SEPTIC SHOCK WITH PURPURA IN CHILDREN:

AN EXPERIMENTAL AND CLINICAL APPROACH

SEPTISCHE SHOCK MET PURPURA BIJ KINDEREN:

EEN EXPERIMENTELE EN KLINISCHE BENADERING

Jan A. HAZELZET

Cover:

Fig: 1. "*The Neisseria Meningitidis*", Ciba.

Fig: 2. Piglet "Onno", Hans de Beer

Fig: 3.+4. Esther, Patient nr 1 in the database

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CHAPTER 1

GENERAL INTRODUCTION

Septic shock with purpura is a rapidly evolving clinical picture characterized by sepsis (tachycardia, tachypnea, fever), shock (hypotension or signs of end-organ failure) and a spectrum of coagulation disorders (ranging from petechiae, purpura to ecchymoses). It is mainly (80 %) caused by *N. meningitidis* (group A, B or C). However, occasionally other micro-organisms such as *Haemophilus influenzae, Haemophilus aegyptius, Streptococcus group A, group B streptococcus, streptococcus pneumoniae, Staphylococcus aureus, Pseudomonas species* and *Capnocytophagus canimorsus* can be detected as a causative agent. Even viruses can under certain conditions cause a comparable clinical picture. Approximately 80 % of the patients is below the age of 18 years, and 50 % below the age of 5 years. The development of the disease in older patients is partly due to the presence of a deficiency in the immune system (complement deficiency, spleen extirpation, diabetes mellitus). The occurrence in children is related to the absence of antibodies, although it is assumed that these children are healthy except for an age-related immaturity of their immune system. The mortality ranges from 15 - 40 %, depending on the selection of the patients.

It is difficult to extrapolate the pathophysiology, clinical findings and therapeutic outcome in adults with sepsis to those in children. In most studies in adults a variety of micro-organisms and underlying diseases has been observed. In contrast, children with meningococcal sepsis form a relatively homogeneous group in which age-related developmental processes in the first years of life probably provide an explanation for the very high mortality in this young age group. The frequent occurrence of this awe-inspiring life threatening clinical picture in the Netherlands encouraged us to perform the studies described in this thesis. To this purpose we prospectively studied the pathophysiology of meningococcal disease, mainly focusing on the role of pro-and counter-inflammatory cytokines, complement activation and coagulation and fibrinolysis. We also initiated investigations on the role of genetic variability in immune response on the course of disease. A large number of the children studied were concomitantly enrolled in a prospective study on the efficacy of HA-1-A, a monoclonal antibody to neutralize circulating endotoxin. Since there were several questions which could not be answered in human studies, we developed a sepsis model in infant and nearly-adult pigs to analyze the presence of age-related differences in the course of the disease of these animals. All patients described in this thesis were admitted to the Pediatric Intensive Care Unit of the Sophia Children's Hospital in Rotterdam. The first child was enrolled in August 1988. The animal experiments were carried out in the Laboratory for Experimental Cardiology, Erasmus University Rotterdam.

CHAPTER 1.1

AIMS OF THE STUDIES

Chapter 1 provides a general overview of the characteristics of sepsis in children **(1.2)** and reviews the current literature on the pathophysiology of septic shock and purpura **(1.3)**.

Chapter 2 mainly contains studies which focus on the effects of LPS and the relation between LPS and cytokines. This chapter describes the binding and clearance of endotoxin in young and old pigs during experimental sepsis **(2.1)**, the different levels of pro-inflammatory mediators (TNF-α, IL-1, and IL-6) in patients with sepsis and the relation between these levels and standard laboratory parameters **(2.2)**, as well as the presence during sepsis of IL-12, a recently described cytokine **(2.3)**. This cytokine induces the production of interferon-γ (IFN-γ) by T cells and natural killer cells. Recently, IL-12 was characterized as a major cytokine in the pathogenesis of gram-negative endotoxemia in mice and in primates.

Chapter 3 describes studies on disorders of coagulation and fibrinolysis in children with septic shock and purpura. In these studies the following questions were discussed:

3.1: How are plasma levels of protein C and S related to presence of disseminated intravascular coagulation (DIC), outcome and severity of disease? This study was carried out in collaboration with the University Hospital of Lille, France.

3.2: Are there age related differences in coagulation parameters between younger and older patients?

3.3: What is the relationship between plasminogen activator inhibitor 1 (PAI-1) and serum cytokine levels in children with septic shock and purpura?

3.4: Is the presence of a 4G/5G promotor polymorphism in the PAI-1 gene related to more severe coagulopathy and fatal outcome?

In **Chapter 4** experimental and clinical features of circulatory failure are discussed. Two major contributing factors to circulatory failure are the capillary leakage leading to a decrease in preload, and the development of myocardial dysfunction. The following issues were studied:

4.1: What is the importance of the complement system in relation to capillary leakage, severity of disease and outcome?
What is the extent of the complement activation, the possible route and regulation of this activation, and the relation of this activation to other inflammatory mediators?

4.2: Since many of the invasive hemodynamic aspects of sepsis can not be assessed in pediatric patients, animals were used to study these items. In this chapter the hemodynamic effects of 2 different dosages of meningococcal toxin are studied in younger and older piglets.

4.3: The clinical and laboratory parameters from 75 patients are evaluated. With these data we developed a potentially useful prognostic score consisting of four simple laboratory parameters.

Chapter 5 In this concluding chapter the results from the previous studies are summarized and put into a future perspective.

CHAPTER 1.2

SEPSIS-RELATED PROBLEMS IN PEDIATRIC PATIENTS

Jan A. Hazelzet and Ronald de Groot

Sepsis, current perspectives in pathophysiology and therapy. 18ed. Berlin Heidelberg, New York; Springer Verlag, 1994: 214-27

1 INTRODUCTION

The physiologic changes that constitute the process of sepsis are induced by microbial agents during bloodstream infection or by the toxic products of pathogens that are released from sites of focal infection. This process involves changes generated by the immune system, and controlled by hormones, cytokines and enzymes. There are many host and microbial factors that may unfavorably influence this complex immune-response. Underlying diseases with immunosuppressive effects, surgery, state of nutrition, immunomodulatory therapy, and site of infection, increase the risk for development of invasive disease and determine the extent of the immune response (10, 20,181). However, sepsis in the pediatric population occurs very frequently in previously healthy children. Also, in comparison with adults, the sepsis is more overwhelming. The mortality is mainly confined to the first 48 hours of the disease. In children vs adults, different pathogens are involved and the quality and quantity of the immune-response depend on the age of the child. These differences are probably caused by developmental aspects of several organ systems and may have special consequences for management and therapy.

2 EPIDEMIOLOGY

The incidence of sepsis in children shows a bimodal distribution. The first peak is in the neonatal period (defined as infants < 1 month of age): 4.3/1000 newborns from which 60% in the first 5 days and an overall mortality of about 20 % (98), and a second peak around the age of 2 years. These periods are two milestones in the development of immunocompetence of the child. In children 1-4 years of age sepsis represents the 9th leading cause of death with an estimated annual mortality rate of 0.5/100.000 population. The pathogens involved in this age group are in the first 2 months mainly *group B Streptococci* (GBS) and *Escherichia coli* (98, 385), and later on: *Neisseria meningitidis*, *Haemophilus influenzae*, *Streptococcus pneumoniae* (all of them encapsulated micro-organisms, which are protected against complement mediated phagocytosis and killing), *Streptococcus pyogenes*, and *Staphylococcus aureus*. In addition an extensive list of other pathogens is involved in nosocomial infections (180). Host factors predisposing to nosocomial infections are similar to those in adults (10, 248).
Sepsis in young children caused by severe meningococcal disease is rapidly progressive, overwhelming and its mortality is high (20-60%) in the first 24-48 hours. Late mortality seems to be rare (44).
There are several clinical syndromes in pediatric patients which show much resemblance with the clinical picture of septic shock. Their relation with toxins is either established or hypothesized. Examples of these syndromes are the toxic shock syndrome (16),

necrotizing enterocolitis (16), and the hemorrhagic shock and encephalopathy syndrome (67).

Clarification of current terminology is important to understand the pathophysiology of the septic process. A definition for sepsis in infants and children is suggested Jafari (181), which is adapted from guidelines proposed for older patients (Table 1). We believe that the diastolic bloodpressure should be included, because in the early phase of sepsis patients are sometimes able to maintain their systolic, but not their diastolic bloodpressure. This results in a low mean bloodpressure and an altered organ perfusion leading to organ dysfunction.

Table 1. Definition for sepsis in pediatric patients [a]

Bacteremia	Presence of viable bacteria in the circulating blood confirmed by culture.
Sepsis	Clinical suspicion of infection and evidence of systemic response to infection (tachycardia, tachypnea, hyperthermia or hypothermia).[b]
Sepsis syndrome	Sepsis plus evidence of altered organ perfusion with at least one of the following: acute change in mental status [c], oliguria [d], elevated blood lactate and hypoxemia.
Septic shock	Sepsis syndrome with hypotension [e] requiring fluid resuscitation and/or vasopressor support.

[a] Adapted from Jafari (181)

[b] Tachycardia and tachypnea > 2 standard deviations (SD) for age, hyperthermia > 38.5 °C; hypothermia < 36 °C.

[c] A reduction by at least 3 points from baseline value in Glasgow coma score.

[d] Diuresis < 0.8 ml/kg/hr.

[e] Systolic or diastolic blood pressure < SD for age.

3 RECOGNITION

Clinical signs and symptoms of sepsis in the pediatric population depend very much of the age of the child. Especially in the first 6 months the symptoms are non-specific. In contrast to most adults or older children, a variety of thermal responses are seen in this age group. Apnea, leading to respiratory insufficiency, is an important sign. Extension of the bacteremia to the meninges is seen in 30 % of the patients with sepsis. Seizures and focal findings are often associated with the presence of meningitis. The hemodynamic signs in patients with sepsis include tachycardia, hypotension (systolic and diastolic) and cold extremities indicating an increased systemic vascular resistance (SVR). The early stages of warm septic shock in infants and older children are heralded by signs of hypoperfusion. The earliest signs are a change in mental state and a decreased urine output. Other signs include tachycardia, a widened pulse pressure with an increase in cardiac output and a decrease in SVR. The rapid progression of the disease and the early exhaustion are

characteristic. The remainder of the clinical picture in this age-group is comparable with that in adults.

4 PATHOPHYSIOLOGY

The interaction between microbial and host factors may partly explain the differences in immune-response between children and adults.

4.1 Microbial factors

Group B streptococci (GBS) are among the leading causes of overwhelming neonatal sepsis. Although other micro-organisms may cause similar clinical syndromes, most investigators have used experimental animal models of GBS infection to study the pulmonary and cardiac hemodynamics of neonatal septic shock. Gram-positive bacteria such as GBS do not have endotoxin. Two different polysaccharide toxins of GBS have been described (164). The polysaccharide capsule may also contribute to the organism's virulence through its protection from host phagocytosis. Although the presence of neutralizing maternal IgG antibodies to the capsular polysaccharide correlates with protection against disease in neonates, the age-related risk of GBS in newborns has not been fully explained.

Severe meningococcal disease is the most frequent cause of sepsis in children above 3 months of age and in teenagers without underlying disease. In several countries in Europe the incidence has increased during the last decade. The clinical picture includes an overwhelming septic shock with purpura. The mortality remains high, in spite of extensive research especially regarding the pathogenetic role of endotoxin (354). The reason for this specific clinical picture is still not completely elucidated. The endotoxin or lipopolysaccharides (LPS) of many non-enteric gram-negative organisms, such as *Neisseria meningitidis* and *Haemophilus influenzae* lack the outer polysacchardide O-chain and contain only the oligosaccharide and the lipid A region often referred to as lipo-oligosaccharides (LOS). The lipid A part is considered to be the most bioactive and toxic part and extensive investigative efforts have been directed towards developing anti-lipid A antibodies. However, in animal experiments there are differences in lethality and in neutralization by polymyxin B between LOS and LPS from *E.coli.* (13) and the lipid A cores of gram-negative organisms are known to be heterogenous. Furthermore, the meningococcus contains surface components and secretes molecules and complex vesicle structures (blebs) that may modulate or deflect the immune system. The bacterium can undergo phase and antigenic variation and is able to use host factors for its own protection and growth (353). These blebs are shed through the body and contain LOS, and also other membrane parts which may also have antigenic properties. Ultracentrifugation

studies on plasma specimens from patients with severe meningococcal disease (SMD) suggest that most of the circulating LOSs are present as large fragments or aggregates (44), which make recognition by the immune system probably more difficult.

4.2 Hostfactors

4.2.1 immune-response

Developmental aspects of the pediatric immune response are still unknown to a large extent. The majority of the work has been performed in the neonate. Much less is known about the growing child. At birth, a child is protected against a variety of bacterial and viral agents by maternal antibodies and remains less susceptible for many infections untill the age of 6 months. The immunoglobulin-synthesis of the infants increases untill at the age of 2 years about 75 % of adult values are reached. The IgG and IgA-synthesis produced after a certain antigenic stimulus is limited until the age of 2- 4 years. Polysaccharide capsules does not give an appropriate immune response and does not induce immunological memory. This is due to an immature T-cell function. These problems can be circumvented by covalently linking the polysaccharide antigens to proteins, the principle used in the production of conjugate vaccines (276, 354). There are many differences between the neonatal polymorphonuclear leukocyte (PMN) and the adult PMN. The response of the neonatal PMN to chemotactic signals initiated by infectious agents is poor. Further disaggregation of PMN and migration to the infected tissues is markedly depressed. Depletion of circulating neutrophils with the exhaustion of the neutrophil storage pool in the bone marrow is more likely to occur because the storage pool is low (14 % of the adult pool) and the turnover rate is high (53). In comparison with adults, there are differences in the cytokine-production of mononuclear cells when they are stimulated in vitro (95). Several studies over the past 5 years have indicated the possible benefit of hematopoietic colony-stimulating factors (CSF). These cytokines may in fact enhance neonatal myeloid progenitor proliferation, modulate neonatal bone marrow neutrophil storage and proliferate pools, and enhance neonatal host defense against overwhelming bacterial infection (52). The characteristics of the PMN of the growing child are not studied in detail.
Results of studies regarding age related differences in susceptibility to and mortality of sepsis between infant and adult animals are controversial and probably depend the experimental model (284, 367), the exact age (284), and the pathogen involved (284).

4.2.2 metabolic-response

Sepsis may lead to pronounced metabolic alterations: elevations of plasma glucose, lactate, insulin, free fatty acids concentrations, and increased muscle protein breakdown

are well known changes during the early phase of sepsis in the adult patient. The differences in metabolic response to sepsis between infants and adults are not completely understood. Muscle protein metabolism data from experimental studies suggest that despite prominent differences in basal protein turnover rates between infant and adult rats the effect of sepsis on muscle protein metabolism is not age dependent (266, 384). The glucoregulatory changes in the developing human and animal are different from the adult. In young infants during sepsis, there is an increased glucose disappearance rate without hyperinsulinaemia (107, 284, 387). In an experimental sepsis model in rats, studying age related differences, the adult rats became hyperglycemic, while young animals were hypoglycemic eight hours after cecal ligation and normalized after 16 hours (266) In the human situation infants with a severe septic shock often have a hypoglycemia which necessitates correction.

4.2.3 cardiovascular system

Clinical studies in adults have shown that myocardial dysfunction begins within hours of sepsis and peaks in 1-2 days with survivors returning to normal function in a 7-10 days period. Hyperdynamic circulatory state in survivors is probably maintained by ventricular dilatation. Again, studies in adults present conflicting results regarding the coronary perfusion during sepsis. Similar studies in children are not available. However, there are several experimental studies, which have shown the presence of age-related differences between neonatal and adult animals in myocardial reserve (which increases because of an improving diastolic compliance), relation between contractile and non-contractile myocardial elements, metabolic substrates (from carbohydrates to free fatty acids), sensitivity for hypoxemia, maturation of the pulmonary vasculature and, sensitivity of sympathic receptors to catecholamines (5, 105, 173, 306) . Our group is preparing an experimental septic shock model in young piglets using meningococcal endotoxin to study the differences between young and adult pigs. Only a small number of studies have been published about the myocardial dysfunction during septic shock in children (38, 238, 242). These studies were performed in pediatric patients with meningococcal sepsis. They revealed hypovolemia and severe disturbances in cardiac function as indicated by left ventricular shortening fraction (echocardiography) and cardiac output (thermodilution). Myocardial dysfunction in septic shock in children reaches a maximum within hours, and is the main cause of early mortality. Recovery takes 1-3 days. Cardiac output (C.O) in infants is classically thought to be more rate- than stroke-volume-dependent, which is probably the reason for cardiac frequencies of sometimes over 180 b/min. It is unknown to what extent ventricular dilatation is useful in the maintainance of a possible hyperdynamic state. It is also not known whether there is a hyperdynamic state in infants (238). C.O in infants is very much preload-dependent. Therefore fluid resuscitation is an important step in the initial

management of children with septic shock to overcome the hypovolemia on the basis of a diffuse capillary leak (58, 59). Altered vascular tone and flow-oxygen utilization systemic relationships are considered a hallmark of sepsis in children. An altered vascular tone leads to decreased peripheral vascular resistance (SVR). This results in a lowered diastolic aorta pressure. It is this pressure in combination with the duration of the diastole which are the two main determinants of coronary perfusion and indeed, coronary ischemia may be seen in children even in the first 24 hours after admission.

