, since preliminary data suggest an improvement in outcome in patients (n = 2) with severe meningococcal shock.

14.4 Genetic factors

Genetic factors of host and pathogen determine the susceptibility to an invasive infection and may be partly responsible for the severity of disease.

Previous investigators showed that defects in the complement system, i.e. in the alternative pathway, C3 and in the terminal complement components C5-C9, coincide with a significantly increased risk for meningococcal disease [145]. This is particularly obvious in patients

contracting meningococcal disease by uncommon serogroups (X, Y, Z, W135) or in subjects with recurrent meningococcal disease. Deficiencies of (specific) antibodies or the presence of IgA antibodies are other risk factors for meningococcal disease. Polymorphism of the Fc receptors (FcγR) for the interaction with IgG on phagocytes may also be crucial. Genetically determined forms of the FcγRIIa (CD32 molecule) express different functional activities. FcγRIIa-R/R131 neutrophils phagocytize IgG2 opsonized microorganisms less effectively than FcγRIIa-H/H131 neutrophils. IgG2 is the major subclass produced in response to bacterial capsular polysaccharides. Bredius et al. found that the homozygous FcγRIIa-R/R131 allotype is significantly more present in survivors of meningococcal septic shock in comparison with a healthy Caucasian population [146]. Thus, the R131 allotype of FcγRIIa, which does not bind IgG2, seems to be a risk factor for invasive meningococcal infections. This is in agreement with epidemiological observations showing a very low incidence of infections caused by encapsulated microorganisms in Japan, where the majority of individuals is FcγRIIa-H/H131 [147, 148], and with observations in children with a history of recurrent infections, where a lower frequency of the FcγRIIa-H/H131 allotype was found in comparison with a healthy population [149]. In contrast to patients with meningococcal septic shock, a higher percentage of FcγRIIa-R/R131 was not found in children with a history of *H. influenzae* meningitis (manuscript in preparation).

Genetic polymorphisms for TNF-α seem to be involved in the regulation of severity of or susceptibility to meningococcal disease. A wide interindividual variability in TNF-α release has been demonstrated after stimulation by endotoxin in vitro of whole blood samples and of peripheral blood mononuclear cells from healthy volunteers [150, 151]. The high initial TNF-α levels in patients who do not survive meningococcal septic shock have been attributed to an exaggerated response to circulating endotoxin [152]. The production of inappropriately large quantities of TNF-α may be due to the presence of a genetic variant in the promoter region of the TNF gene (TNF2 allele) as previously observed in patients with cerebral malaria [153]. The TNF2 allele has been associated with higher constitutive expression and greater secretion of TNF-α after induction [154]. Genetically determined variability in the expression of other proinflammatory cytokines may also be associated with the outcome of serious infectious diseases. A similar variability in the antiinflammatory response certainly may also contribute to the outcome of disease. A reduced expression and release of e.g. IL-10 may enhance or prolong the inflammatory response.

We report in this thesis, that polymorphism of the PAI-1 gene is a determinant for the development and possibly the outcome of children with meningococcal septic shock. PAI-1 concentrations on admission were significantly higher in nonsurvivors at a similar TNF-α concentration, one of the inducers for PAI release as shown in chapter 10. The presence of a single base pair 5G/4G (allele frequency 0.53/0.47) polymorphism in the promoter of the PAI-1 gene has been associated with differences in release of PAI-1 in postoperative patients [155] and in patients with an increased risk of recurrent myocardial infarction [156]. The promoter containing the 4G allele produced significantly more mRNA after stimulation by interleukin-1 than the 5G allele [157]. The 4G allele-specific increase in plasma PAI-1 activity is related to a differential binding of transcription factors to the polymorphic site which increase the basal PAI-1 transcription. Therefore, the 4G/5G polymorphism in the PAI-1 promoter is of functional importance in the regulation of the expression of the PAI-1 gene [157]. These data support our hypothesis proposed in chapter 11 that patients with meningococcemia and a 4G/4G or 4G/5G genotype have a significantly increased production of PAI-1 leading to more severe disease than in children with a 5G/5G genotype. Alternatively children with a 5G/5G genotype may develop milder disease or acquire meningitis. DNA analyses of children with meningococcal septic shock showed no differences in PAI-1 gene promoter allotypes between survivors and non-survivors. However, the study presented in chapter 11 in which 4G/5G polymorphism in the PAI-1 promoter of children with meningococcal septic shock was analyzed and compared with a control group of healthy children clearly indicates that PAI-1 is involved in the development of septic shock with purpura. The frequency of the 4G-allele, which is associated with increased plasma PAI-1 concentrations especially under conditions of stimulation by cytokines, was significantly higher among patients with meningococcal septic shock versus control subjects. The data of Bredius et al. and of our group underscore the importance of further investigations of the role of genetically encoded variability of the host response in the outcome of patients with meningococcal sepsis.

14.5 FINAL REMARKS

Novel approaches to the treatment of bacterial meningitis and sepsis are needed to improve the outcome and reduce the mortality by these disease entities. Thusfar the results of studies with novel immunotherapeutic agents have been disappointing. Occasionally application of new drugs has even been associated with increased mortality. A better understanding of the complex orchestra of mediators involved in the host response against bacterial pathogens will certainly contribute towards the development of new more effective immunotherapeutic regimens. Measurement of intracellular cytokine synthesis by flowcytometry or immunocytochemistry may be applied to localize specific cells responsible for the synthesis of certain mediators in different compartments. A more detailed knowledge on the cellular synthesis of the mediators involved in the host inflammatory response is also needed. Determination of the kinetics of inflammatory mediators and other compounds may contribute towards new insights in the dynamics of the host response and reveal the occurrence of interactions between different inflammatory mediators. Finally, the development and application in large scale clinical studies of a new generation of conjugate vaccines against the major bacteria involved in bacterial meningitis will be the most important scientific breakthrough during the remainder of this century.

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Summary (Samenvatting)

SUMMARY

Bacterial meningitis and meningococcal sepsis are serious infectious diseases which are frequently encountered in previously healthy children. The morbidity and mortality of these clinical entities are relatively high in comparison to those in many other infectious diseases. In this thesis, we present the results of several studies in children with bacterial meningitis caused by *Neisseria meningitidis*, *Haemophilus influenzae* and *Streptococcus pneumoniae* and in children with meningococcal septic shock. Clinical characteristics and host factors such as inflammatory and counterinflammatory mediators and parameters of coagulation and fibrinolysis were investigated to enhance the understanding of the host response.