4.2.4 coagulation system

Especially in meningococcal sepsis, but also in other forms of pediatric and neonatal sepsis, thrombopenia and disseminated intravascular coagulation (DIC) can be found leading to petecchiae and ecchymoses. At birth several clottingfactors are present at serumlevels much lower than in adults. This possibly explains why the balance is easily switched towards coagulation. During the first two years of life these serumlevels increase untill adult values are reached (8, 346). In meningococcal sepsis in children, there is an extreme depletion of anticoagulatory factors like protein C and S (207) as opposed to adults in which a depletion of antithrombine III predominates. Protein C has gained interest because its possible role in sepsis and its protective effect in experimental sepsis (323). The combination of extreme capillary vasoconstriction, caused by mediators like endothelin, and the increased deposition of fibrin, caused by the hypercoagulatory state and a decreased fibrinolysis, will lead to an increased formation of microthrombi and in this way to auto-infarction and gangrene.
Therefore an important part of the volume-suppletion which is administered in our hospital during the acute phase of the sepsis comprises of fresh frozen plasma (FFP), which still is the only balanced substitute of clotting factors in this situation. However there have been no controlled studies to prove the superiority of FFP over other colloids in this situation. On the contrary, recently a study was published in experimental sepsis which indicated the presence of deleterious effects of plasma therapy (51).

5 MANAGEMENT ASPECTS

5.1 Cardiovascular function

The abnormal microcirculation and a decreased aortic diastolic pressure cause a compromised coronary perfusion in children with septic shock. Together with possible depressing mediators this will lead to a severely depressed myocardial function with a decreased stroke-volume. Alfa- and beta-adrenergic support may possibly restore a

normal mean arterial pressure and systemic vascular resistance. To inhibit or improve capillary vasoconstriction, a local vasodilatator could be added. Recently Heyderman (169) presented findings suggesting that a deficiency of prostacyclin (PGI_2) may have a role in the pathogenesis of meningococcal shock and that exogenous PGI_2 may be of therapeutic benefit. There is no consensus on which particular inotropic drugs are most useful. Especially in children the pharmacodynamics of these drugs are still not well known. The combination we use is noradrenaline + dobutamine + prostacyclin, with the lowest possible α-agonist dose to achieve a mean arterial blood pressure which enables an adequate urine output and no further increase in oxygen consumption.

5.2 Respiratory function

More than 60% of critically ill children with septic shock are intubated and ventilated within 24 hours after admission. Indications for intubation are hypoxia, apnea, acidosis, increased work of breathing (W.O.B), and neuromuscular blokkade for invasive procedures. About 40% of these patients develop ARDS. In children with heartfailure, W.O.B can require as much as 25% of oxygen consumption. Extrapolating a simular effect of cardiac dysfunction and increased W.O.B to ARDS in sepsis leads to the general recommendation to intubate and ventilate in an early stage and not to wait untill a disease stage in which intubation becomes a dangerous procedure. Delicate handling of the fluid balance because of increased extravascular lungwater is necessary.

5.3 Fluidresuscitation and Monitoring

The importance of fluidresuscitation to overcome the hypovolemia caused by capillary leak has already been mentioned. Sometimes 100-200 ml/kg of colloids (like FFP) are needed during the first day of treatment. Because of several reasons it is not daily practice to insert pulmonary artery catheters in every patient but central venous pressure monitoring and echocardiography is indicated and frequently used (38, 59, 128, 238). Lactate and several coagulation parameters are used as a tool to monitor the condition of the patient.

6 PROGNOSTIC FACTORS

Several prognostic factors have been related to non-survival:
increased levels of: Serum-endotoxin (44), TNF,IL-1 (362), IL-6 (159, 298, 320, 360), IL-8 (145), lactate (159), plasminogen activator inibitor-1 (42), ICAM-1 (200), procalcitonin (11), elastase-α_1-proteinase inhibitor (314, 326).
decreased levels of: CRP (159, 206), fibrinogen, glucose, protein-C (207), leucocytes, platelets (174), as well as several scoring systems (205, 206, 208, 274).

The morbidity of sepsis in pediatric patients depends on the age and the causative pathogen. In the neonate and infant the neurologic sequelae can be more severe especially if meningitis is present. Chronic renal failure after a septic period is rare in children. In particular in SMD the morbidity is relatively low and is limited to skin lesions and amputations of extremities.

7 FUTURE THERAPY

Prevention is still superior to treatment. Progress has been made in the field of active immunization. In several countries in Europe immunization has been started against *Haemophilus influenzae*. It will take at least another 5 years to develop a vaccin against *N. meningitidis* group B. Passive immunization using either pooled plasma or pathogen specific-immunoglobulins has been used in neonates. A limited number of studies have been performed and they suggest a beneficial effect in GBS-infections (104). Administration of neutrophils to infants with significant neutropenia has been suggested as approach (98). The European multicenter trial using HA-1-A in pediatric patients with septic shock and purpura is still ongoing. More than 170 patients are included. Until this trial has been concluded, a recommendation for the use of HA-1-A in these patients can not be given. Concerning the extensive list of other possible immunomodulative therapies, it is important to realize that on admission of a septic child, the process is in a well advanced stage, so that only influencing the momentary endotoxin-level will have a limited influence on outcome. Trials should be directed to influence the micro-circulation. Currently, a trial with recombinant protein C is in preparation.

8 CONCLUSIONS

- There is only limited epidemiological and clinical data available on children with septic shock. There is a need for a universal definition in children with sepsis. This definition may be adapted from guidelines proposed for adults.
- Septic shock in neonates and infants is more overwhelming than in adults. The highest mortality is in the first 48 hours and does often occur in previously healthy children.
- Specific sepsis-related differences between adults and pediatric patients are probably caused by developmental aspects especially in the cardiovascular and coagulation system, and the immune-, and metabolic response.
- Pathophysiological aspects of sepsis especially in the infant-age range should be subject of further research.

CHAPTER 1.3

PATHOPHYSIOLOGY OF MENINGOCOCCAL SEPSIS IN CHILDREN

Ester D. de Kleijn, Jan A. Hazelzet, René F. Kornelisse and Ronald de Groot

Eur J Pediatr, in press

1. INTRODUCTION

Meningococcal disease, first described by Vieusseux in 1805 as epidemic cerebrospinal fever, remains a major health problem in many countries (356). Clinical manifestations vary from self-limiting bacteremia to meningitis or fulminant sepsis (figure 1).

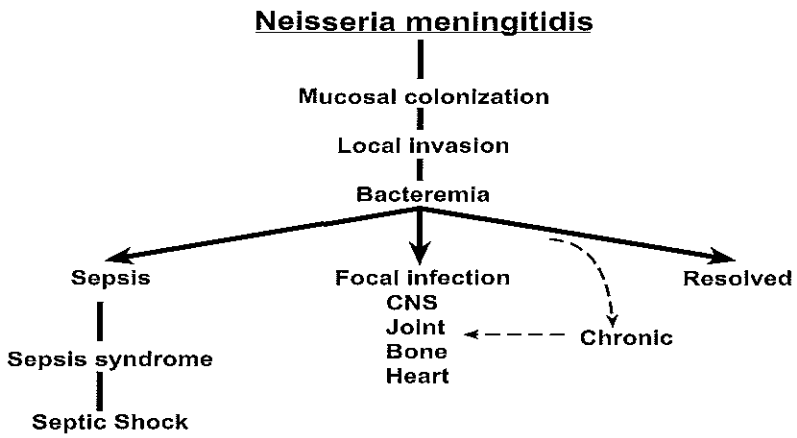

Figure 1. Clinical evolution of infections by *Neisseria meningitidis*.
Bacteremia: presence of viable bacteria in the circulation blood confirmed by culture
Sepsis: clinical suspicion of a severe infection and evidence of systemic response to infection (tachycardia, tachypnea, hyperthemia or hypoterhmia)
Sepsis syndrome: sepsis plus evidence of altered end-organ perfusion with at least one of the following: acute changes in mental status, oliguria, elevated lactate and hypoxemia
Septic shock: sepsis syndrome with hypotention requiring fluid resuscitation and/or vasopressor support
CNS: central nervous system.

Meningococcal sepsis is characterized by a rapid onset of disease, fever, purpura and ultimately shock. Non-specific presenting symptoms such as a flu-like picture and a rash are commonly observed. The diagnosis of meningococcal disease becomes obvious when petechiae develop. Meningococcal disease is predominantly seen in children with a peak incidence around 2 years of age. A second peak is noted among teenagers. The overall mortality rate of meningococcal septic shock varies between 20% and 50%, is higher in infants than in older children and has not changed significantly over the past three decades despite improvements in management and therapy (44, 121, 146, 158, 250, 279, 343, 380).

The systemic inflammatory response in patients with meningococcal disease aims to

neutralize microorganisms and their toxic products, but it may also overreact and thus induce serious tissue damage to the host. Three pathways characterize the intravascular inflammatory response: 1) activation of cascade systems, 2) the release of intercellular pro- and anti-inflammatory mediators and 3) altered function of endothelial cells in the vascular wall. In meningococcal infection coagulation, fibrinolysis, complement and kallikrein-kinin systems, cytokine production, and activation of neutrophils and platelets, are all apparently upregulated by native lipopolysaccharides (LPS) in a dose-dependent manner (45) (figure 2). This review provides an overview of the systems involved in the pathophysiology of meningococcal sepsis and of possible therapeutic interventions.

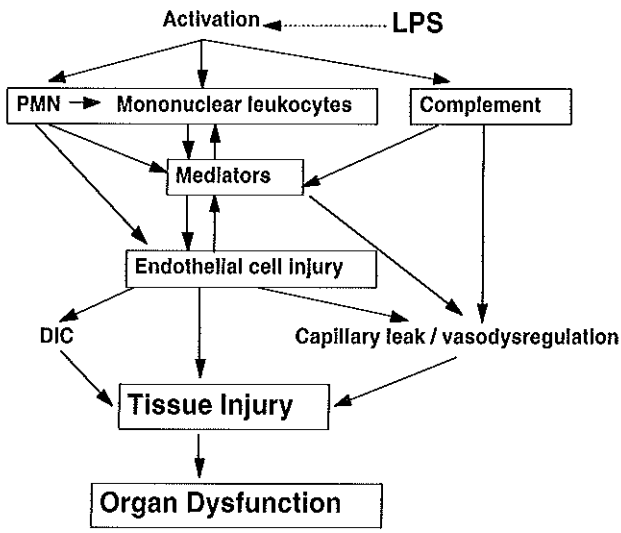

Figure 2. Pathophysiology of meningococcal sepsis
LPS: Lipopolysaccharide, PMN: Polymorphonuclear leukocytes, DIC: disseminated intravascular coagulation.

1.1 Role of LPS in meningococcal disease

Meningococci enter and replicate in the bloodstream, after crossing the epithelial barrier without causing an intense local inflammatory response. They liberate various amounts of endotoxin (lipopolysaccharides)-containing outer cell wall fragments partly in the form of blebs (88). Blebs bind antibodies that would otherwise attach to whole bacteria and probably play a crucial role in the pathogenesis of this form of septic shock. High levels of circulating endotoxins correlate with fatal outcome and with severity of disease(45, 47, 360, 361) . 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* (354). High density lipoproteins, complement factors, antibodies, albumin, transferrin and lipopolysaccharide binding protein (LBP) have the ability to complex with LPS. Several of these proteins appear to have a detoxifying effect (152, 234, 327). LBP however, amplifies the effect of LPS. After complexation with LBP, LPS is presented to the CD14 receptor of macrophages and PMN's (Figure 3). This interaction leads to cellular activation at a much lower concentration of LPS than without LBP (234). High levels of LPS can also directly activate CD14 receptors. Bactericidal/permeability-increasing protein (BPI) is a potent bactericidal protein produced by polymorphonuclear leukocytes (PMN). It is stored in the azurophilic granules and also expressed on the cell surface. The bactericidal activity of BPI is caused by the strong affinity of BPI for LPS (368). In addition to bactericidal capacity, BPI also neutralizes LPS activities in vitro and vivo (232).

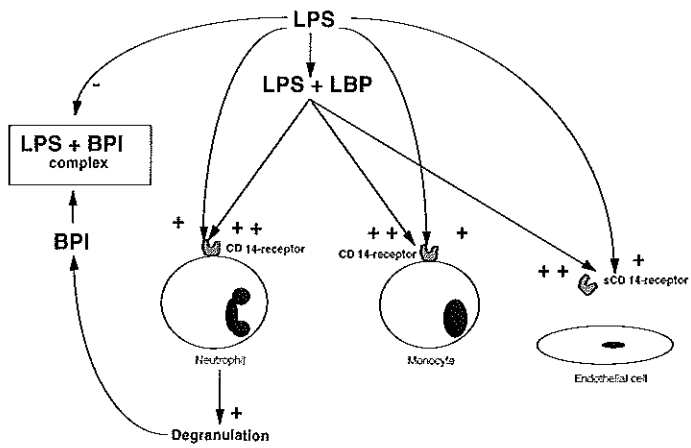

Figure 3. Competition between BPI and LBP in the binding of LPS
LPS: lipopolysaccharides, BPI: bactericidal/permeability=increasing protein, LPB: lipopolysaccharide binding protein, CD14: CD14 membrane bound antigen, sCD14: soluble CD14 antigen, +: activation, -" neutralization.

1.2 Pro- and counterinflammatory mediators

Lipopolysaccharides induce a release of proinflammatory mediators in gram-negative sepsis. These mediators are synthesized and released by macrophages, monocytes, and endothelial cells. Cytokines are paracrine agents: they act locally on a variety of tissues

signalling information to adjacent cells. Experimental and clinical data have shown that tumor necrosis factor (TNF)-α and interleukin (IL)-1ß are the key mediators in meningococcal sepsis. TNF-α and IL-1ß exert their effects by different mechanisms including the induction of other cytokines, activation of neutrophils and leukocytes, enhancement of adherence of PMN and monocytes to endothelium, generation of prostaglandins and production of nitric oxide. The release of other mediators (IL-6, IL-8, LIF, IL-12 and IFN-γ) is triggered by LPS, TNF-α, and IL-1ß. These cytokines are elevated in meningococcal sepsis and their levels correlate with severity of disease. Interleukin (IL)-6 is a major pyrogen, stimulates the synthesis of acute-phase proteins (159) and has the ability to induce proliferation and antibody production by B-cells. Interleukin (IL)-8 is a potent chemoattractant, activates neutrophils and is thought to be involved in neutrophil-mediated vessel-wall injury (12, 161). Leukemia inhibitory factor (LIF) has multiple actions, many of which are shared with TNF-α, IL-1 and IL-6 (13, 366). IL-12 is a recently described proinflammatory cytokine, which seems to play a key role in the differentiation of Th1 cells and induces the production of interferon (IFN)-γ by T-cells and natural killer (NK)-cells (65, 163, 182, 333, 334, 336, 381, 157). The levels of IL-12 in meningococcal septic shock are related to outcome and severity of disease (157). Interferon-γ (IFN-γ) activates other cytokines. Plasma levels of IFN-γ are increased in sepsis, although not consistently (49, 54, 129, 157, 162, 193, 360).

The short peak of pro-inflammatory cytokines is directly followed by an increase of counterregulatory cytokines like IL-1 receptor antagonist (IL-1Ra), IL-10, soluble TNF receptors (sTNFRs) and soluble IL-6 receptor (sIL-6R). These mediators except sIL-6R are considered to be anti-inflammatory because they reduce mortality in experimental endotoxemia (124, 257, 347). 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 (343). The role of sTNFR is complex. It is believed that sTNFR is released in the circulation after binding of TNF-α on the target cell. This shedding may protect the cell against ongoing stimulation of TNF-α (202). In contrast sIL-6R seems to stimulate the biologic activity of IL-6. Septic patients had significantly lower concentrations of sIL-6R compared to healthy volunteers (115, 116, 388). Interleukin 10 is released in massive amounts into the systemic circulation during the initial phase of fulminant meningococcal septic shock and high serum levels of IL-10 are related to outcome in children with this disease (85, 211, 281). IL-10 is a potent inhibitor of cytokine production (177, 124, 182, 363). It also suppresses the procoagulant activity induced by LPS at the surface of human monocytes (281). IL-10 stimulates the production of IL-1Ra and induces the release of sTNFRs by monocytes (209).

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

with outcome and severity of disease (45, 121, 159, 360, 366, 372). These cytokines are cleared from plasma rapidly. A negative correlation has been reported between the initial levels of cytokines and the time between first appearance of petechiae and admission (159, 195). This shorter duration of petechiae in non-survivors suggests a shorter disease course and associated higher levels of cytokines. The earlier admission of non-survivors may indicate a higher production of LPS per time span, thus triggering mediator systems more intensively or may be explained by a higher responsiveness to lipopolysaccharides or to proinflammatory cytokines (195). Westendorp et al. found a genetically encoded anti-inflammatory cytokine profile during the initial phase of infection, which decreased the non-specific host response and favored growth of micro-organisms. Families from meningococcal disease patients characterized by low TNF production in an ex vivo whole blood stimulation setup, had a tenfold increased risk for fatal outcome, whereas high IL-10 production increased the risk 20-fold. Families with both characteristics had the highest risk (373). However, others have studied the genotype of both surviving and non-surviving patients with severe sepsis and found support for the hypothesis that individuals with a genotype for high TNF production have a worse outcome (37, 244, 318). Their results showed an association between a polymorphic variation in the TNF-α gene promotor region and death from meningococcal disease (244).

1.3 Endothelial damage and capillary leakage

Endothelial cells are not merely a selective permeability barrier between blood and underlying tissues, but actively play an important role in maintaining homeostasis. During sepsis, endotoxin and several other mediators activate vascular endothelial cells and initiate a rapid alteration of structure and function of these cells (figure 4). Finally endothelial damage is leading to a severe capillary leak syndrome. Capillary leakage and subsequently edema are the result of: destructive changes of endothelium (glycosaminoglycan component (190)), active separation of tight junctions between endothelial cells (201) and high molecular protein leakage.

These processes are partly induced by circulating mediators (TNF-α, IL-1, IL-8, platelet activating factor (PAF), leukotrienes, thromboxane A_2, thrombin, vascular permeability factor, complement factors, kinins (34,75), and the adherence of neutrophils and platelets (133, 351, 365). Activated leukocytes are primed to initiate phagocytosis and microbial killing by degranulation and release of proteolytic enzymes and toxic oxygen radicals, but in this way they also play a role in endothelial damage (31, 133, 351, 365). Elastase is one of these degranulation products of activated neutrophils. Elastase-α 1-antitrypsin complexes in plasma appear to be of prognostic significance: levels are higher in non-survivors (254). Ultimately, increased vascular permeability leads to profound interstitial

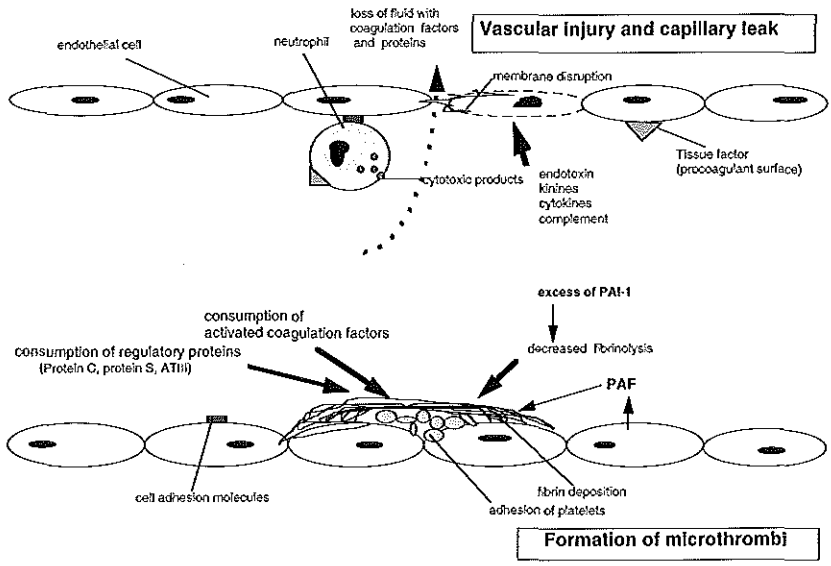

Figure 4. Disseminated intravascular coagulation, characterized by microvascular thrombosis and bleeding diathesis. PAI: plasminogen actovator inhibitor; PAF: platelet activating factor.

edema with diffuse parenchymal cell injury and persistent hypovolemia followed by organ dysfunction.

1.4 Complement

The complement system plays a key role in host defense mechanisms, resulting in lysis of bacteria, enhancement of phagocytosis by monocytes or polymorphonuclear leukocytes or neutralization of endotoxin (113, 114). This system is an essential element in the maintenance of homeostasis not only of several immunological functions, but also of the coagulation and fibrinolysis system, vascular permeability and vascular tonus. Fulminant meningococcal sepsis is associated with excessive complement activation (47,113). Complement peptides generated during activation, have pro-inflammatory effects such as stimulation, aggregation and degranulation of neutrophils and induction of expression of selectins on the endothelial surface. However, overstimulation or inadequate inhibition of the complement system may lead to an inappropriate reaction and ultimately to tissue injury (135). The complement system can be activated through the classical and the alternative pathway. The classic pathway requires recognition and binding of bacterial antigens by specific antibodies. In vitro studies indicate that lipid A and the polysaccharide side chain may complex with C1q and factor B to initiate activation of the classical pathway

without involvement of antibodies. The alternative complement pathway can be activated by a variety of substances, including polysaccharides, bacterial endotoxins, cytokines and immune complexes (31, 296). Normally, intravascular clearance of bacteria is mediated through the deposition of complement components: C3b for phagocytic clearance (294); the membrane attack complex or C5b-C9 for lysis (113, 114). Previous studies indicate that the degree of complement activation in sepsis is related to the amount of circulating native lipopolysaccharides (119, 220, 243). 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 (47). The presence of C4bc, C4bd and Bd point to activation of both the classical and alternative pathways. Brandtzaeg et al. suggest that complement activation is predominantly caused by alternative pathway activation (41). In contrast, we could prove an important contribution of the classical pathway in the activation of the complement system in patients with meningococcal sepsis. This activation was related to outcome and severity of disease (155). Brandtzaeg and colleagues showed that complement activation may persist during the first 12-24 hours of disease, when production of other inflammatory mediators is already downregulated (47). Complement deficient individuals point to the importance of the complement system in meningococcal disease. Meningococci cause 80% of all systemic bacterial infections in these individuals, although in late-complement-component deficient individuals mortality is five to ten fold lower than in complement sufficient individuals (84). The complement and the coagulation system share the same protein as their inhibitor in plasma, i.e. C1-esterase inhibitor (C1-INH). C1-INH levels may increase up to two-fold during uncomplicated infections (139). During sepsis C1-INH levels were found to be normal or even decreased especially in non-survivors (252). In our patients we also found decreased levels of C1-INH (155, 156). Hack et al. hypothesized that increased degradation of C1-INH in sepsis may result in an insufficient control of the complement and coagulation systems (139).

1.5 Coagulation and fibrinolysis

Coagulation disorders and abnormalities of fibrinolysis are common in patients with meningococcal sepsis. The most severe manifestation is disseminated intravascular coagulation (DIC), characterized by microvascular thrombosis and bleeding diathesis (table 1). Widespread microvascular thrombosis does contribute substantially to organ dysfunction and survival. The production of thrombin and the conversion of fibrinogen to fibrin, may be activated by the intrinsic pathway (through factor XII) or by the extrinsic pathway (through factor VII/tissue factor) activation (figure 5). Activation of the coagulation system in sepsis occurs predominantly through the extrinsic route. The importance of the extrinsic route of coagulation was shown in an experimental model of baboons in which infusion of monoclonal antibodies against tissue-factor protects against lethal shock by

Table 1. Factors involved in the pathophysiology of DIC

Deposition of micro-thrombi
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)

E. coli. and attenuates coagulopathy (325). 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 (270).

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 (261). Through kallikrein, activation of factor XII results in the generation of bradykinin, complement, plasmin and elastase. In this way, factor XII mediates inflammation, fibrinolysis, and tissue damage as well as clot formation. Hypotension is probably partly mediated by generation of kinins such as bradykinin. Factor XII levels are lowest in patients with septic shock (168, 253).

Levels of natural inhibitors of coagulation are markedly altered during meningococcal - sepsis. Several studies confirmed that antithrombin III (AT-III) levels (46, 110-112, 207), protein S, and notably protein C (46, 100, 110, 112, 207, 279, 280), 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 (158, 279). Elevated initial levels of the extrinsic pathway inhibitor (EPI), another inhibitor

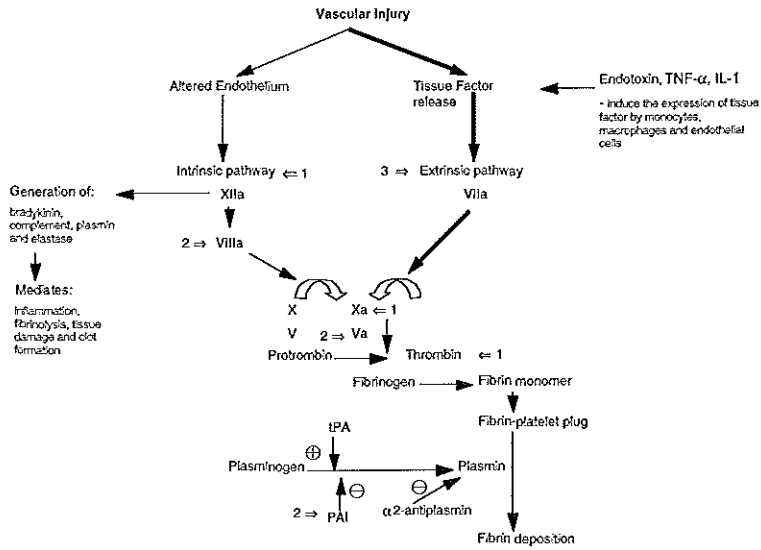

Figure 5. TNF-α: tumor necrosis faxtor a; IL-1: interleuking-1
V: coagulation factor V; X: coagulation factor X and Xa, when activated;
tPA: tissue plasminogen actovator; PAI: plasminogen activator inhibitor.
The intrinsic pathway involves the activation of coagulation factor XII, and VIII. The extrinsic pathway involves the activation of factor VII by tissue factor.

Natural inhibitors:
1: antithrombin III ⟹
2: activated Protein C
3: tissue factor pathway inhibitor

of coagulation, were found in patients with fulminant meningococcemia (46). 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 (46).

The fibrinolytic system becomes activated by tissue plasminogen activator (tPA) during the early course of meningococcal sepsis. Subsequently, fibrinolysis is inhibited by increased levels of plasminogen activator inhibitor (PAI-1) (42, 158, 195). In patients with sepsis and septic shock tPA levels are increased and related to outcome and severity of disease (268, 358). However, we could not detect significant differences in the initial levels of tPA in survivors and nonsurvivors with meningococcal septic shock (158, 195). In adult patients with non-meningococcal septic shock, levels of plasminogen and alpha-2-antiplasmin are low in septic shock but not related to outcome (168, 358). In contrast in children with

meningococcal septic shock, alpha-2-antiplasmin levels as well as the ratio PAI-1/tPA were related to outcome in patients with meningococcal septic shock (42, 100, 158). These changes in fibrinolytic parameters result in an ineffective fibrinolysis. Of interest, a genetic polymorphism in the promotor of the PAI-1 gene is suggested to explain higher PAI-1 levels in non-survivors at a similar TNF stimulus (195).

The massive consumption coagulopathy is characterized by low levels of coagulation factors VII, X, V, prothrombin, fibrinogen, and platelets. Because of the massive demand of anticoagulation factors due to widespread activation of the anticoagulant pathway, the host's endogenous anticoagulants are depleted causing purpura fulminans (279). This depletion is possibly age-related (158, 279). Platelets also interact with neutrophils through a selectin-mediated mechanism. These interactions may bring activated platelets close to the endothelium (147). Even without adherence to the endothelium, platelets can profoundly affect endothelial function by the release of vasoactive substances from platelet storage granules. Such substances include adenosine diphosphate (ADP), serotonin, PAF and lipoxin A4, which may mediate vasodilatation (175). Platelet adhesion to vascular endothelium is mediated by coagulation factor VIII (von Willebrand factor), which is found in plasma, endothelial cells and in the storage granules of platelets. Platelets also form and release thromboxane A2 and leukotriene C1, which may mediate vasoconstriction (293). The result is a procoagulant state which is reflected in formation of microthrombi in the skin, the extremities and the adrenals. The excessive consumption of coagulation factors results in a hemorrhagic diathesis and local tissue hemorrhages.

1.6 Circulatory dysfunction

Shock or circulatory collapse in patients with fulminant meningococcal sepsis are caused by a combination of an inappropriate vascular tone, myocardial dysfunction, capillary leakage and intravascular microthrombosis. Initially, patients with meningococcal sepsis present with intense vasoconstriction. Subsequently, the systemic vascular resistance falls due to vasodilatation in the course of the treatment requiring volume suppletion and vasopressors. A dysbalance between forces causing vasodilatation and those causing vasoconstriction of the blood vessels results in generalized vasodilatation and hypotension, but in some capillary beds like the skin and the pulmonary circulation it leads to vasoconstriction. Vasoconstrictor substances that are elevated in patients with shock include catecholamines, renin, aldosterone, thromboxane A2 and endothelin(34, 192, 321, 357). A deficiency of PGI_2 synthesis by the endothelium is also involved in vasoconstriction during meningococcal disease (169). On the other hand, bradykinin and nitric oxide are potent vasodilator compounds leading to hypotension (221, 295, 329).

The major mechanism of death in meningococcal sepsis is circulatory collapse resulting from a combination of capillary leak, intravascular volume depletion, myocardial failure and

vasodilatation (238). As compensatory mechanisms fail, hypotension ensues, perfusion of vital organs becomes inadequate and the resulting hypoxia and acidosis contribute to myocardial dysfunction. There is only a limited number of hemodynamic studies in pediatric patients with meningococcal sepsis (38, 328, 242). Probably the most detailed is the one of Mercier et al. The results of this study suggest a different cardiovascular pattern in children with meningococcal sepsis than in adult patients with sepsis. All of the non-survivors had a decreased cardiac index (CI) and nearly all of the survivors had a normal CI in stead of the increased CI which can be encountered in adult patients with sepsis (238). The timespan in which the circulatory failure takes place is different in meningococcal sepsis in comparison with other forms of sepsis. Mortality happens in the first 48 hours, while recovery seemed much longer in adults (7-10 days) than in children (1-3 days) (38). The mechanism of the myocardial failure in human sepsis remains partly speculative. Circulating myocardial substances like TNF-α , IL-1 and endotoxin itself and not myocardial hypoperfusion is probably responsible for this depression (197, 198, 210, 265). However, also other aspects of the systemic inflammatory response including circulating cytokines, tissue damaging, leukocyte-endothelial cell- myocyte interaction leading to severe myocardial edema and mismatch of oxygen supply and demand in cardiac microvasculature will cause this myocardial depression (238, 364). To what extent these mechanisms are also applicable to meningococcal sepsis is not clear yet.

2 THERAPEUTIC INTERVENTIONS

The most important complication requiring urgent intervention is the development of shock in the presence of a profound capillary leak. The aim of the whole treatment is to provide sufficient fluid to maintain the intravascular volume and electrolyte balance, while minimizing the accumulation of extravascular fluid. When shock continues despite aggressive correction of the volume deficit, inotropic support should be given (189). Recently, extracorporeal membrane oxygenation (ECMO), is suggested as a support therapy for patients with severe cardiorespiratory failure. Venoarterial ECMO provides both circulatory and respiratory support and could assist septic patients if conventional medical managment is failing. The potential risk of haemorrhage is of concern, because of the necessity for anticoagulation during ECMO. The little experience of ECMO for meningococcal disease is encouraging with two-thirds of the patients surviving and most survivors leading functionally normal lives (19, 64, 134). The question remains whether these patients would have survived without ECMO.

New therapies directed towards modulating the systems involved in the pathophysiology, have so far shown little benefit in clinical practice (table 2). To neutralize circulating endotoxin, antibodies against the lipid A moiety of endotoxin (HA-1A) have been studied.