Chapter 2 reviews the current knowledge of the host inflammatory response in patients with and animal models of bacterial meningitis. In addition, the epidemiology and diagnosis of bacterial meningitis are discussed. Pathophysiological alterations in the CSF compartment of patients with bacterial meningitis result from the combined effects of the microorganism, its cell wall products, and the host inflammatory response. Recent advances in understanding of the host inflammatory response have resulted in the development and application of new therapeutic strategies. These compounds aim to modulate the inflammatory response and reduce the incidence of sequelae and death. The use of steroids in children with bacterial meningitis has beneficial effects although the available data are still controversial. Additionally, studies in experimental models of bacterial meningitis indicate that non-steroidal anti-inflammatory drugs and monoclonal antibodies against bacterial products, cytokines and CD18 on leucocytes may reduce the extent of the meningeal inflammation.
The introduction of the *H. influenzae* type b vaccine in immunization programs has resulted in a rapid decline in the incidence of *H. influenzae* meningitis. Effective vaccines against *S. pneumoniae* and *N. meningitidis* will probably become available within a few years.

Chapter 3 describes a retrospective study on the outcome of pneumococcal meningitis in 83 children admitted to the Sophia Children's Hospital during a 25-year period. The median age of the children was 8 months. The most common associated infection was otitis media in 42% of all cases. The most frequently isolated capsular serotypes and/or serogroups of *S. pneumoniae* were 6, 14, 18, 19 and 23. These isolates accounted for 62% of all cases. Approximately one third of the study population consisted of secondary referrals usually for intensive care treatment. A marked

difference in mortality was observed between primary and secondary referrals: 7% and 35% respectively. An overall mortality rate of 17% was observed. Children often die of neurological sequelae. The higher mortality rate (33%) in adults, mostly elderly, as observed in another Dutch retrospective study, is frequently due to the development of cardiorespiratory failure. The frequency of sequelae in the pediatric population was 35%: hearing loss (≥30 dB) in 19% and neurological sequelae in 25%. Coma, respiratory distress, shock, a cerebrospinal fluid (CSF) protein level ≥2.5 g/L, a peripheral white blood cell count $<5x10^9$/L, and a serum sodium level of <135 mmol/L during admission were clinical findings with the strongest predictive value for sequelae or death in children.

In **Chapter 4**, a study is presented on the molecular characterization of pneumococcal strains obtained from children with meningitis admitted to the Sophia Children's Hospital between 1975 and 1992. Pneumococcal isolates were characterized by serotyping and two genotyping methods, BOX-fingerprinting and restriction fragment end labeling, to evaluate whether clonal strains were present within the serotypes/-groups. Pneumococcal isolates from 44 of 68 patients were still available for analysis. All strains were susceptible to penicillin. Serotypes/-groups 6, 14, 19, and 18 represented 56% of all isolates. The results of the molecular typing methods demonstrate complete absence of clonal relatedness between isolates from children with pneumococcal meningitis.
The following three chapters discuss the contribution of pro- and anti-inflammatory mediators in the host response of children with bacterial meningitis.

Chapter 5 describes the presence of anti-inflammatory compounds interleukin (IL)-10, soluble tumor necrosis factor (TNF)-α receptors p55 (sTNFR-55) and sTNFR-75 in children with bacterial meningitis. These compounds downregulate the host inflammatory response and are therefore important targets for future therapeutic interventions. Pro- and anti-inflammatory cytokines were measured in serum and CSF from 37 children with a bacterial meningitis. CSF concentrations of IL-10, sTNFR-55 and sTNFR-75 and of the cytokines TNF-α, IL-6, IL-8 were markedly elevated and were with the exception of the sTNFRs significantly higher in CSF than in serum. CSF concentrations of sTNFR-55 and sTNFR-75 were exclusively associated positively with concentrations of IL-10. This is in agreement with the recent observation that IL-10 increases the synthesis and cell-surface turnover of sTNFRs from monocytes. IL-10 thus may suppress TNF-α activity in the CSF compartment by an inhibition of TNF-α secretion, stimulation of the release of sTNFRs and down-regulation of the

expression of surface TNFRs. Surprisingly, CSF glucose levels correlated highly with IL-10, sTNFR-55 and sTNFR-75. CSF levels of cytokines decreased rapidly in time while sTNFRs levels remained elevated for at least 24 hours.

Chapter 6 describes the role of IL-12, a novel cytokine with proinflammatory effects, in the CSF compartment of patients with bacterial meningitis. IL-12 also stimulates the production of interferon (IFN)-γ which in turn enhances the function of macrophages and polymorphonuclear leukocytes by stimulation of non-specific defence mechanisms such as phagocytosis and secretion of reactive oxygen intermediates. The release of IFN-γ is costimulated by TNF-α and IL-1ß, and inhibited by IL-10. Serum and CSF levels of bioactive IL-12 (p70) and the inactive subunit p40 and IFN-γ were measured in 35 patients and 10 control subjects. CSF concentrations of IL-12 p40 as well as those of IFN-γ were markedly elevated whereas IL-12 p70 was hardly detectable. CSF levels of IFN-γ correlated positively with IL-12 p40 and TNF-α but not with the other proinflammatory cytokines IL-6, IL-8 or IL-10. CSF levels of IFN-γ were significantly higher in patients with pneumococcal meningitis than in children with meningitis by *H. influenzae* and *N. meningitidis*. This could be explained by a higher TNF-α to IL-10 ratio in the CSF compartment in children patients with gram-positive bacterial meningitis in comparison with those with gram-negative bacterial meningitis. These data suggest that IFN-γ production in the CSF compartment is induced by IL-12 and the co-stimulator TNF-α during the acute phase of bacterial meningitis.

Chapter 7 discusses the role of nitric oxide (NO) in the pathophysiology of bacterial meningitis. NO, a short-lived free radical, which can be produced in vitro by microglia and astrocytes after stimulation with cytokines (TNF-α and IL-1ß) and lipopolysaccharides, has attracted attention as a potential neurotoxic compound. NO production results from the conversion of L-arginine to L-citrulline by the enzyme NO synthase (NOS). NO decomposes to the stable endproducts nitrate and nitrite. Therefore, serum and/or CSF concentrations of the precursor (L-arginine) and degradation products of NO (nitrate, nitrite) and TNF-α were measured in patients and control subjects. CSF nitrate levels were significantly elevated in children with bacterial meningitis. This was mainly due to an increased blood-brain barrier permeability. CSF nitrate levels are therefore not a good parameter for the endogenous NO production in the CSF compartment. CSF NO/nitrite levels were significantly elevated in children with meningitis. NO/nitrite levels decreased in time. CSF levels of NO/nitrite correlated with those of TNF-α and glucose. CSF levels of L-arginine were significantly lower in patients in comparison to controls

which may be explained by the oxidation of L-arginine to NO by NO-synthase. We conclude that enhanced NO production may be partly responsible for the anaerobic glycolysis and the occurrence of neurological damage in children with bacterial meningitis.