Table 2. Experimental treatment in meningococcal septic patients

author	experimental treatment	type of study	number of patients	conclusion
Beca J [19]	ECMO	retrospective, descriptive study	n=9 children (5 survivors)	ECMO supported the circulation successfully.
Goldman AP [134]	ECMO	retrospective, descriptive study	n=12 children (8 survivors)	ECMO might be considered to support patients with meningococcal disease.
Champion MP [84]	ECMO	descriptive	n=2 (both survivors)	ECMO may have a role in refractory shock.
van Deuren M [342]	plasma or whole blood exchange (PEBE)	descriptive, with historical controls	n=15 children and young adults (12 survivors)	early initiation of PEBE may improve the rate of survival.
Reeves JH [268]	hemofiltration plasmafiltration	retrospective case notes	n=18 children (5 survivors) n= 9 children (3 survivors)	hemofiltration or plasmafiltration is safe, but their effect on outcome remains unknown.
Best C [22]	early hemo-diafiltration	descriptive	n=4 children (all survivors)	use for treatment is speculative.
Frieling JTM [116]	plasma or whole blood exchange	prospective, descriptive	n=9 children (6 survivors)	plasma or whole blood exchange increase the concentration of soluble IL-6 receptors.
Westendorp RGJ [371]	leuka-plasmapheresis	open, prospective with historical controls	n=13 children (10 survivors)	leuka-plasmapheresis might be of value.
Gerard, P [125]	heparin	descriptive	n=19 children (17 survivors)	heparin must be considered as an adjunctive therapeutic agent.
Haneberg B [149]	heparin	randomized therapeutic trial	n=26: heparin: 9/11 survivors, no heparin 13/15 survivors	heparin has no influence on outcome.
Kuppermann N [196]	heparin	retrospective, descriptive study	n=24: heparin: 3/6 survivors, no heparin 10/18 survivors	heparin may limit digit and extremity necrosis.
Gerson WT [127]	protein C	descriptive study	n=1 child (survived)	could be instrumental in survival.
Rivard GE [202]	protein C	pilot study	n=4 children (all survivors)	safe, possible contribution in decreasing morbidity and mortality rates.
Smith OP [311]	protein C, heparin and	pilot study hemo/dia-filtration	n=12 children and young adults (all survivors)	decrease in mortality and morbidity, call for a double blind randomized controlled trial.
Fourrier F [111]	antithrombin III	descriptive	n=5 young adults (all survivors)	could contribute in decreasing morbidity and mortality.
Keeley SR[157]	tissue plasminogen activator	descriptive	n=1 child (survived) (rt-PA)	possibility to decrease the morbidity of amputations.
Zenz, W [389]	rt-PA	descriptive	n=2 children (both survivors)	rt-PA may be an additional approach.
Zenz W [390]	rt-PA	descriptive	n=2 children (all survivors)	should be considered as an adjuvant therapeutic option.
Aiuto LT [6]	rt-PA	descriptive	n=1 child (survived)	improvement of organ perfusion and cardiac performance.
Giroir BP [132]	r-BPI	open, dose escalation, with historical controls	n=26 children (25 survivors)	marked biological effect of rBPI.

HA-1A has been evaluated in adults (236, 391) and recently in children with meningococcalseptic shock. In a European multicenter trial in children with meningococcal septic shock, the morbidity and mortality were not statistical significant different between children in the HA-1A and in the placebogroup (manuscript in preparation). New studies to evaluate the efficacy of recombinant-BPI and high density lipoproteins (HDL) in children with meningococcal sepsis, are recently started. A phase 2 trial of rBPI-21 showed a mortality of 4% in patients with a predicted mortality range of 20-50% (132). Early haemofiltration with plasma or whole blood exchange may be useful in the management of meningococcal sepsis in children (22, 273, 288, 324). However, Frieling et al. showed that plasma or whole blood exchange did not significantly influence IL-6 concentrations but as a possible side effect, did increase the soluble IL-6 receptor concentration directly after an exchange session followed by a rapid decrease (117). Although, several authors underlined the possible value of this treatment in meningococcal sepsis, no controlled studies are available. Inhibition of cytokines by anti-TNF-antibodies or IL-1-receptor antagonist showed no efficacy in patients with sepsis or septic shock (4, 72, 106, 260, 289). As agents to control disseminated intravascular coagulation in patients with fulminant meningococcemia heparin, antithrombin III concentrate, fresh frozen plasma and protein C concentrate have been studied (111, 112, 125, 127, 149, 153, 199, 292, 311). However with the exception of fresh frozen plasma and protein C, which have some fibrinolytic properties, these drugs cannot add to the dissolution of fibrin clots. Recombinant tissue plasminogen activator (rt-PA) is a new fibrinolytic drug. It induces a clot-selective fibrinolysis that is associated with only a little decrease of fibrinogen. Preliminary experience with rt-PA in two patients suggests that rt-PA should be considered as an investigational therapeutic option in patients with lifethreatening disease and no response to conventional treatment (6, 187, 389, 390). However, severe bleeding can be a serious side effect, because titration of the dosage is difficult in the acute phase since PAI-1 levels are high in the initial phase, but rapidly decrease in time. A new trial to evaluate the efficacy of protein C in the treatment of children with meningococcal septic shock has recently started. Possible future treatment modalities include r-BPI (phase 3 trial has started), C1 esterase inhibitor, protein C, rt-PA and tissue factor pathway inhibitor.

CHAPTER 2

ENDOTOXIN AND INFLAMMATORY MEDIATORS

CHAPTER 2.1

AGE RELATED DIFFERENCE IN ENDOTOXIN CLEARANCE AND CYTOKINE RESPONSE DURING AN IN VIVO CHALLENGE TO MENINGOCOCCAL ENDOTOXIN IN PIGS

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1 INTRODUCTION

Meningococcal sepsis is a rapidly progressive, life threatening form of septic shock occurring mainly in children below the age of 5 years. Mortality remains high especially in the children below the age of 3 years despite improvements in intensive care treatments (158). There is no explanation for the previously documented difference in mortality between young and older children. Low levels of antibodies (290), immaturity of cell mediated immunity (375) and age related differences in coagulation response have all been suggested as possible explanations for this age-related difference in mortality. The presence of high amounts of endotoxin linked to membrane fragments (vesicles or blebs) of the meningococcus is a characteristic feature in patients with meningococcal sepsis. The plasma concentrations of endotoxin are correlated with mortality and severity of disease (44). Previously, several authors observed an age related increase in sensitivity to endotoxin in rodents (48, 68, 71, 171, 322). The toxicity of endotoxin depends upon two mechanisms: 1. the recognition and binding of endotoxin and 2. the endotoxin induced cytokine production. We studied in vivo responses to rough meningococcal endotoxin in young (3 weeks) and old (4 months) pigs to confirm the presence of age related differences. In this paper we report the findings on hematological parameters and pro- and counter-inflammatory cytokine responses. The hemodynamic consequences of the challenge with endotoxin have been reported earlier (Hazelzet et al. Manuscript accepted for publication).

2 MATERIALS AND METHODS

2.1 Endotoxin

Outer membrane (OM) blebs were prepared from *Neisseria meningitidis* group B strain H44/76 using a standard method (275). Briefly, bacteria were treated with 0.2 M LiCl and 0.01 M EDTA at pH 7.0 during 2 h at 45°C. The mixture was centrifuged at 15,000 rpm for 10 min to remove bacteria and the supernatant ultracentrifuged to peel of the outer membranes. The pellet was resuspended in saline with 2% sucrose and 0.02% merthiolate and stored in vials at 4-8°C. LPS (blebs) was analyzed for KDO (2-keto-3-deoxy-D-manno-octonate) content (186), and quantified by electrophoresis (SDS-PAGE) following silver staining (338). The concentration of protein in the blebs mixture was 50µg/ml.

2.2 Assays

2.2.1 endotoxin

Endotoxin-free equipment and chemicals were used through all experiments. Before determination, plasma samples were thawed and diluted 1:10 in distilled pyrogen free water. All samples, standards and controls were heat-inactivated for 12 min at 75°C and cooled to room temperature. The chromogenic limulus lysate assay (BioWhittaker, Brussels, Belgium) was used. The detection limit was 0,1 EU/ml with the standard curve 0.1-1.0 EU/ml (1 EU=0.1 ng). Duplicate plasma samples were tested in separate runs and the mean value was used.

2.2.2 cytokines (TNF, IL-6, IL-10)

Tumor necrosis factor (TNF-α) was measured with an ELISA (Predicta TNF-α, Genzyme, Boston, MA). Each sample was assayed in duplicate in dilutions of 1:2 and 1:10 according to manufacturers' instructions. Cross-reactivity of this assay with pig TNF-α has been demonstrated (350).
Interleukin 6 (IL-6) was determined with the IL-6 dependent mouse hybridoma cell line B13.29 clone B9 (2). Plasma samples were heat inactivated at 56°C for 30 min and serial dilutions were incubated for 72 h with B9 cells in microtiter plates. During the last 4 h of incubation 0.5 µCi of 6-^{3}H thymidine (Amersham, UK) was added. The cells were harvested simultaneously with a 96 well sample harvester (Pharmacia/LKB/Wallac, Turku, Finland). The complete filters containing 96 spots were placed in plastic bags. Scintillation liquid (Pharmacia/LKB/Wallac, Turku, Finland) was added and the bags were sealed. Radioactivity incorporated into DNA was measured simultaneously for 30 s with a LKB/ Wallac 1205 Betaplate counter (Genterm Pharmacia/LKB/Wallac, Turku, Finland). A software package was used to operate the system and to calculate mean cpm and SD of 6 replicate cultures. Recombinant IL-6 (CLB, Amsterdam, The Netherlands) was included as a standard. The detection limit was 0.5 pg/ml of the rIL-6.
Interleukin 10 (IL-10) was measured with an ELISA (Medgenix, Belgium) developed for measuring human IL-10 (80). For this pig assay Mab clone 4C4 was used for the capture and 1A9 for the detection. The cross-reactivity for pig IL-10 has been established in a swine whole blood stimulation experiment. Detection limit is 3 pg/ml, plasma levels in healthy volunteers were 7.8 pg/ml.

2.2.3 chemical analysis

Serum lactate (Sigma Diagnostics, St Louis, USA) was measured by enzymatic end-point determination. White blood cell and platelet counts were determined using a cell counter (OSM-2, Sysmix, Kobe, Japan).

2.3 Animals

Land-race x Yorkshire domestic piglets of 4 months of age, 40.5±0.8 kg (n=19) and of 3 weeks of age, 7.2±0.3 kg (mean±SEM) (n=16) were studied. The animals were randomly assigned to a group receiving either a low (1 µg per kg body weight) or a high (10 µg per kg body weight) dose of endotoxin, yielding a total of 4 groups of animals: old low dose (OL) and old high dose (OH), and young low dose (YL), and young high dose (YH).
All experiments were performed in accordance with the "Guiding Principles in the Care and Use of Laboratory Animals" as approved by the Council of the American Physiological Society and with the approval of the Animal Care Committee of the Erasmus University Rotterdam.
The animals were fasted for 15 h prior to the surgical procedure. This procedure and the experimental sepsis protocol are described in detail previously (Hazelzet et al. manuscript submitted). Briefly: older animals were sedated with intramuscular (im) injection of 25 mg/kg ketamine (Ketalin®, Apharmo, Arnhem, The Netherlands), followed by induction of anesthesia with an intravenous (iv) injection of 10 mg/kg methomidate (Hypnodil®, Janssen Pharmaceutical, Tilburg, The Netherlands) through an ear-vein cannula. The younger animals were sedated by mask inhalation of a mixture of O_2/N_2O mixture (1:2) and isoflurane (3%), followed by induction of anesthesia with iv methomidate. All animals were intubated and ventilated. Anesthesia was maintained with an O_2/N_2O mixture (1:2) and isoflurane (0.25-3%). To maintain fluid and metabolic balance, 5 ml/kg/hr glucose 5% in NaCl 0.225% was administered iv continuously. Under sterile conditions both external jugular veins and common carotid arteries were surgically exposed through a ventral paramedian incision. On the right side a catheter was inserted and positioned in the descending aorta for blood sampling and measurement of arterial blood pressure; and through the jugular vein a thermodilution catheter (Arrow®, Reading, USA) was introduced and inserted into the pulmonary artery to monitor the mean pulmonary artery pressure (MPAP), cardiac output (CO), and central venous pressure (CVP), and to sample mixed venous blood. The left carotid artery was cannulated with a pressure transducer-tipped catheter (Sensodyn MTC®, Braun Medical, Uden, The Netherlands), which was positioned in the left ventricle to measure the left ventricular pressure. A separate lumen in this catheter was used to inject micro spheres. A catheter was inserted into the external jugular vein for drugs and fluid administration. Once the proper position of the catheters was verified, using fluoroscopy, arterial blood was sampled for in vitro analysis and all catheters were tunneled subcutaneously to the dorsal side of the neck and secured with sutures. After weaning from anesthesia and ventilation, the animals were extubated and allowed to recover from surgery. Catheters were protected with a vest (Tubigrip®, Seton Health Care group, Oldham, UK) and flushed with heparin-solution (500 IU/ml) once a day. Prophylactically, the animals received 25 mg/kg amoxicilline (Clamoxyl®, SB-Farma,

Rijswijk, The Netherlands) and 5 mg/kg gentamicin (A.U.V. Cuyk, The Netherlands) iv once daily starting on the day of the surgical procedure. Every day, the animals were adapted to the laboratory facilities (7-10 days) to ensure hemodynamic stability. The experimental protocols were executed when the animals could rest unrestrained and quietly for up to 4 h, and systemic hemodynamic parameters therefore remained stable.

2.4 Experimental sepsis

On the day of the endotoxin protocol the needed amount of endotoxin (OM-blebs) was diluted with saline to a total volume of 10/20 ml (young/old). The dose of endotoxin was estimated from experience obtained in pilot experiments. The low dose (1 µg/kg) was previously found to be non-lethal, and the high dose sub-lethal (50% mortality). Because of the rapid increase in pulmonary vascular resistance (259) in the early phase of endotoxin infusion, oxygen (3-5 l/min) was supplemented to prevent hypoxia in the initial phase. When the mean pulmonary artery pressure (MPAP) increased threefold (usually at 10 min), the endotoxin infusion was interrupted until MPAP had decreased to below 2 times the baseline value (usually at 30 min). At that time the infusion was restarted at a higher rate to end the infusion within 1 h. During the whole protocol 5 ml/kg/h fluid was iv administered (glucose 5% in NaCl 0.225%). After the endotoxin infusion a period of 1-2 h was allowed to establish a stable hemodynamic situation before starting an infusion of 30 ml/kg plasma (Haemaccel®, Behring Pharma, Amsterdam, The Netherlands) infused over 30 min. After completion of the volume loading a washout period of 2 h was taken, before termination of the protocol. At regular intervals (10, 30, 60, 90, 120, 180, 240, 300 min) arterial blood was sampled for blood gas, white blood cell and platelet count determination or centrifuged and stored at -70 °C for later analysis. Moribund animals during the protocol were humanely euthanized.

2.5 Statistical analysis

Statistical analysis was performed using (SPSS®, Chicago, USA) Wilcoxon signed rank test or paired Student *t*-tests to compare values at baseline with subsequent time points within groups. Differences between groups were tested with repeated measurements ANOVA. When appropriate, logarithmically transformed values were used. Factors related to survival were tested with multiple regression analysis. Data are expressed as mean value ± SEM, or median (range). P values below 0.05 were considered significant.

3 RESULTS

3.1 In vivo response

3.1.1 survival and General Manifestations

The clinical picture which evolved during infusion of endotoxin consisted of restlessness and even agitation in the period of sudden increase in pulmonary vascular resistance (10-30 min after start of the infusion), tachypnea (breathing frequencies >80/min), vomiting, shivering, reduced reaction to stimuli and finally lethargy. This clinical picture was similar in all groups. The core temperature increased approximately 2°C in all four groups. There was no mortality in the low dose, young animal group, but 3 animals died in high dose, young animal group (mortality 37.5 %), 1 old animal died in the low dose group (12.5 %) and 4 old animals in the high dose group (44 %). The moment of death was 149 ± 13 min after the start of the endotoxin infusion.

3.1.2 blood cell counts, endotoxin and cytokine concentration

The baseline WBC was 11.6 (8.0-17.2) and 14.5 (10.5-22.4) 10^9/L for the young and old animals respectively (p=0.005). In Fig: 1 the relative change from baseline is shown for the different age and dose groups. Already during the ET infusion there was a steep decrease in WBC, the lowest level was reached about 3 h after the start of the ET infusion.

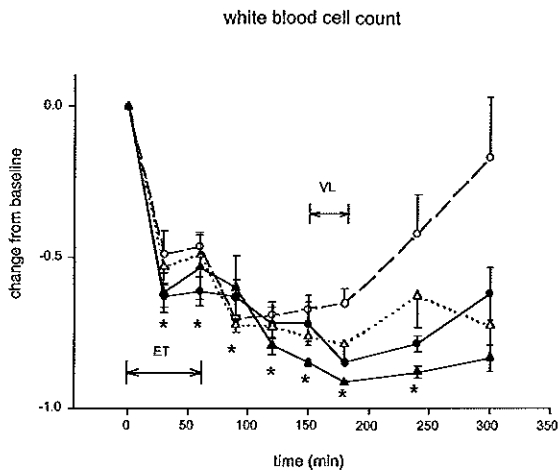

Figure 1. Time course of white blood cell count, relative change from baseline, from young (circles) and old pigs (triangles), low (open) and high dose (closed). ET= endotoxin infusion, VL= volume loading. Data are mean ± sem; *= significant change from baseline.

From that moment the WBC in the younger animals started to increase again, while in the older the numbers remained low.

The platelet count showed a comparable pattern (Fig: 2); the median levels at baseline were 197 (96-430) and 247 (140-529) 10^9/L for the young and old animals respectively (not significantly different). Similar to the white blood cell count, the platelet count started to decrease during the ET infusion and the lowest level was reached at 180 min.

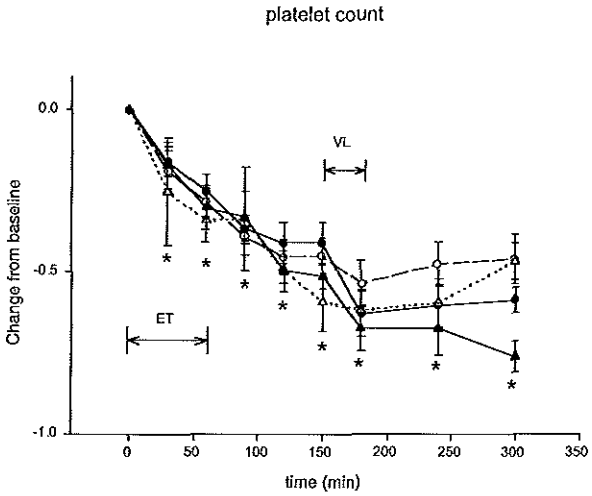

Figure 2. Time course of platelate count, relative change from baseline, from young (circles) and old pigs (triangles), low (open) and high dose (closed). ET = endotoxin infusion, VL = volume loading.

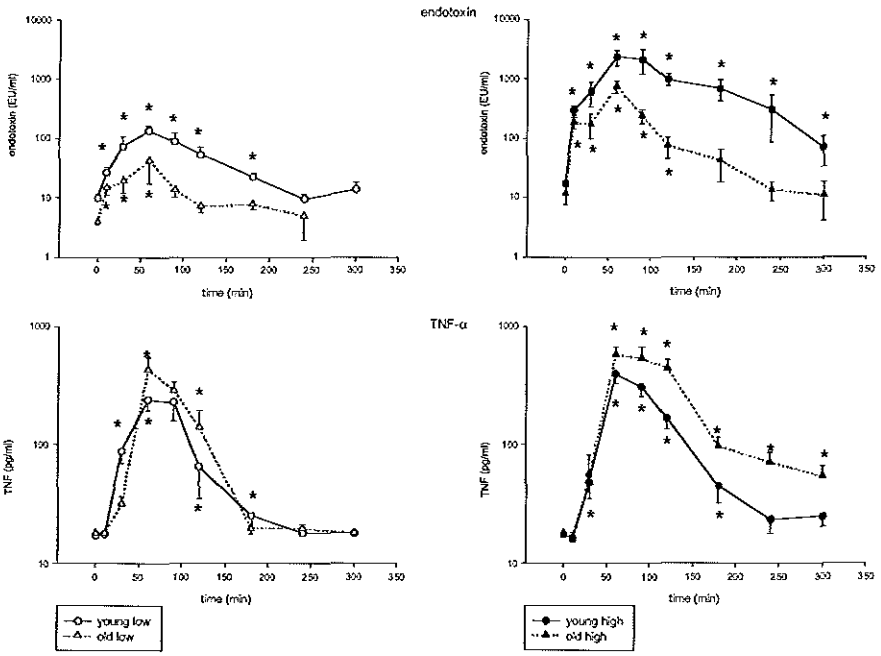

Figure 3. Time course of levels of endotoxin (upper panels) and TNF-α (lower panels) from young (circles) and old pigs (triangles), low (open) and high dose (closed).

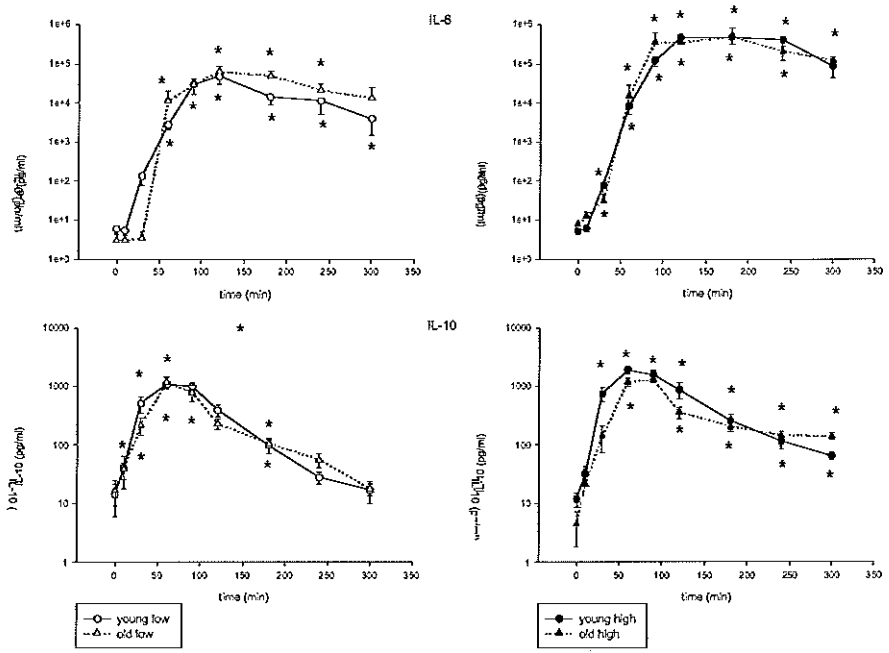

Figure 4. Time course of levels of IL-6 (upper) and IL-10 (lower) from young (circles) and old pigs (triangles), low (open) and high dose (closed).

The endotoxin levels are shown in Fig: 3. Maximal levels were reached for all groups at about 60 min, which is the end of the infusion. Medians of the maximal levels were different between the groups: 128 (60-315) and 2480 (354-8675) EU/ml for low vs high in the young animals ($p < 0.001$); 23 (5-200) and 641 (110-2190) EU/ml ($p < 0.001$) for low versus high in the old animals. In the low dose group of the young animals endotoxin levels were still elevated at 240 min; in the old this period was shorter. Also in the high dose groups this was different between the two ages. Repeated measurements ANOVA showed age and dose related differences at all time points ($p < 0.001$), except for 10 and 30 min. In the young animals the median of the maximal ET levels was 4.4 times higher than in the older animals. Maximal TNF levels were reached at about 60 min. Median maximal levels were: 224 (90-488) and 323 (275-800) for the young animals; 375 (162-1031) and 581 (218-1088) for low vs high in the old animals (differences not significant). In the high dose old animals, the TNF levels remained increased for a longer period. The pattern of IL-6 response was similar between young and old (Fig: 4). Maximal levels were reached at about 100 min, and notably in the high dose groups the levels remained high during the whole experiment. Median maximal levels were: 35.7 (9.0-131) ng/ml and 1000 (27.5-1500) for low vs high in the young animals; 57.0 (6.0-161.3) and 243.0 (3.4-2000) ng/ml for low vs high in the old

animals (differences not significant). Levels were at all time points affected by dose (RmANOVA, p=0.017). IL-10 reached maximal levels at about 60 min and also remained elevated, but not as high as the IL-6 levels. RmANOVA evaluating the levels until volume loading showed age (p=0.047) and dose (p=0.016) related differences. At 30 min and at 120 min the median IL-10 levels in the younger animals were significantly higher as compared to the older animals. Median maximal levels were 1353 (498-1682) pg/ml and 2363 (912-2831) pg/ml for low and high dose in the young animals; 1403 (397-2051) pg/ml and 1444 (412-2185) pg/ml for low vs high in the old animals (no significant difference between the low dose groups, p=0.043 for the high dose groups). Except for the IL-6 levels in the young animals, there was no relation between endotoxin or cytokine levels and survival.

4 DISCUSSION

The present study was designed to examine the presence of differences in cytokine responses in young and near adult piglets to an in vivo meningococcal endotoxin challenge. The major findings of the in vivo studies were that (i) there was a dose-related, but not an age-related difference in mortality, (ii) the same dose on weight base of endotoxin resulted in higher levels and longer presence of endotoxin in blood of young animals, (iii) despite these higher endotoxin levels in the young animals the levels of TNF and IL-6 were similar to the old animals, and the levels of IL-10 were slightly, but significantly higher in the young animals.

The importance of the level of endotoxin in relation to severity of disease in meningococcal disease has well been established (43, 44, 46). Also the difference in structure (40, 81) and bioactivity (13) as compared to other forms of LPS has been noticed. This is the first sepsis model using rough meningococcal endotoxin. Purified meningococcal LPS has been used in small laboratory animals to study the pathophysiology of meningococcal meningitis and sepsis. Age related sensitivity for LPS has been studied in mice, rats and rabbits (71, 91, 98, 171, 178, 251, 284, 312, 322, 367). The conclusions of those studies were not uniform, although with the exception of neonatal animals there seems to be an age dependent increase in sensitivity to endotoxin. This difference in sensitivity has previously been explained by differences in cytokine production. In vitro LPS stimulation of monocytes from preterm and term neonates as well as adults resulted in lower production of IL-6 in the younger individuals (301, 382). Antigen and mitogen induced production of IL-2, IL-4, IL-6 and IFN-γ by peripheral blood mononuclear cells from children was lower than from adults (216); Similarly, TNF-α, IL-4, and IFN-γ production after LPS stimulation was lower in mononucleated cells from cord blood versus from adult blood. In in vitro whole blood stimulation experiments with meningococcal LPS, we found a lower IL-6 production in young animals in comparison with old animals. However, this difference disappeared when

adult plasma was added to blood cells from young animals. Hence, LPS binding capacity of plasma probably plays a role in this difference in sensitivity to endotoxin. This was confirmed in our in vivo experiment in which an equal amount on weight base of LPS resulted in higher serum levels and different clearance of endotoxin in the young animals. Whether there also is an age related difference in cytokine production capacity is difficult to estimate, but at least the pattern of cytokines is different between young and old. Peak values of TNF were somewhat lower, although not significant, and IL-10 levels higher in young animals. these data were in contrast with the findings of Tadeda et al. who found higher levels of both TNF and IL-10 in aged animals (322). The endotoxin levels in the young animals were about 10 fold higher than reported in patients with meningococcal sepsis (44). However, it is likely that the native endotoxin binding and clearance capacity is exceeded more gradually in patients. In the old animals LPS was eliminated from the circulation with a half-life of about 1-2 h; in the young animals this was longer (3-4 h). About the same (1-2h) elimination rate was found in patients with meningococcal sepsis (44). Whether this longer elimination time has clinical consequences is not clear, but in our study there was no difference in mortality. LPS is probably cleared from the circulation by the liver and spleen. It is interesting to notice that in our hemodynamic comparison between young and old animals we found in the young animals after infusion with meningococcal endotoxin a lower increase in flow to the liver and a higher decrease in flow to the intestines and spleen than in the old animals (Hazelzet et al. Manuscript in press). Major plasma factors capable of binding endotoxin are LPS-binding protein (LBP) (55) bactericidal/permeability-increasing protein (BPI), and lipoprotein (notably HDL) (168). LBP amplifies the activity of LPS, in contrast to BPI and HDL, which neutralize the effects of LPS. It has been suggested that endogenously derived plasma levels of BPI are likely to be inadequate to compete for LPS binding to the much more abundant LBP in the circulation, at least in adult volunteers (55). Not much is known about LBP levels in pigs, but it is most likely that the differences in endotoxin levels between young and old animals are caused by differences in endotoxin binding capacity between young and old. There is also no knowledge about LBP, BPI or HDL levels in meningococcal sepsis patients, but the result of a phase 1-2 study with rBPI were promising(132), so a phase 3 study has recently been started. Further studies will be necessary to examine whether these age related differences in endotoxin binding and clearance will have consequences for the choice of therapy in patients with meningococcal sepsis.

CHAPTER 2.2

RELATION BETWEEN CYTOKINES AND ROUTINE LABORATORY PARAMETERS IN CHILDREN WITH SEPTIC SHOCK AND PURPURA

Jan A. Hazelzet, , Edwin van der Voort,, Jan Lindemans, Paul G.J. ter Heerdt and Herman J. Neijens

1 INTRODUCTION

Septic shock combined with purpura is a clinical syndrome with a sudden onset and a rapid progression. Most patients are children under the age of 10 years and the infection is mostly caused by meningococci. The severity of the clinical condition on admission in the hospital and the progression with which the disease is going on may be difficult to estimate. For this reason several scoring systems including laboratory parameters have been proposed to predict the prognosis of the disease. Recently Leclerc et al (206) presented a study in which they indicated that there was a good correlation between the serum C-reactive protein (CRP) level and the prognosis of children with severe infectious purpura and shock. Giraud et all (131) showed that in 35 adult patients with meningococcal purpura, low fibrinogen level (1.5 g/l) was the sole variable with prognostic value.
The last decade, a new group of proteins, called cytokines, has been identified as important mediators of the host response to sepsis. Most extensively studied are probably tumor necrosis factor (TNF) and interleukine 1 (IL-1), especially in relation to meningococcal infections (56, 76, 129, 360). More recently it has become clear that another cytokine, interleukin 6 (IL-6), is also involved in the systemic changes during severe infections (140). The biological activities of IL-6 partly overlap those of IL-1 and TNF, but IL-6 is probably also involved in the regulation of TNF and IL-1 production (302). IL-6 is secreted by macrophages, fibroblasts, and endothelial cells, it stimulates the synthesis of acute-phase proteins and is an endogenous pyrogen.
During acute admission of a patient with septic shock several laboratory parameters are routinely determined to estimate the clinical condition of the patient, for instance: lactate as end-product of anaerobic metabolism, a marker of cellular hypoxia, C-reactive protein (CRP) as acute-phase protein a marker for inflammation and tissue injury, and fibrinogen, an acute-phase protein and coagulation factor.
The aims of this study were to investigate the relation between cytokines and these established laboratory parameters on admission and the association of these parameters to the severity of the disease. This relation so far, has not been studied in children with septic shock and purpura.

2 PATIENTS AND METHODS

The study protocol was approved by the Institutional Review Board, and this Board waved the need for informed consent.
patients. The patients who were studied were all admitted to the Pediatric Intensive Care Unit from the Sophia Children's Hospital in Rotterdam with the suspicion of severe infectious purpura i.e a clinical diagnosis of sepsis (manifested by two or more of the

following conditions: temperature > 38.5°C or < 36°C; heart rate above 2 s.d of the age related mean normal value; respiratory rate > 25 breaths per minute; and white blood cell count > 12.000/mm^3, or < 4000/mm^3), in combination with petechiae and/or pupura. The patients were divided into a shock-group and a nonshock-group, according to standard criteria (two or more of the following features: systolic blood pressure below 2 s.d of the age-related mean normal value, diuresis less than 0.8 ml/kg/hr, capillary refill time more than 3 s, need for inotropic support, sudden change in mental state). The PRISM-score (274) was recorded using the most abnormal value of each parameter recorded in the first 4 hours after admission.

assays. On admission arterial blood was sampled as soon as possible: one ml was collected in fluoride-oxalate for measurement of lactate, one ml was taken in citrate for determination of fibrinogen, two ml were allowed to clot and serum stored at -70°C for measurement of cytokines and CRP in a later stage.

Lactate was measured by enzymatic end-point determination (228), CRP by immuno-nephelometric assay (315), and fibrinogen by standard procedures (69).

TNF and IL-1 were measured using an immunoradiometric assay for quantative measurement (IRMA, Medgenix Diagnostics Benelux), and IL-6 by using an enzyme amplified sensitivity immuno-assay (EASIA, Medgenix Diagnostics Benelux). These assays measure free and recptor-complexed cytokines.

statistical analysis. Differences between patient groups were analyzed using the unpaired two-tailed Student's t-test with separate variance estimation. The logarithmic values of the cytokine concentrations were used in this test. Relationships of cytokines to laboratory parameters were determined by calculating the Spearman correlation coefficient and a two-tailed significance.