Chapter 8 reviews the pathophysiological backgrounds of meningococcal sepsis. The ability of *N. meningitidis* to circumvent the immune system, and the rate of multiplication within the circulation leading to production of LPS, appear to determine the clinical symptomatology and outcome. The massive release of LPS induces the production of high levels of proinflammatory cytokines. The coagulation, fibrinolytic, complement and kallikrein-kinine cascade systems also become activated by these cytokines. Neutralization of endotoxin or inhibition of proinflammatory cytokines in human studies did not result in a better outcome in patients with septic shock. However, beneficial effects were documented in experimental animals.

Current meningococcal vaccines use capsular polysaccharides of serogroup A, C, W-135 and Y meningococci. Since the capsular polysaccharide of group B meningococci lacks immunogenicity, vaccines based on outer membrane proteins are under investigation. An effective meningococcal vaccine will probably be available in the near future.

In **chapter 9** the clinical characteristics and outcome of patients with septic shock and purpura are summarized. Additionally, prognostic factors were examined and a new scoring system was developed. Eighty-seven children with septic shock and purpura were included in this study. The median age was 3.2 years (range 3 weeks - 17.9 years. Blood- and/or CSF cultures grew *N. meningitidis* in 75 children and *H. influenzae* in two patients. Cultures remained sterile in 10 patients. The most common phenotype of *N. meningitidis* was B:4:P1.4 (27%). The mortality rate was 25% (95% confidence interval 15% - 34%). Patients admitted primarily to Sophia Children's Hospital died more often than those referred from other hospitals. However, this difference was not significant. Serious sequelae were observed in 15% of the survivors: skin necrosis requiring skingrafts or amputation (n = 9) and/or neurologic sequelae (n = 2). Serum calcium levels were significantly lower in patients with seizures. Disseminated intravascular coagulation occurred in 75% of the patients and its presence was associated with an increased mortality. Logistic regression analysis of laboratory parameters identified four independent variables for predicting the likelihood of survival: the serum levels of C-reactive protein (CRP), potassium, base excess, and platelet count. The outcome was predicted correctly in 87% of the patients which is higher than in any of the previously reported scoring systems.

In **Chapter 10**, the balance between the levels of proinflammatory (TNF-α, IL-6, IL-8) and counterinflammatory compounds (IL-10, sTNFR-55, sTNFR-75) was studied. The relationship with indicators of hemostasis was also investigated. Blood samples were obtained from 38 children who were consecutively admitted with meningococcal septic shock. The mortality rate was 29%. Serum levels of pro- and counterinflammatory compounds and plasma levels of plasminogen activator inhibitor (PAI)-1 were significantly higher in nonsurvivors. The interval between appearance of petechiae and blood sampling was significantly shorter in nonsurvivors than in survivors. A strongly negative correlation was found between the initial levels of these mediators and the interval between the onset of petechiae and blood sampling. In addition, CRP levels, which indirectly reflect the duration of illness, were significantly correlated with this interval. The significantly shorter interval and the lower level of CRP in nonsurvivors suggest a shorter disease course. This may explain the higher levels of cytokines. The earlier PICU admission of nonsurvivors points to an accumulation of more native lipopolysaccharide in a shorter time which triggers inflammatory mediators more intensively resulting in more severely ill patients earlier in the course of disease. Alternatively, a greater responsiveness to lipopolysaccharides or proinflammatory cytokines may have caused a more rapid deterioration leading to an earlier admission. However, with the exception of PAI-1 levels, differences between concentrations of these mediators disappeared after adjustment for the interval mentioned above. PAI-1 levels correlated with TNF-α concentrations and were nearly two times higher in nonsurvivors at a similar concentration of TNF-α. The increased PAI-1 response to TNF-α may be associated with fatality probably because of polymorphism in the PAI-1 promoter region.

In **chapter 11** we questioned whether the significantly increased production of PAI-1 in nonsurvivors with meningococcal septic shock versus survivors could be attributed to a common 4G/5G polymorphism in the promoter of the PAI-1 gene. The presence of the 4G allele is associated with higher basal production of PAI-1 and an increased responsiveness to stimulation with cytokines and endotoxin. Other investigators previously showed, that 4G/5G polymorphism in the promoter of the PAI-1 gene is associated with differences in PAI-1 activity in adults with non-insulin-dependent diabetes mellitus, coronary artery disease and myocardial infarction. We therefore hypothesized that higher production of PAI-1 in patients with meningococcemia might be a contributing factor towards more severe disease. To this purpose, DNA samples from 37 Dutch children who suffered from meningococcal sepsis were compared with DNA specimens

from healthy control subjects. Genetic analysis of the PAI-1 promoter polymorphism was documented by means of allele specific oligo-melting. The results of this study show that the genotype distribution was not different between survivors and nonsurvivors of meningococcal septic shock. However, the 5G/5G genotype is significantly less present in children with meningococcal sepsis in comparison with control subjects. We propose that children with meningococcemia and a 4G/4G or 4G/5G genotype have a significantly increased production of PAI-1 leading to more severe disease than in children with a 5G/5G genotype.

In **chapter 12**, the role of IL-12 and IFN-γ in the pathophysiology of septic shock with purpura was investigated. IL-12 plays a role in the host defence against microbial infections and has been shown to induce production of IFN-γ. Levels of IFN-γ are increased in experimental as well as clinical sepsis. Plasma levels of IL-12, were measured together with IFN-γ and other cytokines in 46 children with septic shock and purpura. The plasma IL-12 p40, TNF-α, IL-6, IL-8, IL-10 levels on admission were significantly higher in nonsurvivors compared to survivors. IL-12 p70 levels were elevated in only 9 patients and were associated with IL-12 p40. Only twelve (29 %) of the patients had detectable levels of IFN-γ. Circulating levels of IL-12 p40 and to a lesser extent those of IL-12 p70, are elevated in children with septic shock and purpura, and correlate with severity of disease and outcome.

In **Chapter 13,** we studied age-related differences in outcome and severity of clotting abnormalities in children with septic shock and purpura. Parameters of coagulation and fibrinolysis and plasma levels of cytokines were prospectively measured in 79 patients on admission. The mortality rate was 27%. The median age of the study population was 3.1 years and survivors were significantly older than nonsurvivors. The patients were divided in two age groups for analysis: younger and older than median age. The mortality in children ≤ 3.1 years was 40% versus 13% in children > 3.1 years (P = .006). With the exception of FVII, vWF and t-PA, parameters of coagulation and fibrinolysis, as well as plasma cytokine levels were related to outcome. In contrast to cytokine levels, which were not different between the two age groups, plasma levels of fibrinogen, prothrombin, factors V, VII, VIII, vWF, protein C, ATIII, FDP, and PAI-1/t-PA were related to age, indicating a more severe coagulopathy in children ≤ 3.1 years despite a similar degree of inflammatory response. A relative deficiency of coagulation factors due to an immature state of the clotting system, as well as an inadequate fibrinolytic response, both related to age may have caused this more severe coagulative response in younger children, and may have contributed to the higher mortality rate.