3 RESULTS

From August 1989 till July 1990 seventeen patients fulfilled the criteria. In 15 patients *Neisseria meningitidis* and in 2 patients *Haemophilus influenza* was isolated in blood and/ or cerebrospinal fluid. Inotropic support was started only in one patient within 0.5 hour before admission, in the other patients when necessary after admission. According to the defined criteria 9 patients were in shock of whom 4 did not survive. The latter 4 patients were considered a separate group.

The values of lactate, fibrinogen, CRP, TNF, IL-1, and 6 for the different groups are presented in Fig. 1. Significant differences exist between all three groups concerning lactate, TNF, and IL-6. Fibrinogen, CRP and IL-1 discriminate only between survivors and non-survivors. Regarding the PRISM-scores, there were only significant ($p<0.01$) differences between survivors (10.1±6) and non-survivors (30.5±10)

A highly significant correlation (Table 1) exists between cytokines, the PRISM-score and lactate (TNF: r=0.69, IL-1: r=0.56, IL-6: r=0.65, PRISM: r=0.65). A significant inverse correlation exists between cytokines and CRP (TNF: r=-0.55, IL-1: r=-0.64, and Il-6: r=-0.56), and IL-6 and fibrinogen (r=-0.65). No significant correlations are found between TNF, IL-1, PRISM and fibrinogen, and PRISM and CRP.

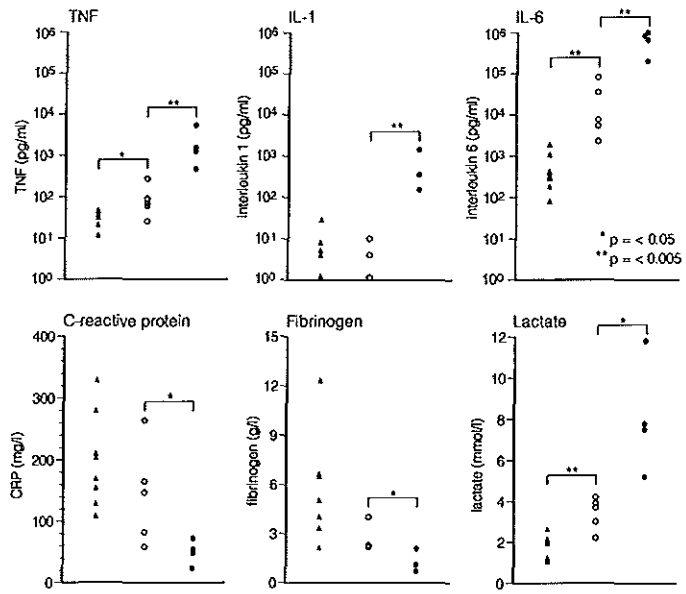

Figure 1. Serum concentrations for cytokines (TNF, IL-1, and IL6) and routine laboratory data (CRP, fibrinogen, and lactate) on admission for the 3 groups (triangles represent non-shock patients, open circles shock patients who survived, and the closed circles are shock patients who did not survive).

Table 1. Non-linear correlation-coefficients (Spearman) between routine laboratory parameters, PRISM-score and cytokines, and linear correlation coefficients between laboratory parameters and PRISM-score.

	lactate	CRP	fibrinogen	PRISM
TNF	0.71**	-0.50*	-0.43	0.66**
IL-1	0.56*	-0.64**	-0.05	0.49
IL-6	0.67**	-0.51*	-0.65*	0.79***
PRISM	0.65**	-0.50	-0.57	-

* p=0.05
** p=0.01
*** p=0.001

4 DISCUSSION

Septic shock with purpura is a life threatening clinical syndrome with an overwhelmingcourse and is frequently fatal. Most often it is caused by severe meningococcal disease (SMD) and the highest incidence is found in children under the age of 10 years. In The Netherlands the attack rate of meningococcal sepsis has increased from 100 cases in 1986 to 320 in 1991 on a total number of 15 million inhabitants. This increase has also been reported in other countries in Europe. On admission of those patients, it is not always easy to estimate the severity of the clinical situation. Nevertheless, one has to decide on the necessity for aggressive therapy and the level of supportive care. As we are more and more aware, septic shock is initiated by peripheral cellular alterations that lead to systemic dysfunction and organ failure. Some of these functional alterations may be caused directly by the infectious organisms or their products. An important portion of the pathogenesis however is mediated by endogenous host factors, particularly cytokines (56). In clinical studies, especially in SMD, the presence and concentration of TNF, IL-1, and IL-6 in serum turned out to be related to severity of disease and survival (57, 76, 109, 129, 131, 140, 249, 320, 360). Also the results in this study confirm the existence of a clear relation between the serum levels of TNF and IL-6 and both severity and survival of pediatric SMD-patients. A practical drawback of cytokines is the time required for the performance of the assays, taking several hours or even a day. Routine laboratory parameters like lactate, CRP and fibrinogen are easier and much faster to determine, but their relations to the level of cytokines and the severity of the disease have not been completely elucidated in SMD. Lactate, an end-product of anaerobic metabolism and in this way a marker of cellular dysfunction and tissue hypoperfusion (240), proved in this study to correlate well with severity of disease. Most of the studies regarding lactate in critically ill patients are in agreement with these results (140, 240, 285). However in a recently published study no significant difference in lactate was found between survivors and non-survivors in septic patients (90).

In our study a negative correlation was found between CRP-level and severity of disease with a significant difference between survivors and non-survivors. This is in agreement with the study of Leclerc (206). Other clinical studies on septic shock patients (90, 140), burn patients (249, 383), and experimental studies (109), have presented positive correlations between CRP or other acute phase proteins and survival. CRP has been demonstrated to bind to damaged tissue in vivo. When bound, CRP is capable of activating the complement system via the classical pathway (383). In addition, CRP stimulates the secretion of TNF, IL-1, IL-6, resulting in a self-amplifying inflammatory sequence (56, 383) and in this way plays a critical role in the inflammatory response to infection and injury. Nevertheless, a negative correlation between CRP and the level of cytokines was found. An explanation for this phenomenon might be either that in SMD, CRP is massively bound in the initial phase

of the illness or the fact that CRP may not yet have had enough time to increase in severe SMD. Peltola (267) described a group of patients with epiglottitis in whom the CRP level was related to the duration of the history. Leclerc found no relation between CRP level and the time between measurement and onset of symptoms (206).
We found significant differences in the fibrinogen level between survivors and non-survivors. There was a negative, non-significant correlation between fibrinogen and cytokines. These results are also described in other studies (129, 131). Fibrinogen is an acute phase protein, which is increased during infections and inflammation. It is an important clotting factor and the level is decreased during diffuse intravascular coagulation (DIC). In SMD the level of fibrinogen probably reflects more the severity of the DIC, than the extent of the acute phase protein reaction.
The serum levels of cytokines and lactate correlated well with the PRISM-score, which in another way suggests their relation with the severity of the disease.

5 CONCLUSIONS

A log-linear, positive relation exists between cytokines and arterial plasma lactate in children with septic shock and purpura. In this condition we observed a negative, exponential relation between cytokines and CRP, and fibrinogen on admission. Cytokines, as well as lactate, CRP and fibrinogen have a good prognostic value for survival. However lactate, TNF and IL-6 correlate significantly with the PRISM-score and differentiate also for the severity of the disease. These parameters can be used to select patient groups for the use of new therapeutic modalities, to follow the effect of these therapies, and to compare patient groups.

CHAPTER 2.3

INTERLEUKIN-12 LEVELS DURING THE INITIAL PHASE OF SEPTIC SHOCK WITH PURPURA IN CHILDREN: RELATION TO SEVERITY OF DISEASE

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1 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. Lipopolysaccharide (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 (360), IL-6 (154, 366), IL-8 (145), and IL-10 (85, 195), are increased in children with septic shock and purpura. Severity of disease is related to the initial plasma levels of LPS (44) and of these cytokines.

Interleukin 12 (IL-12) is a recently described cytokine (191, 333) 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: (65, 332, 333, 335-337)). 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 (65, 218), and in the host defense against bacterial, parasitic and viral infections (333). IL-12 also induces the production of interferon (IFN)-γ by T-cells and natural killer (NK)-cells (163, 381). The plasma levels of IFN-γ are increased in experimental models for sepsis (49, 162, 129) as well as in human sepsis (54, 129), although not consistently (360). Recently, IL-12 was characterized as a major cytokine in the pathogenesis of gram-negative endotoxemia in mice (163) and in primates (182). 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.

2 RESULTS

2.1 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,

HA-1-A (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 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.

2.2 Clinical and laboratory parameters

Clinical and laboratory parameters obtained on admission (PRISM-score, arterial lactate, WBC, 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)	Non-survivors* (N = 7)	p value**
Age (years)	3.8 (0.5-17.9)	4.9 (0.5-17.9)	2.2 (1.4-12.3)	0.259
Male / female	29/17	9/8	3/4	0.653
PRISM (score)	13 (1-38)	9 (1-20)	21 (17-25)	<0.001
Lactate (mmol/l)	5.0 (1.1-20.0)	4.2 (1.1-15.5)	7.2 (4.0-20.0)	0.047
WBC ($x10^9$/l)	9.2 (1.3-44.4)	17.0 (6.1-44.4)	4.9 (1.3-8.2)	<0.001
Glucose (mmol/l)	5.6 (1.0-14.2)	8.4 (1.9-14.2)	2.8 (1.0-10.1)	n.s
CRP (mg/l)	110 (34-250)	167 (39-250)	70 (38-162)	0.002

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 U-test).

2.3 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=< 0.001).The median (range) plasma IL-12 p40 level on admission (Fig. 1) was 457 (244-2677) pg/ml in non-survivors versus 189 (40-521) pg/ml in survivors (p= < 0.001). 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= 0.007) 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) (Fig. 2).

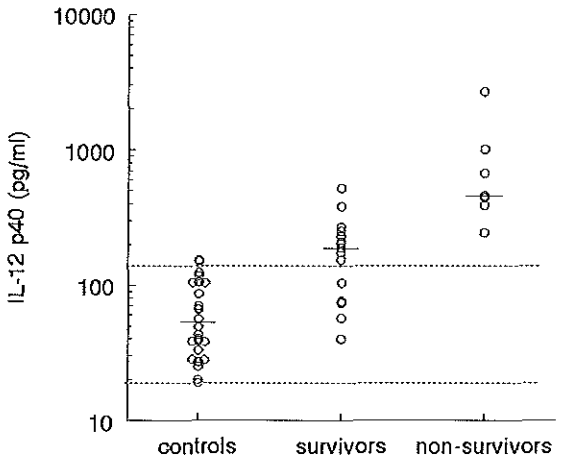

Figure 1. Plasma levels of IL-12 p40 on admission of children with septic shock with purpura (placebo treated, n=24) and of healthy controls (n=26). Solid lines represent median values. Dotted lines indicate the range of normal values.

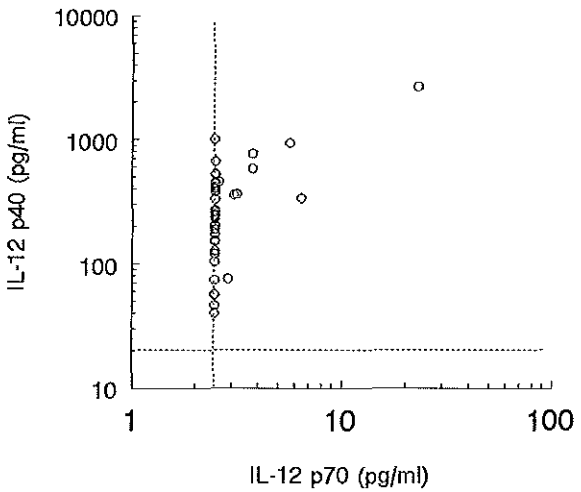

Figure 2. Scattergram of IL-12 p40 versus IL-12 p70. Data represent the levels on admission. Dotted lines represent the lower detection limits.

2.4 Relation between IL-12 and other cytokines on admission

IL-12 p40 plasma levels on admission were positively correlated with TNF- , 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 = 0.042) and IL-12 p40 (p = 0.007) levels than patients with undetectable levels of IL-12 p70.

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

	IL-12 p40 (N = 41)	
	r*	p value
TNF-α	0.45	0.003
IL-6	0.56	<0.001
IL-8	0.60	<0.001
IL-10	0.51	0.001

* Spearman's rank coefficient of correlation.

2.5 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 = 0.019).

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 value
PRISM	0.42	0.005
Lactate	0.16	0.286
WBC	-0.56	<0.001
Glucose	-0.39	0.01
CRP	-0.33	0.028

* Spearman's rank coefficient of correlation.

2.6 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 ($p < 0.005$) increased in comparison with patients with undetectable levels of IFN-γ. In addition, those 12 patients had significantly ($p < 0.005$) lower WBC and a significantly ($p < 0.05$) 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 ($p=0.08$).

3 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 (191) heterodimeric cytokine, which appears to play an important role as a functional bridge between natural resistance and adaptive immune response (332). 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 (163), thus protecting against lethality (262). Similar findings were reported in a model of a generalized Shwartzmann reaction in mice (381). In baboons challenged with *Escherichia coli* (182), 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 (65, 78) and polymorphonuclear cells (63) as well as in septic baboons (182) 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 (235). Studies by Ling et al. (217) 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 (126). Nevertheless, in vitro an in vivo studies have clearly shown that the production of p40 is linked to that of IL-12 (182, 333), and hence elevated levels of p40 subunit in our patients probably reflected the production of bioactive IL-12. Consistent herewith 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 (63, 79, 122). 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 (182). 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 (44, 195) 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 ß (191). The role of IFN-γ during in vitro LPS-challenge (77), in vivo endotoxemia in mice (44, 162, 163, 193, 381), or the generalized Shwartzmann reaction in mice (26, 27, 262), 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 (541) and in children with meningococcal septic shock (360). 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 (129). 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 (182).

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 (274). Serum lactate is related to the degree of circulatory failure (159). WBC and CRP are negatively correlated with the fulminant evolution of meningococcal septic shock (205, 206). Low serum glucose levels are reported by some authors (93), 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.

3.1 Patients and Methods

3.1.1 study protocol

Children above 3 months and below 18 years of age with septic shock and petechiae/ 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 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, 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 (274) 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.

3.1.2 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 45000 g for 30 minutes at +4°C. Plasma was stored at -70°C until tests were performed.

3.1.3 assays

White blood cell count (WBC), as well as lactate, glucose and C-reactive protein (CRP) levels were determined routinely. WBC 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 (315).
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.

3.1.4 assays of IL-12

IL-12 p40 antigen was measured with an ELISA (278). Briefly, mAbC11.79 and biotinylated mAbC8.6, both directed against the IL-12 p40 subunit (78), 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 (182). Shortly, mAb20C2, which has relative specificity for the IL-12 p70 heterodimer (78), 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.

3.1.5 statistical analysis

Results are expressed as medians (range) unless otherwise specified. Differences between groups were tested with the Mann-Whitney U-test or Fisher's exact test in case of percentages. Correlation coefficients given are Spearman's. Two-tailed p values ≤ 0.05 were considered statistically significant.

CHAPTER 3

COAGULATION AND FIBRINOLY DISORDERS

CHAPTER 3.1

PROTEIN C AND S DEFICIENCY IN SEVERE INFECTIOUS PURPURA OF CHILDREN: A COLLABORATIVE STUDY OF 40 CASES

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Intens Care Med 1992; 18:202-5

1 INTRODUCTION

Protein C (PC) and protein S (PS) are vitamin K dependent plasma proteins synthesized in the liver. In vitro studies have shown that, when PC is cleaved by thrombin in the presence of thrombomodulin, the activated PC thus formed is a potent anticoagulant and promotes fibrinolysis; PS serves as the cofactor for activated PC anticoagulant and fibrinolytic activities (70).

Microvascular thrombosis and skin necrosis with disseminated intravascular coagulation occur in severe infectious purpura (204, 205) in which PC and PS deficiencies have been reported (46, 222, 230, 280), and in homozygous PC deficiency (231) and homozygous PS deficiency (224).

The aim of this study was to examine, in severe infectious purpura of children, the relationships between PC and PS levels determined on admission, and shock, DIC, and outcome.

2 METHODS

Forty consecutive children, hospitalized with severe infectious purpura in Rotterdam (n = 22) and Lille (n = 18) between January 1988 and February 1990, were prospectively studied. Mean age was 52 months (ranging from 6 -185 months). Purpura on admission was petechial in 11, ecchymotic in 20, and necrotic in 9. *Neisseria meningitidis* was recovered from 29 children and *Hemophilus influenzae* from one. No organism was identified in the remaining children, but in these cases antibiotics were started before admission.

Shock was defined (205) by two of the following criteria: systolic blood pressure less than 2 standard deviations of normal (144), capillary refill time longer than 3 s, and urine output less then 1 ml/kg/h.

The hemostatic studies, on admission, consisted of platelets, fibrinogen, factors II, V, VII, X, fibrin degradation products, and fibrin monomer measurements. We also determined on admission, antithrombin III (AT III) activity and PC activity by chromogenic test (StagoFrance), PC antigen and total PS by ELISA (Stago-France); in 16 children from Lille, free PS was determined by ELISA (Stago-France). Results were expressed as % of normal adult values. DIC was defined by the combination of 3 of the following features: platelet count less than $150x10^9$/l, fibrinogen less than 2g/l, factor V less than 60%, and presence of fibrinogen degradation products or fibrin monomers.

Statistical analysis was performed using Wilcoxon rank-sum test, and analysis of covariance for age-adjusted comparisons, with a general linear model procedure (Statistical Analysis System, Cary, NC).

3 RESULTS

Mean age, frequency of shock and DIC, between children from Rotterdam and those from Lille were not statistically different. Mean age between shock and non-shock children, between children with and without DIC, and between survivors and non-survivors, were not statistically different.

Of the 30 children in shock, 20 had DIC. All children with DIC, and 10 without DIC were in shock. Of 20 children who were in shock and had DIC, 7 died (2 from Rotterdam and 5 from Lille: difference not significant) and 3 survived with amputation; no child without shock or DIC died.

Common hemostatic variables are shown in Table 1. In our children as a whole, factor II levels correlated with PC antigen, PC activity, and total PS levels ($p<0.005$); factor VII levels correlated with PC activity levels ($p< 0.05$), but did not correlatie with PC antigen, and total PS levels; factor X levels correlated with PC antigen, PC activity, and total PS level ($p< 0.05$). PC antigen, PC activity and total PS levels in the different groups of children are shown in Figs. 1 - 3.

Table 1. Hemostatic variables (mean ± SD) on admission in 40 children with severe infectious purpura

Variable	Shock + (N = 30)	Shock - (N = 10)	DIC + (N = 20)	DIC - (N = 20)	Non-survivors (N = 7)	Survivors (N = 33)
Platelets (10^9/l)	103 ± 14 **	217 ± 29	60 ± 37 ***	203 ± 81	44 ± 18 **	*150* ± 95
Fibrinogen (g/l)	2.8 ± 2.7 **	*5* ± 1.4	1.3 ± 1 ***	5.4 ± 2.1	0.3 ± 0.4 ***	4 ± 2.4
Factor II (%)	*50* ± 27 *	66 ± 13	36 ± 18 ***	72 ± 16	25 ± 18 **	60 ± 22
Factor V (%)	44 ± 39 *	74 ± 30	21 ± 14***	80 ± 32	11 ± 7 **	60 ± 37
Factor VII (%)	25 ± 20	32 ± 17	19 ± 11	33 ± 22	20 ± 14	28 ± 20
Factor X (%)	58 ± 39	56 ± 25	41 ± 18 **	73 ± 41	32 ± 20	63 ± 35

DIC, disseminated intravascular coagulation; +, present; - absent; *p<*0.05;* **p<*0.005;* **p<*0.0001*

PC antigen and PC activity were significantly decreased in shock children, in children with DIC, and in non-survivors. Age-adjusted levels of PC antigen and PC activity were also significantly decreased in shock children, in children with DIC, and in non-survivors (PC antigen: shock children/non-shock children ($p<0.05$), children with DIC/children without DIC ($p<0.0005$)), non-survivors/survivors ($p<0.05$); PC activity: shock children/non-shock children ($p<0.05$), children with DIC/children without DIC ($p<0.0005$), non-survivors/ survivors ($p<0.005$). Total PS was not different between shock and non-shock children but was significantly decreased in children with DIC and in non-survivors. Age-adjusted levels

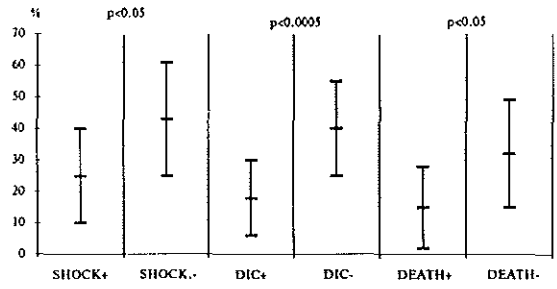

Figure 1. Protein C antigen levels on admission (mean ±SD) expressed as % of normal adult values in severe infectious purpura, DIC, disseminated intravascular coagulation; +, present; -, absent.

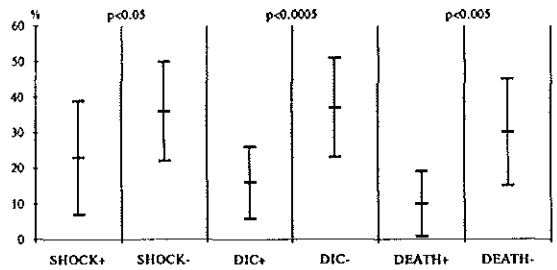

Figure 2. Protein C activity levels on admission (mean ±SD) expressed as % of normal adult values in severe infectious purpura, DIC, disseminated intravascular coagulation; +, present; -, absent.

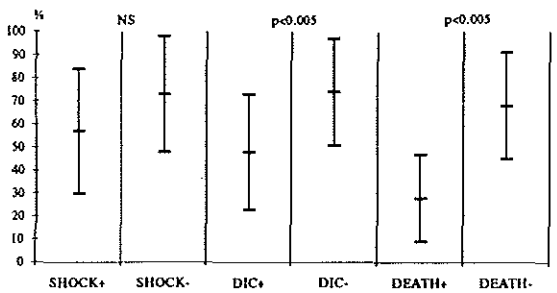

Figure 3. Total protein S levels on admission (mean ±SD) expressed as % of normal adult values in severe infectious purpura, DIC, disseminated intravascular coagualation; +, present; -, absent.

of total PS were also significantly decreased in children with DIC ($p < 0.005$), and in non-survivors ($p < 0.005$). In the 16 children in whom free PS levels were determined, free PS level (mean ± SD = 36±15) correlated ($p<0.005$) with total PS levels (mean ± SD = 39 ± 18); because of the small number of free PS level determinations, comparisons between the different groups were not performed. AT III activity was also significantly decreased in shock children (shock children: 55±21/non-shock children: 73±19; $p<0.05$), in children with DIC (children with DIC: 47±16/children without DIC: 72±20; $p<0.0005$), and in non-survivors (non-survivors: 42 ± 22/survivors: 63 ± 20; $p<0.05$).

4 DISCUSSION

On admission of our patients, PC deficieney was always present, even in the absence of shock and DIC; PC deficiency could not be explained by age because Nardi et al. showed that PC levels were in the adult range by 1 year of age (246): only 4 of our 40 children were younger than 1 year. Moreover, PC antigen and PC activity levels in our patients were under 75.8 % ± 11 %, the value given by Nardi et al. in children between 8-12 months of age (246). Acquired PC deficiency was reported by Griffin et al. in adult patients with DIC: 6 of 9 adult patients with severe infection in the absence of liver disease had decreased PC level (patients who died had a lower PC level than those who survived, but there was no significant difference); however 5 of 15 patients without fibrinogen degradation products had a decreased PC level (136). PS deficiency was also reported in adult patients with DIC (160).

PC and PS deficiencies were reported by Fourrier et al. in 5 adult patients with severe infectious purpura who survived (112). Since the report from Marlar et al. (1 case with meningococcal sepsis) (230), Powars et al. reported acquired PC and PS deficiencies in 6 children with meningococcemia and purpura fulminans (280). Brandtzaeg et al. studied 13 children with fulminant meningococcal septicemia; in these children PC levels were lower than in children with meningococcal meningitis and meningococcemia with shock, but PC levels were not different between survivors and non-survivors (46). Blanco et al. reported PC deficiency in 23 children with meningococcal sepsis; surprisingly, PC levels were not different in children with DIC as compared with those without DIC (28). Madden et al. recently reported 2 children with infectious purpura fulminans and PC and PS deficiency (222). In 1 patient of Powars et al. (280), in the 2 patients of Madden et al. (222), and in 4 of our patients, PC level was measured after recovery: it rose to normal value; this excluded congenital PC deficiency revealed by an infection as reported by Ozsoylu et al. (263).

PC level depression in DIC is probably due to ongoing consumption of the protein and not to impaired synthesis: Heeb et al. have demonstrated that in many cases of DIC, PC is activated and complexed with a PC inhibitor and then cleared from circulation (160). As PC antigen and PC activity levels were also decreased in our children without DIC, an impaired synthesis cannot be ruled out; however, these results might also be explained by the drastic definition of DIC we chose.

In our patients who had patent DIC, PC was far more decreased than PS, suggesting a peculiar implication of PC in the hemostatic abnormalities. This selective consumption was also noted by Madden et al. (222).

Taylor et al. have shown that exogenously added activated PC prevents the coagulopathic response and lethal effects of E. Coli injections in baboon; in additional experiments they found that blocking PC activation in vitro led to a more severe pathologic response to

sublethal concentration of *E. coli* organisms: this response was prevented by coinfusion of activated PC: the mechanism by which activated PC may function in this shock model is as an anticoagulant (323). Activated PC probably also functions as a profibrinolytic agent; Okajima et al. have suggested that PC might regulate the plasminogen activator inhibitor 1 activity under some conditions such as in septicemia in which PAI-1 activity increases remarkably (258). Furthermore, Pralong et all. in patients with septic shock (282), and Brandtzaeg et al. in children with systemic meningococcal disease (42) have shown that PAI-1 levels are significantly higher in patients with a fatal outcome than in survivors.
In the literature, congenital PC deficiency has been treated by fresh frozen plasma or factor IX concentrates (231). However infusion of large volumes of fresh frozen plasma achieves a plasma PC level of only 10 % of normal, and thromboembolic complications following administration of factor IX concentraties have oceasionally been reported (359). A severe congenital PC deficiency was successfully treated by Vukovich et al. with a concentratie of human PC and PS given every 48 h (359). Nowadays, advances in recombinant DNA technology permit the production of pure PC and activated PC. Madden et al. have shown that recombinant PC has similar properties to the plasma form of PC (223).
We conclude that PC and PS levels were decreased in our children with severe infectious purpura of proved or suspected bacterial origin, and that PC levels were significantly decreased in shock children, in children with DIC, and in non-survivors. As PC has anticoagulant and profibrinolytic actions, PC supplementation, and AT III supplementation (which has been proposed in aduit meningococcemia (112)) should be evaluated in children with severe infectious purpura with shock and DIC.

CHAPTER 3.2

AGE-RELATED DIFFERENCES IN OUTCOME AND SEVERITY OF DIC IN CHILDREN WITH SEPTIC SHOCK AND PURPURA

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1 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 *N. meningitidis*. 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 (25, 268, 212, 319, 330). Natural anticoagulants, such as antithrombin III (ATIII) and protein C and S play an important role in this process (96, 97, 110, 112). Levels of protein C and S are decreased in septic shock and purpura, which may explain the severe procoagulant state (46, 100, 112, 127, 167, 207, 279, 280, 307). 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 (279). 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 TNF-α , IL-6, IL-8, IL-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.

2 PATIENTS AND METHODS

2.1 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 (274) 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 degradation products.
The study protocol was approved by the Medical Ethics Committee of the University Hospital Rotterdam.

2.2 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 20000 g for 30 minutes at +4°C. Plasma was stored at -80°C until tests were performed.

2.3 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 (315). 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 (APTT) was measured with the Neotromtin reagent, in which vegetable phospholipids and ellagic acid as a particulate activator are used. (N 28-40 sec.). Fibrinogen was determined with a modified Clauss method (69) (N > 2 g/l). The activities of coagulation prothrombin, factors VII, V and X were measured by one stage clotting assays, based on the thromboplastin time, 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 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 III (AT III), the plasminogen as well as the alpha-2 antiplasmin activities were determined in quantitative kinetic tests by a synthetic chromogenic substrate method (N 70-140%). 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 electroimmunodiffusion (Laurell rocket technique). (N 68-140%, ± 250 µg/ml). Plasminogen activator inhibitor-1 (PAI-1) antigen was determined using an ELISA and expressed as ng/ml. Thrombin-antithrombin III complexes (TAT), plasmin- 2-antiplasmin complexes (PAP), and tissue-type plasminogen activator (t-PA) were measured with ELISA's (TAT, t-PA) or radioimmunoassay (PAP) previously described (32). Results were expressed as nmol/l (PAP), µg/l (TAT) or ng/ml (t-PA).

2.4 Statistical analysis

Results are expressed as medians (range) unless otherwise specified. Differences between groups were tested with the Mann-Whitney U-test or Fisher's exact test in case of percentages. Correlation coefficients given are Spearman's. Two-tailed p-values ≤ 0.05 were considered as statistically significant.

3 RESULTS

3.1 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=0.006) 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-1-A (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.

3.2 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=0.013) 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: 32-50ml) 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.

3.3 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 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)	Non-survivors (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 m/f	47/32	35/23	12/9	26/14	21/18
Need for inotropics	74 (94%)	53 (91%)	21&&	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	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	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	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	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≤ 0.05, **=p≤ 0.01, &=p≤ 0.005, &&=p≤ 0.001.

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

Cytokine	Survivors (N=35)	Non-survivors (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)	15200 (36-1.5x10^5)	280000 (87-2.0x10^5)&	66000 (36-2.0x10^5)	15000 (87-1.5x10^5)
IL-8 (pg/ml)	1100 (13-0.38x10^5)	88000 (23-0.37x10^5) &&	6500 (13-0.37x10^5)	1100 (23-0.38x10^5)
IL-10 (pg/ml)	2260 (41-0.11x10^5)	19000 (30-0.10x10^5) *	7200 (41-0.10x10^5)	2260 (30-0.11x10^5)

Values are medians (range); significance of the difference between groups (Mann-Whitney U-test):
*=p≤ 0.05, &=p≤ 0.005, &&=p≤ 0.001.

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

Coagulation parameter	Survivors (N=57)	Non-survivors (N=19)	≤ 3.1 Years (N=38)	> 3.1 Years (N=38)	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/)	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
F V (%)	40 (5-116)	16 (2-48) &&	25 (2-116)	38 (3-77) *	70-120
F VII (%)	19 (3-41)	21 (5-41)	25 (5-41)	18 (3-34) *	70-120
F VIIIc (%)	84 (3-286)	22 (2-62) &&	42 (4-133)	87 (2-286) &&	70-150
F vW (%)	422 (200-711)	394 (227-629)	376 (227-570)	454 (200-711) *	80-120
F vW/VIIIc	5.0 (1.9-147)	17.5 (5.7-264) &&	8.6 (2.4-30.6)	5.2 (1.9-264) *	1
F IX (%)	50 (16-118)	40 (22-86) &	48 (16-86)	47 (23-118)	70-120
F X (%)	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≤ 0.05, &=p≤ 0.005, &&=p≤ 0.001.

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=57)	Non-survivors (N=19)	≤ 3.1 Years (N=38)	> 3.1 Years (N=38)	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
AT III (%)	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
$_2$-Antiplasmin (%)	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 U-test): *=p≤ 0.05, **=p≤ 0.01, &=p≤ 0.005, &&=p≤ 0.001.

3.4 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, VIIIc, 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 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.
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 ATIII, antiplasmin, 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 ($p=0.011$) different between children above and below 3.1 years.

3.6 Relationship of cytokines to coagulation parameters

APTT, fibrinogen, FVIIIc, 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. The results are summarized in Table 5. Only those parameters are listed which showed significant correlation with 3 or more cytokines. Differences between the correlation patterns for both groups were: the strong negative correlations between fibrinogen, FVIIIc, 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.

4 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 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 (100, 207, 274, 280). There were no differences between PC antigen and PC activity. Increased levels of PC inhibitor did, therefore, not explain the decreased PC levels (230). 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 (323). 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 (70). 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 (30), studies in volunteers (319), and human septic shock patients (268), 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 (358). In our study t-PA levels, though increased, were not associated with outcome and only correlated weakly with serum lactate ($r=0.45$, $p=0.001$) and WBC ($r=0.31$, $p<0.05$). The levels of t-PA did correlate weakly with the TAT complexes ($r=0.34$, $p < 0.05$), 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. α2-Antiplasmin levels were significantly lower in non-survivors, as was previously reported (42, 168). This presumably reflects consumption of this antifibrinolytic protein, since nearly all patients had increased circulating PAP-complexes. Decreased α2-antiplasmin levels facilitates 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 (30), and in human sepsis (42, 94, 168, 268, 282) and are associated with a decrease in levels of PAP-complexes (272). 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 (195).