In **Chapter 14**, the results of this thesis are critically discussed in the context of recent literature. Suggestions for future research are given.

SAMENVATTING

Bacteriële meningitis en meningococcensepsis zijn op de kinderleeftijd frequent voorkomende ernstige infectieziekten bij voorheen veelal gezonde kinderen. De morbiditeit en mortaliteit ten gevolge van deze ziektebeelden zijn hoog in vergelijking met die van andere infectieziekten. In dit proefschrift worden de resultaten gepresenteerd van een aantal studies bij kinderen met bacteriële meningitis veroorzaakt door *N. meningitidis*, *H. influenzae* en *S. pneumoniae* en bij kinderen met septische shock door *N. meningitidis*. Klinische aspecten en gastheer factoren, zoals ontstekingsmediatoren en parameters van coagulatie en fibrinolyse werden bestudeerd teneinde het inzicht in de pathofysiologie van de gastheerrespons te vergroten.

Hoofdstuk 2 geeft een overzicht van de huidige inzichten in de ontstekingsreactie bij patiënten en proefdieren met bacteriële meningitis. Daarnaast wordt een beschrijving gegeven van de epidemiologie en de diagnostiek bij kinderen met bacteriële meningitis. De pathofysiologische veranderingen in het liquor compartiment van patiënten met bacteriële meningitis zijn het gevolg van het effect van het micro-organisme en zijn celwandproducten enerzijds en de ontstekingsreactie van de gastheer anderzijds. De toegenomen kennis op het gebied van de gastheerrespons heeft mede bijgedragen aan de ontwikkeling èn toepassing van nieuwe behandelingsmodaliteiten. Nieuwe therapeutische interventies hebben als doel de ontstekingsreactie te onderdrukken en de morbiditeit en mortaliteit te verminderen. Zo lijkt het gebruik van corticosteroïden bij kinderen met bacteriële meningitis gunstige effecten te hebben bij patiënten met meningitis door *H. influenzae*, alhoewel sommige studies deze resultaten niet kunnen bevestigen. Onderzoek in proefdieren met bacteriële meningitis heeft aangetoond dat ontstekingsremmende middelen die niet tot de groep van corticosteroïden behoren en monoclonale antilichamen tegen celwandproducten van bacteriën, cytokines en het CD18 molecuul op leukocyten de ernst van de meningeale ontstekingsreactie verminderen.

De introductie van het *H. influenzae* type b vaccin in het Rijksvaccinatieprogramma heeft geresulteerd in een snelle afname in de incidentie van meningitis door *H. influenzae*. Effectieve vaccins tegen *S. pneumoniae* en *N. meningitidis* zijn waarschijnlijk spoedig beschikbaar.

Hoofdstuk 3 betreft een retrospectieve studie naar de gevolgen van pneumococcen meningitis bij 83 patiënten, die gedurende de afgelopen 25 jaar in het Sophia Kinderziekenhuis opgenomen waren. De mediane leeftijd van de kinderen was 8 maanden. De meest voorkomende geassocieerde

infectie was otitis media (42%). De meest geïsoleerde kapsel serotypen en/of serogroepen van *S. pneumoniae* waren 6, 14, 18, 19 en 23. Tezamen vormen deze 62% van alle isolaten. Ongeveer 30% van alle kinderen waren verwezen vanuit een ander ziekenhuis wegens de noodzaak tot intensive care behandeling. Het verschil in mortaliteit tussen primaire en secondaire verwijzingen was respectievelijk 7 en 35%. De mortaliteit in deze groep was 17%. Kinderen overleden meestal ten gevolge van neurologische problemen. De hogere mortaliteit bij volwassenen, meestal bejaarden, was hoofdzakelijk het gevolg van cardiopulmonale complicaties. De morbiditeit ten gevolge van pneumococcen meningitis was 35%: gehoorverlies (≥ 30dB) werd gevonden bij 19% en neurologische restverschijnselen bij 25% van de patiënten. De prognose was het slechtst bij kinderen, die bij opname de volgende kenmerken vertoonden: coma, shock, respiratoire insufficiëntie, een liquor eiwit gehalte ≥ 2.5 g/L, een leukocyten getal < 5.10^9/L en een serum natrium gehalte < 135 mmol/L.

In **hoofdstuk 4** worden de resultaten gepresenteerd van de moleculaire karakterisering van pneumococcen isolaten afkomstig van kinderen met meningitis opgenomen in het Sophia Kinderziekenhuis tussen 1975 en 1992. Deze stammen werden gekarakteriseerd door middel van serotypering en twee genotyperingsmethoden: "BOX fingerprinting" en "restriction fragment end labeling". De aanwezigheid van clonaliteit binnen serotypen/groepen werd onderzocht. Pneumococcen isolaten van 44 van de 68 patiënten waren beschikbaar voor analyse. Alle stammen waren gevoelig voor penicilline. Serotypen/groepen 6, 14, 19 en 18 vertegenwoordigden 56% van alle isolaten. De moleculaire typeringsmethoden toonden geen genetische verwantschap tussen de diverse isolaten van patiënten met pneumococcen meningitis.

In **hoofdstuk 5** wordt de aanwezigheid bestudeerd van de anti-inflammatoire mediatoren interleukine (IL)-10, soluble tumor necrosis factor receptoren p55 (sTNFR-55) en sTNFR-75 bij kinderen met bacteriële meningitis. Deze mediatoren verminderen de omvang van de ontstekingsreactie en zijn daarom mogelijk belangrijke kandidaten voor gebruik in toekomstige interventiestudies. Pro- en anti-inflammatoire mediatoren werden gemeten in liquor en serum van 37 patiënten met bacteriële meningitis. De liquor concentraties van IL-10, sTNFR-55 en 75 en van de proinflammatoire cytokines TNF-α, IL-6, IL-8 waren sterk verhoogd. De concentraties van deze mediatoren waren, met uitzondering van die van sTNFR-55 en 75, significant hoger in het liquorcompartiment dan in het serum. De liquor concentraties van sTNFR-55 en sTNFR-75 waren alleen gecorreleerd met de concentraties van IL-10. Deze observatie is in overeenstemming met recente gegevens die tonen dat IL-10 de synthese en turnover

van TNFRn op het celoppervlak van monocyten verhoogt. IL-10 kan dus de TNF-α activiteit onderdrukken door remming van de TNF-α produktie en stimulatie van de afgifte van sTNFRn en vermindering van de expressie van TNFR op het celoppervlak. De glucose concentratie in de liquor cerebrospinalis was positief geassocieerd met de concentraties van IL-10, sTNFR-55 en sTNFR-75. De liquor concentraties van alle cytokines dalen snel gedurende de eerste uren na meting, terwijl de concentraties van sTNFR verhoogd blijven gedurende tenminste 24 uur.

In **hoofdstuk 6** wordt de functie bestudeerd van IL-12 in het liquor compartiment van patiënten met bacteriële meningitis. IL-12 is een relatief nieuw cytokine met proinflammatoire eigenschappen. IL-12 stimuleert de produktie van IFN-γ dat op zijn beurt de functie van macrofagen en granulocyten verbetert door stimulatie van niet-specifieke afweermechanismen, zoals fagocytose en de secretie van zuurstofradicalen. De afgifte van IFN-γ wordt mede gestimuleerd door TNF-α en IL-1ß en geremd door IL-10. Serum en liquor concentraties van bioactief IL-12 (p70) en de inactieve subunit p40 en van IFN-γ werden gemeten bij 35 patiënten en 10 controle personen. Zowel de liquor concentratie van IL-12 p40, als die van IFN-γ waren verhoogd, terwijl IL-12 p70 nauwelijks meetbaar was. De liquor concentraties van IFN-γ waren positief gecorreleerd met concentraties van IL-12 p40 en TNF-α maar niet met die van andere cytokines, zoals IL-6, IL-8 en IL-10. De liquor concentraties van IFN-γ waren significant hoger bij patiënten met pneumococcenmeningitis dan bij kinderen met meningitis veroorzaakt door *H. influenzae* en *N. meningitidis*. Een mogelijke verklaring hiervoor is de hogere TNF-α/IL-10 ratio bij kinderen met een Gram-positieve bacteriële meningitis dan bij kinderen met een Gram-negatieve bacteriële meningitis. De resultaten van dit onderzoek suggereren dat de produktie van IFN-γ in het liquor compartiment gedurende de acute fase van bacteriële meningitis wordt geïnduceerd door IL-12 en TNF-α.

In **hoofdstuk 7** wordt de rol bestudeerd van stikstofoxide (NO) in de pathofysiologie van bacteriële meningitis. NO, een vrij radicaal, kan worden geproduceerd door microglia en astrocyten na stimulatie door cytokines (TNF-α en IL-1ß) en lipopolysaccharides en heeft neurotoxische eigenschappen. NO produktie vindt plaats door de omzetting van L-arginine naar L-citrulline via het enzym NO synthase. NO wordt vervolgens afgebroken naar de stabiele eindprodukten nitraat en nitriet. Serum en liquor concentraties van het substraat (L-arginine) en de afbraakprodukten van NO (nitraat, nitriet) en TNF-α werden gemeten bij patiënten en bij een controlegroep. Liquor concentraties van nitraat waren significant verhoogd bij kinderen met bacteriële meningitis. Deze verhoging was hoofdzakelijk

het gevolg van de toegenomen doorlaatbaarheid van de bloedhersenbarrière. Liquor nitraat concentraties waren derhalve geen goede maat voor endogene NO produktie in het liquor compartiment. Liquor NO/nitriet concentraties waren significant verhoogd bij kinderen met meningitis en namen af in de tijd. De liquor concentraties van NO/nitriet waren gecorreleerd met die van TNF-α en glucose. De liquor concentraties van L-arginine waren significant lager bij patiënten dan bij controles, hetgeen kan worden verklaard door de oxydatie van L-arginine naar L-citrulline en NO via NO synthase. Wij concluderen dat de toename in NO produktie in het liquor compartiment van patiënten met bacteriële meningitis tenminste gedeeltelijk verantwoordelijk is voor de anaërobe glycolyse en het optreden van neurologische schade bij patiënten met bacteriële meningitis.

Hoofdstuk 8 geeft een overzicht van de pathofysiologische mechanismen, die betrokken zijn bij meningococcensepsis. Het vrijkomen van lipopolysacchariden induceert de produktie van proinflammatoire cytokines. De coagulatie, fibrinolyse, complement en kallikreine-kinine systemen worden tevens geactiveerd. Studies bij de mens met geneesmiddelen die endotoxines en proinflammatoire cytokines neutraliseren leveren nauwelijks een verbetering op van de prognose bij patiënten met een septische shock. Dierexperimentele studies tonen daarentegen wel gunstige effecten. De thans beschikbare meningococcen vaccins (serogroep A, C, W-135) zijn gericht tegen kapselpolysaccharides. Aangezien het kapselpolysaccharide van groep B meningococcen nauwelijks immunogeen is, zijn vaccins gebaseerd op membraaneiwitten in ontwikkeling. Een effectief meningococcen vaccin zal waarschijnlijk binnenkort beschikbaar komen.

Klinische karakteristieken en de prognose van patiënten met septische shock en purpura worden beschreven in **hoofdstuk 9.** Een nieuwe prognostische score werd ontwikkeld. Zevenentachtig kinderen met een septische shock en purpura werden geëvalueerd. De mediane leeftijd was 3.2 jaar (range 3 weken - 17.9 jaar). Bloed en/of liquor kweken toonden *N. meningitidis* bij 75 kinderen en *H. influenzae* bij 2 patiënten. De kweken bleven steriel bij 10 patiënten. Het meest voorkomende fenotype van *N. meningitidis* was P:4:P1,4 (27%). De mortaliteit bedroeg 25% (95% betrouwbaarheidsinterval 15% - 34%). Patiënten primair opgenomen in het Sophia Kinderziekenhuis overleden frequenter dan diegenen die verwezen waren vanuit andere ziekenhuizen. Dit verschil was echter niet significant. Ernstige restverschijnselen werden gezien bij 15% van de overlevenden; huidnecrose (n = 9), en/of, neurologische restverschijnselen (n = 2). Serum calcium concentraties waren significant lager bij patiënten met convulsies. Gedisseminеerde intravasale stolling werd waargenomen bij 75% van de

patiënten en was geassocieerd met het optreden van een hogere mortaliteit. Bij logistische regressieanalyse werden 4 onafhankelijke laboratorium parameters gevonden die de kans op overlijden voorspellen nl. de serum concentratie van "C-reactive protein" (CRP), het serum kaliumgehalte, de "base excess" en het aantal thrombocyten in arterieel bloed. De mortaliteit werd correct voorspeld bij 87% van de patiënten, hetgeen hoger is dan bij andere scoringssystemen.

De balans tussen proinflammatoire (TNF-α, IL-6, IL-8) en anti-inflammatoire mediatoren (IL-10, sTNFR-55, sTNFR-75) bij patiënten met meningococcen septische shock werd bestudeerd in **hoofdstuk 10**. Tevens werd de relatie met parameters van coagulatie en fibrinolyse bestudeerd. Bij 38 achtereenvolgende patiënten opgenomen met meningococcen septische shock werden bij opname bloedmonsters verkregen. De mortaliteit bij de patiëntengroep was 29%. Serum concentraties van pro- en anti-inflammatoire mediatoren en plasma concentraties van "plasminogeen activator inhibitor" (PAI)-1 waren significant hoger bij patiënten die kwamen te overlijden. Petechiae waren bij opname significant korter aanwezig bij patiënten die overleden dan bij overlevenden. Concentraties van het CRP, die indirect de duur van de ziekte weerspiegelen, waren significant gecorreleerd met de duur van de aanwezigheid van petechiae. De significant kortere duur van petechiae en de lagere CRP concentratie bij patiënten die overleden suggereren de aanwezigheid van een kortere ziekteduur hetgeen een verklaring vormt voor de aanwezigheid van hogere cytokine concentraties bij opname van patiënten die uiteindelijk overleden. Het kortere interval tussen de ontwikkeling van ziekteverschijnselen en klinische opname bij patiënten die overleden, kan verklaard worden door de hogere produktie van lipopolysacchariden (LPS) of door de aanwezigheid van een sterkere ontstekingsreactie. Anderzijds kan een grotere gevoeligheid voor LPS dan wel cytokines de snellere klinische achteruitgang van patiënten die overleden zijn, verklaren. Met uitzondering van plasma PAI-1 concentraties werden na correctie voor het bovengenoemde tijdsinterval geen verschillen gevonden tussen overlevende en overleden patiënten in de concentraties van cytokines. De PAI-1 concentraties waren positief gecorreleerd met de serumspiegels van TNF-α. Daarnaast waren de PAI-1 concentraties bijna tweemaal hoger bij patiënten die overleden dan bij de overlevenden bij eenzelfde serum TNF-α concentratie. De hogere PAI-1 produktie is geassocieerd met een hogere mortaliteit hetgeen mogelijk veroorzaakt wordt door een polymorfisme in de PAI-1 promotor regio.

In **hoofdstuk 11** werd onderzocht of de significant hogere PAI-1 produktie in overleden kinderen met meningococcen septische shock kan worden verklaard door de aanwezigheid van een 4G/5G polymorphisme in

de promotor van het PAI-1 gen. De aanwezigheid van de 4G allel is geassocieerd met een hogere basale produktie van PAI-1 en een toegenomen PAI-1 respons na stimulatie met cytokines en endotoxines. In eerdere studies werd reeds aangetoond dat het 4G/5G polymorphisme in de promotor van het PAI-1 gen geassocieerd is met verschillen in PAI-1 activiteit bij volwassenen met coronair ziekte en myocard infarcten. De hypothese geformuleerd in **hoofdstuk 10** was dat hogere PAI-1 produktie bij patiënten met een meningococcemie kan bijdragen tot ernstiger beloop van de ziekte. DNA monsters van 37 Nederlandse kinderen met meningococcen sepsis werden vergeleken met DNA monsters van gezonde controles. Genetische analyse van het PAI-1 promotor polymorfisme werd gedocumenteerd aan de hand van allel specifieke oligo-melting. Er werd geen verschil in genotype verdeling gevonden tussen overledenen en overlevende patiënten met meningococcen septische shock. Echter, het 5G/5G genotype was significant minder frequent aanwezig bij kinderen met een meningococcensepsis dan bij de controle populatie. Wij veronderstellen dat kinderen met meningococcemie en de aanwezigheid van het 4G/5G of 4G/4G genotype significant meer PAI-1 produceren, hetgeen aanleiding geeft tot een ernstiger ziektebeloop dan bij kinderen met het 5G/5G genotype.

In **hoofdstuk 12** werd de rol van IL-12 en IFN-γ in de pathofysiologie van septische shock met purpura onderzocht. IL-12 speelt een rol bij de afweer tegen invasieve micro-organismen en stimuleert de produktie van IFN-γ. De concentraties van IFN-γ zijn verhoogd bij zowel experimentele vormen van sepsis als bij patiënten met sepsis. Plasma concentraties van IL-12, IFN-γ en andere cytokines werden gemeten bij 46 kinderen met septische shock en purpura. Plasma concentraties van IL-12 p40, TNF-α, IL-6, IL-8, IL-10 waren bij opname significant hoger bij kinderen die overleden dan bij overlevende patiënten. IL-12 p70 concentraties waren slechts meetbaar bij 9 patiënten en de serumspiegels waren positief geassocieerd met die van IL-12 p40. Twaalf patiënten (29%) hadden meetbare concentraties van IFN-γ. Circulerende concentraties van IL-12 p40 en in mindere mate van IL-12 p70 waren verhoogd bij kinderen met septische shock en purpura. De serum concentraties waren positief gecorreleerd met de ernst van de ziekte en met de prognose van de ziekte.

In **hoofdstuk 13** werden leeftijdsafhankelijke verschillen in de uitkomst en de ernst van de stollingsstoornissen bij kinderen met een septische shock en purpura bestudeerd. Parameters van coagulatie en fibrinolyse en plasma concentraties van cytokines werden gemeten bij 79 patiënten. De mortaliteit in deze studie bedroeg 27%. De mediane leeftijd van de patiënten was 3.1 jaar. Overlevende kinderen waren significant

ouder dan de overleden patiënten. Ten behoeve van verdere analyse werden de patiënten verdeeld in twee leeftijdsgroepen: jonger en ouder dan de mediane leeftijd. De mortaliteit bij kinderen ≤ 3.1 jaar was 40% versus 13% bij kinderen > 3.1 jaar (P = .006). Met uitzondering van factor (F) VII, van Willebrand factor (vWF) en tissue plasminogeen activator (t-PA) waren de parameters van coagulatie en de concentraties van cytokines geassocieerd met de prognose. De cytokine concentraties waren echter niet verschillend tussen beide leeftijdsgroepen terwijl de plasma concentraties van fibrinogeen, prothrombine, FV, VII, VIII, vWF, protein C, antithrombine III, fibrinogeen afbraakprodukten en de ratio PAI-1/t-PA geassocieerd waren met de leeftijd. De stollingsstoornissen waren ernstiger bij kinderen ≤ 3.1 jaar ondanks de aanwezigheid van gelijke cytokine concentraties. Een relatieve deficiëntie van stollingsfactoren door een nog onrijp stollingssysteem en een inadequate fibrinolyse waren gerelateerd aan de leeftijd. De sterkere stollingsstoornissen bij jongere kinderen kan bijdragen aan de hogere mortaliteit in deze leeftijdscategorie.

In **hoofdstuk 14** worden de resultaten van het in dit proefschrift beschreven onderzoek kritisch bediscussieerd. Suggesties voor verder onderzoek worden gegeven.

List of abbreviations

List of abbreviations

ADH	: antidiuretic hormone
AIDS	: acquired immune deficiency syndrome
APPT	: activated partial thromboplastin time
ASO melting	: allele-specific oligo melting
BAEP	: brain stem auditory evoked potentials
BBB	: blood brain barrier
bp	: basepair
BPI	: bactericidal/permeability-increasing protein
°C	: degree Celcius
C1-INH	: C1-esterase inhibitor
CD	: cluster of designation/cluster of differentiation
CI	: confidence interval
CNS	: central nervous system
CRP	: C-reactive protein
CSF	: cerebrospinal fluid
CTAB	: cetyl-trimethyl-ammoniumbromide
DIC	: disseminated intravascular coagulation
DNA	: deoxyribonucleic acid
ELISA	: enzyme linked immuno sorbent assay
EPI	: extrinsic pathway inhibitor
Fc	: crystallizable fragment
FcγR	: Fc receptor for IgG
FDP	: fibrin/fibrinogen degradation products
g	: unit of gravity
h	: hour
H	: *Heamophilus*
Hib	: *Haemophilus influenzae* type b
IFN	: interferon
Ig	: immunoglobulin
IL	: interleukin
L-NAME	: N-nitro-L-arginine methyl ester
LBP	: lipopolysaccharide binding protein
LIF	: leukemia inhibitory factor
LPS	: lipopolysaccharide
mAb	: monoclonal antibody
mRNA	: mesenger mRNA
MSS	: meningococcal septic shock
n	: number in study or group
N	: *Neisseria*
NK-cell	: natural killer cell

NO : nitric oxide
NOS : nitric oxide synthase
OMP : outer membrane protein
PAF : platelet activating factor
PAI : plasminogen activator inhibitor
PAP : plasmin antiplasmin complex
PC : protein C
PCR : polymerase chain reaction
PG : prostaglandin
PICU : pediatric intensive care unit
PMNL : polymorphnuclear leukocytes
PRISM : pediatric risk of mortality
r : recombinant (e.g. rt-PA)
R : receptor
RFEL : restriction fragment end labeling
RNA : ribonucleic acid
RR : relative risk
rt-PA : recombinant tissue plasminigen activator
S : *Streptococcus*
SD : standard deviation
SIADH : syndrome of inappropriate secretion of antidiuretic hormone
SOD : superoxide dismutase
sTNFR : soluble tumor necrosis factor receptor
t-PA : tissue plasminogen activator
TAT : thrombin-antithrombin complex
TGF : transforming growth factor
TNF : tumor necrosis factor
UPGMA : unweighted pair group method using arithmetic averages
vWF : von Willebrand Factor
WBC : white blood cell
WHO : World Health Organization

Dankwoord

DANKWOORD

Het afsluiten van een periode van onderzoek met een proefschrift is veelal het resultaat van een intense samenwerking met velen. Aan het einde van dit boekje wil ik dan ook een ieder bedanken die een bijdrage heeft geleverd. Een aantal mensen wil ik in het bijzonder bedanken.

Dr. R. de Groot, co-promotor, beste Ronald. Onze samenwerking begon met als uiteindelijk doel een promotie. Hoewel een promotieonderzoek en de benodigde financiën aanvankelijk nog niet beschikbaar waren, werd dit ruimschoots gecompenseerd door je enthousiasme en niet aflatende stroom aan ideeën. Mede dankzij jouw grote werkdrift werden deze ideeën omgezet in voortreffelijke onderzoeksprojecten die hebben geleid tot dit proefschrift. Ondanks je drukke werkzaamheden was je altijd bereikbaar voor overleg. Eventuele hindernissen werden snel genomen. Naast wetenschappelijk inspanningen was er ook tijd voor ontspanning ('Even een biertje drinken'). De periode die achter mij ligt heb ik ervaren als uiterst plezierig en leerzaam. Bedankt voor alles.

Prof. Dr. H.J. Neijens, promotor, beste Herman, ik wil je hartelijk bedanken voor de gelegenheid en de vrijheid die je mij hebt geboden om onderzoek te doen binnen de subafdeling infectie- en immuunziekten van het Sophia Kinderziekenhuis te Rotterdam. Dank voor je kritisch beoordeling van het manuscript en voor de leerzame adviezen tijdens onze gesprekken.

De overige leden van de kleine commissie, Prof. Dr. J.J.M. van Dongen, Prof. Dr. J.W.M. van der Meer en Prof. Dr. H.A. Verbrugh wil ik bedanken voor hun bereidheid het manuscript op snelle wijze kritisch te beoordelen.

Dr. E.D. Wolff, beste Eric. Bij jou heeft mijn interesse voor het wetenschappelijk onderzoek wortel geschoten. Ik wil je hiervoor dan ook hartelijk bedanken.

Beste Henriëtte van Vuuren, research-verpleegkundige van het eerste uur, je was in de beginfase betrokken bij het beschreven onderzoek. Het meningitis-onderzoek en later het sepsis-onderzoek zijn mede dankzij jouw inspanningen geleidelijk van de grond gekomen. Nadat je de moedige stap hebt genomen de opleiding tot IC-verpleegkundige te beginnen, heeft Marja Suur je taken op uitstekende wijze overgenomen. Beste Marja, je organisatorische talenten en inzet zijn van ongekende hoogte. Groots is je prestatie om naast het "gewone werk" de graad van Master of Science te behalen. Ik ben jullie zeer dankbaar voor alle inspanningen en het vervullen van bereikbaarheidsdiensten. Mijn dank gaat ook uit naar Toos Langeveld, Yolanda van den Ouden, Alma van Zantwijk, Atie Bruggeling en Ester de Kleijn die ook deelnamen aan het patientgebonden onderzoek.

Beste semafoon, ik mis je niet, maar dankzij de vele keren dat je onze rust hebt verstoord, hebben wij veel kostbaar materiaal kunnen verzamelen dat de basis vormde voor het beschreven onderzoek. Ik realiseer mij dat het dragen van een semafoon buitengewoon lastig kan zijn.

Vervolgens gaat mijn dank uit naar de kinderartsen en de arts-assistenten van de ziekenhuizen die deelnamen aan het meningitis-onderzoek, te weten het Reinier de Graaf Gasthuis te Delft, het Zuiderziekenhuis en het Ikazia Ziekenhuis te Rotterdam, en het Juliana Kinderziekenhuis te Den Haag. Zonder jullie aanmelding van patiënten zou het onderzoek zeker niet zo'n succes zijn geworden.

Het sepsis-onderzoek werd uitgevoerd op de subafdeling Extra Zorg, later Intensive Care Pediatrie. Drs. J.A. Hazelzet en Drs. E. van der Voort, beste Jan en Edwin, ik wil jullie bedanken voor de prettige wijze waarmee onderzoek op jullie afdeling mogelijk werd gemaakt. Jan, ik hoop dat ook jij binnenkort je proefschrift kan afronden.

Mijn dank gaat ook uit naar ouders die toestemming gaven voor deelname van hun ernstig zieke kind aan het beschreven onderzoek.

Plezierig, nuttig en efficiënt was de samenwerking met de afdeling Immunologie aan de Erasmus Universiteit te Rotterdam. Dr. Huub Savelkoul, Dr. Hedda Koning, Astrid Vredendaal en Miranda Baert wil ik hartelijk bedanken voor de prettige manier waarop jullie mij geleerd hebben cytokines te bepalen. De goede samenwerking maakte het mogelijk om met geringe hoeveelheden bloed en liquor vele cytokines tegelijkertijd te bepalen. Bedankt.

Dr. Peter Hermans en Marcel Sluijter wil ik bedanken voor mijn inwijding in het moleculaire werk.

Dr. W.C.J. Hop, beste Wim, met genoegen denk ik terug aan onze discussies ten aanzien van de statistische analyses uitgevoerd in het kader van al het onderzoek. Ik wil je hartelijk bedanken voor de snelle service en alle goede adviezen.

Ook ben ik dank verschuldigd aan Dr. Lodewijk Spanjaard (Referentie-laboratorium voor Bacteriële Meningitis te Amsterdam), Dr. Klaas Hoekman, Dr. Jelle Visser (Vrije Universiteit te Amsterdam), Prof. Dr. Erik Hack, Gerard van Mierlo (CLB te Amsterdam), Dr. Jan Huijmans en Robin Janki (Erasmus Universiteit te Rotterdam) en de medewerkers van het klinisch chemisch laboratorium en de speciële hematologie (Sophia Kinderziekenhuis te Rotterdam). Ik wil hen bedanken voor de vele bepalingen die zij hebben verricht. Tevens wil ik hen bedanken voor alle nuttige adviezen. De subafdeling hematologie/oncologie van het Sophia Kinderziekenhuis wil ik hartelijk bedanken voor hun bereidheid op snelle wijze vele liquor en serummonsters ten behoeve van normaalwaarden te verzamelen.

De studenten, Alex Borsboom, Mark van Iperen, Andy Spoor, Annemieke Ruitenberg, Carla Westerbeek, Mariëlla Withagen hebben actief deelgenomen aan een aantal facetten van het genoemde onderzoek. Hen wil ik bedanken voor hun interesse en inzet.

Riet Visser en Jaqueline de Vogel, dank voor de goede regulatie van de geldstromen en voor jullie interesse in de voortgang van het onderzoek.

Mijn (ex-)collega's tijdens mijn periode als onderzoeker, Wouter de Waal, Hans van Goudoever, Eric Sulkers, Theo Sas, Svatti Patandin, Jan-Erik Bunt, Corine Koopman, Marjon Cnossen, Hanneke IJsselstein, Arne van Teunenbroek, Arjen van Esch, Niels de Bruin, Eveline Nieuwhof, Henna Pollack, Ineke Groothuis, Karin Vos, Annemarie Ferwerda, Freek van den Heuvel, Margriet Stuijvenberg, Anneke Boerlage, Annemieke Boot, Rijk van Ginkel, Janneke van Nieuwkasteele wil ik bedanken voor de prettige werksfeer en de gezelligheid tijdens en naast het werk.

Beste Anneke van Duuren, ik wil je hartelijk bedanken voor de razendsnelle secretariële ondersteuning bij het gereed maken van het manuscript.

Vanaf 1 oktober 1995 moesten mijn werkzaamheden als fulltime onderzoeker plaatsmaken voor patiëntenzorg en de kliniek in het kader van mijn opleiding tot kinderarts. De overgang van onderzoek naar klinische taken was groot. Zelfstandigheid moest plaats maken voor leemte aan kennis en vaardigheden.

Mijn collega's arts-assistenten en de kinderartsen in het Sophia Kinderziekenhuis en in het Sint Franciscus Gasthuis te Rotterdam wil ik bedanken voor de plezierige werksfeer. Tevens ben ik hen dankbaar voor de extra vrije tijd die mij is geboden om het proefschrift voor 1997 te kunnen voltooien.

Verder wil ik iedereen bedanken die heeft meegeleefd naar de voltooiing van dit proefschrift.

Mijn ouders wil ik bedanken voor de opvoeding die zij mij gaven. Tevens ben ik hen dankbaar dat zij mij in de gelegenheid hebben gesteld te studeren. Daarnaast wil ik mijn ouders en schoonouders bedanken voor hun steun en om daar waar het nodig was bij te springen. Ik kan altijd op jullie rekenen.

Lieve Monique, zonder jouw steun en vertrouwen zou ik de combinatie van opleiding tot kinderarts, afronding van een proefschrift en een gezinsleven onmogelijk hebben kunnen volhouden. Ik ben je hier dan ook heel dankbaar voor. Lieve Anne, het boekje is klaar en als papa niet bij de zieke kindjes is kan hij nu meer tijd met jou en mama doorbrengen.

René

Oktober 1996

Curriculum vitae

List of Publications

CURRICULUM VITAE

René Frank Kornelisse was born in Rotterdam, The Netherlands, on January 6, 1964. He passed his secondary school exam (VWO) in 1982 at the Albert Einstein Lyceum in Hoogvliet. In 1982, medical training was started at the Medical Faculty of the Erasmus University Rotterdam. In June 1989 the Medical Degree (cum laude) was obtained. From January 1989 until the end of 1990, a research project was performed entitled "Protein metabolism in rats with renal failure", at the Department of Pediatrics, division of Nephrology (head: Dr. E.D. Wolff), Sophia Children's Hospital, Rotterdam. At the end of 1990 the research presented in this thesis was initiated under the supervision of Dr. R. de Groot in the Division of Infectious Diseases and Immunology (head: Prof. Dr. H.J. Neijens). This project involved several studies on clinical aspects and host response in patients with bacterial meningitis and meningococcal sepsis. Between 1990 and 1995 the data of the work described in this thesis were collected. In October 1995 he enrolled in the residency program in Pediatrics at the Sophia Children's Hospital, Rotterdam (head: Prof. Dr. H.K.A. Visser/Prof. Dr. H.J. Neijens). At present he is continuing his specialist training at St. Franciscus Gasthuis, Rotterdam under the supervision of Dr. R. Spritzer. Since 1992 he has been married to Monique Vogelezang, and they have one daughter: Anne.

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