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 (8). Protein C is somewhat unusual since it does not reach adult levels until the fourth year of life (246). 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 (279). 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 coagulation response in the younger children. We suggest that this more severe coagulation response was due to the immature state of their clotting system, in particular of the anticoagulant pathways, resulting in extensive consumption of coagulation factors. 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 2 groups. Plasma levels of PAI-1 of both age groups correlated strongly with cytokine levels, as well as with TAT-complexes ($r=0.73$, $p<0.001$; $r=0.80$, $p<0.001$). 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 ($r=-0.05$, $p=0.81$), there was a very strong correlation found in the older age group ($r=0.80$, $p=<0.001$). 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 ≤ 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, WBC, glucose, and CRP) 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 opsono-phagocytic 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 microthrombosis 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 the activation of the coagulation was not influenced (270). However, the same group established that protein C treatment prevented the coagulopathic as well as the lethal effects (323). The same combined effect was described for tissue factor pathway inhibitor (60). 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.

CHAPTER 3.3

THE RELATIONSHIP BETWEEN PLASMINOGEN ACTIVATOR INHIBITOR-1 AND PRO-INFLAMMATORY AND COUNTER-INFLAMMATORY MEDIATORS IN CHILDREN WITH MENINGOCOCCAL SEPTIC SHOCK

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1 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 (44, 129, 146, 205, 331, 349).
Lipopolysaccharides (endotoxin), a component of the outer membrane of gram-negative bacteria, induces the release of pro-inflammatory 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 (36, 47, 340, 341, 344). The production of pro-inflammatory cytokines and the extent of the inflammatory response is downregulated by counter-inflammatory 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 (363). The biologic activity of TNF-α is also neutralized by sTNFR-55 and -75 (82, 194, 203, 219, 300). 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 277.
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 (319). Fibrin deposition and complement activation cause extensive endothelial damage and are associated with multiple organ failure (61, 141, 161).
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 pro-inflammatory cytokines (TNF-α, IL-6, IL-8) and counter-inflammatory 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.

2 METHODS

2.1 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 (274). 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.

2.2 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 (315).

2.2.1 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.

2.2.2 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 (69) (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 (348) 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 (207).

2.3 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 non-survivors. Two-tailed P values ≤ .05 were considered statistically significant.

3 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.

3.1 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 non-survivors 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 non-survivors. Serum CRP levels were also significantly lower in non-survivors 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).

The parameters of coagulation and fibrinolysis are summarized in Table 2. DIC was observed in all non-survivors 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 non-survivors. Plasminogen levels were similar in survivors and non-survivors. The t-PA, PAI-1 antigen, and FDP levels were higher in non-survivors than in survivors.

Table 1. Characteristics of patients with meningococcal septic shock.

Characteristic	Survivors (n = 27)	Non-survivors (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.

3.2 Pro-inflammatory cytokines and counter-inflammatory compound levels at admission

At admission, serum levels of pro-inflammatory cytokines and counter-inflammatory 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 non-survivors 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).

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

Characteristic	Reference range	Survivors (n = 27)	Non-survivors (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).

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

	Survivors (n = 27)	Non-survivors (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.

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.

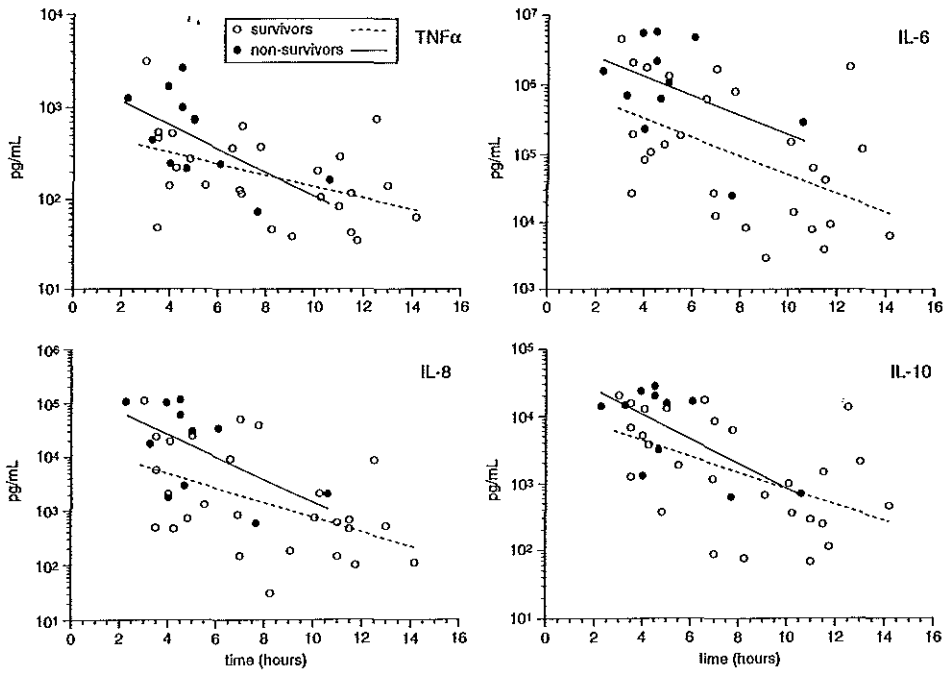

Figure 1. Relation between initial serum concentrations of inflammatory compounds (tumor necrosis factor, TNF-α , interleukin IL-6, -8, -10) and interval between onset of petechiae and blood sampling in 38 children with meningococcal septic shock. Solid and dotted lines indicate regression through values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

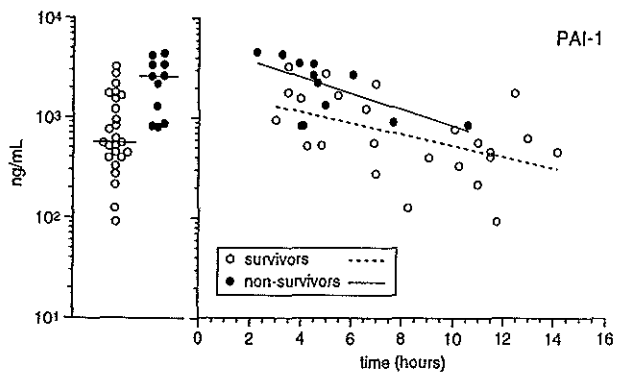

Figure 2. Initial plasma levels of plasminogen activator inhibitor (PAI-1; $n = 36$). Left, relation between PAI-1 levels and survival. Right, initial PAI-1 plasma levels in relation to interval between onset of petechiae and blood sampling. Slope between survivors and non-survivors did not significantly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

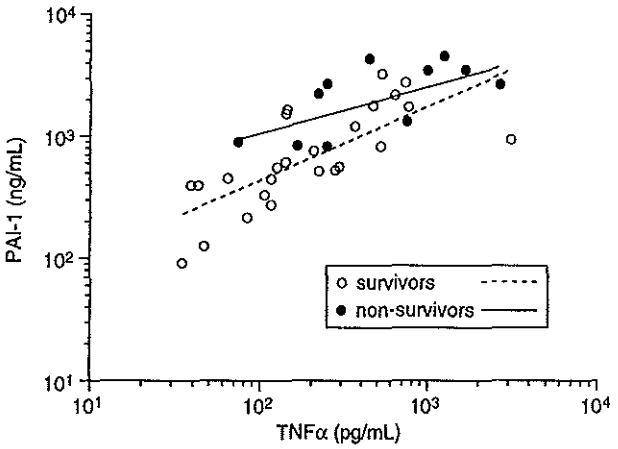

Figure 3. Relation between initial levels of tumor necrosis factor (TNF)-α and plasminogen activator inhibitor-1 (PAI-1) in 36 patients with meningococcal septic shock. Slope between regression lines of survivors and non-survivors did not significantly deviate from parallelism ($P = .31$).

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 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.

3.3 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 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 non-survivors 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 non-survivors ($P = .01$) 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 $r = .60$, $P < .001$).

4 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 pro-inflammatory 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 (44, 129, 130, 145, 159, 211, 343).

A wide inter-individual variability in TNF-α release after stimulation by endotoxin has been demonstrated in vitro in whole blood samples and peripheral blood mononuclear cells isolated from healthy volunteers (87, 241). 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 (372). 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 (237). The TNF2 allele has been associated with higher constitutive expression and greater secretion of TNF-α after induction (374).

The initial concentrations of TNF-α in this and previous studies were significantly higher in MSS non-survivors than in survivors. However, the magnitude of TNF-α serum levels and of other pro-inflammatory 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. (85, 90, 211, 260). 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 (85, 129, 145). In addition, in the present study, CRP levels, which indirectly reflect the duration of illness (267), 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 non-survivors suggest a shorter disease course and may therefore explain the higher levels of cytokines. The earlier PICU admission of non-survivors 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, non-survivors may have been admitted earlier because of a more rapid deterioration due to greater responsiveness to lipopolysaccharides or pro-inflammatory cytokines.

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 (239, 344). In experiments in baboons infused continuously with *Escherichia coli*, peak levels of TNF-α also occured very early in the course of disease (103, 239, 287). 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 non-survivors disappeared after adjustment for the time between onset of petechiae and blood sampling. These data suggest that the survivors and non-survivors in the present study may have had similar releases of inflammatory cytokines (TNF-α, IL-6, IL-8).

The pro-inflammatory cytokines are counteracted by counter-inflammatory compounds. We observed significantly higher concentrations of IL-10 in non-survivors with MSS. Lehmann et al. (211) recently reported that high IL-10 levels in patients with meningococcal disease are associated with fatality. In contrast, Derkx et al. (85) 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 non-survivors. IL-10 acts as a potent inhibitor of the release of the pro-inflammatory cytokines TNF-α , IL-1ß, IL-6, and IL-8 from T-cells (355), polymorphonuclear leukocytes (62), and monocytes and macrophages (33, 102, 363). 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 (17, 124, 176). Chernoff et al. (66) 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 pro-inflammatory cytokines TNF-α, IL-6, and

IL-8 in survivors and non-survivors suggests the presence of an adequate IL-10 response to down-regulate the production of pro-inflammatory cytokines.

In addition, sTNFR-55 and -75 can neutralize the biologic activity of TNF-α. Girardin et al. (130) found that high levels of the sTNFRs are associated with an increased likelihood of fatality, and Froon et al. (118) 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 (21). The higher serum creatinine levels of non-survivors ($P = .08$) 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 (14, 271). 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 (14, 23, 24, 247, 348). 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 non-survivors ($P = .003$).

Endotoxin, TNF-α, and IL-1ß modulate the fibrinolytic system to secrete both t-PA and PAI-1, which respectively activate and inhibit fibrinolysis (74, 92, 151, 213, 272, 303, 345). Moreover, fibrin and thrombin formed during coagulation are also potent inducers for the release of t-PA and PAI-1 (123, 177). 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 non-survivors. This finding together with decreased protein C activity result in insufficient fibrinolytic activity during a markedly procoagulant state that is associated with vital organ microembolization (61, 141, 161, 219). Administration of recombinant t-PA may therefore be considered an adjuvant therapeutic option in patients with fulminant meningococcemia as suggested by Zenz et al. (389).

In previous studies, PAI-1 levels rapidly decreased after hospitalization (42, 94), and PAI-1 and TNF-α levels were strongly associated (121, 233). 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 non-survivors at a similar TNF-α concentration. We questioned whether inter-individual 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 (264) and in patients with an increased risk of recurrent myocardial infarction (148).

The promotor containing the deletion allele produced six times more mRNA than the insertion allele in response to IL-1ß (79). The insertion/deletion polymorphism in the PAI-1 promotor is of functional importance in the regulation of the expression of the PAI-1 gene (79). 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 (236).

We conclude that high levels of pro-inflammatory cytokines and counter-inflammatory 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 non-survivors 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.

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CHAPTER 3.4

THE PLASMINOGEN ACTIVATOR INHIBITOR-1 4G/5G PROMOTER POLYMORPHISM AFFECTS PLASMA LEVELS OF PAI-1 AND OUTCOME OF MENINGOCOCCAL DISEASE

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CHAPTER 4

EXPERIMENTAL AND CLINICAL FEATURES OF CIRCULATORY FAILURES

CHAPTER 4.1

COMPLEMENT ACTIVATION IN RELATION TO CAPILLARY LEAKAGE IN CHILDREN WITH SEPTIC SHOCK AND PURPURA

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1 INTRODUCTION

The complement system is involved in many aspects of the inflammatory response in patients with sepsis: amongst others it mediates chemotaxis of neutrophils and opsonization of particles and micro-organisms, stimulates the release of granules from leucocytes, induces vasodilatation, and enhances vascular permeability, either or not via its influences on the cytokine network and coagulation system (31). Septic shock with purpura in children is mainly caused by meningococci. It is generally accepted that this condition results from the release of large amounts of lipopolysaccharide (LPS) into the circulation leading to a capillary leakage, severe hypotension, micro-thrombosis, and ultimately to organ failure and death (39, 158). The capillary leakage which occurs during sepsis, is considered to be induced by the release and activation of endogenous inflammatory mediators such as cytokines and activated components of the complement and contact systems. Genetic deficiencies of the complement system, in particular those of the membrane attack complex, lead to a higher susceptibility for and recurrent infections with *N. meningitidis* (99). However, most children with severe meningococcal disease (SMD), are complement sufficient (18,170). Thus, excessive complement activation, and not complement deficiency, is responsible for low levels of native complement components in most patients with severe meningococcal sepsis (31, 35, 47, 89, 141, 329). The degree of complement activation in SMD is related to the concentration of lipopolysaccharide (LPS) in plasma (41, 47). Patients with mild disease (low or undetectable LPS plasma levels) have a low degree of complement activation, whereas patients with septic shock and purpura are characterized by high circulating levels of LPS as well as excessive complement activation.
This study was designed to assess the extent of complement activation, and the possible route and regulation of this activation in children with septic shock and purpura. Moreover, we investigated the relation between complement activation and severity of disease, as reflected by the degree of capillary leakage, and by circulating levels of several inflammatory mediators.

2 PATIENTS AND METHODS

2.1 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. Patients were

eligible for inclusion when they met both 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 suppletion or evidence of adequate intravascular volume [defined by the presence of a palpable liver, a cardio-thoracic 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 (274) was calculated using the most abnormal value of each variable recorded during the first 4 hours after admission at the PICU. The exact amount of volume suppletion (human plasma or fresh frozen plasma) administered during the stay in the ICU to restore the hemodynamic condition of the patient, was recorded. All patients received maximal supportive therapy: antibiotics, volume-administration, inotropic support, and mechanical ventilation.
The study protocol was approved by the Medical Ethics Committee of the University Hospital Rotterdam.

2.2 Collection of blood

Arterial blood samples were collected within two hours after admission, and after 24 and 72 hours. Blood for analysis of complement parameters was collected in vials containing 3.8% trisodium citrate, immediately chilled on ice, and centrifuged at 4000 g for 10 minutes. Subsequently, platelet-poor plasma was obtained by centrifugation of the supernatant at 20000 g for 30 minutes at +4°C. Plasma was stored at -80°C until tests were performed.

2.3 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 (315).
C3a levels were determined by a radio-immuno-assay (RIA) described previously (143) and expressed as nM. Levels of C3a in healthy persons are below 5 nM. Activation of C4 was assessed with an ELISA as described previously (377). In brief, mAb anti C4-1, which

recognizes a neo-epitope on the C4 activation products C4b, C4bi, and C4c, together referred to as C4b/c, and not on native C4, was used as a catching Ab. Biotinylated polyclonal rabbit anti-human C4 Abs were used as detecting Abs. Results were expressed in nM. Activation of C3 was assessed with a similar ELISA (377). In brief, mAb anti-C3-9 recognizing a neo-epitope on C3b, C3bi, and C3c (C3b/c) was used as catching Ab and biotinylated polyclonal rabbit anti-human C3c Abs were used as detecting Abs. Results were expressed in nM. Complement-CRP complexes were determined using a novel method (377). In short, purified complement complexes were quantified by differential antibody ELISAs. In these ELISAs monoclonal antibodies directed against C4d and C3d were used to capture complexes. These antibodies capture C4b/C4bi/C4d and C3b/C3bi/C3d, respectively. CRP-complement complexes were detected by biotinylated monoclonal antibodies against CRP. Results were expressed as pM of complement fixed to CRP. Levels of both types of complexes in normal healthy volunteers were below the limit of detection (4 pM).
Functional and proteolytically inactive C1-Inh (fC1-Inh and iC1-Inh, respectively), were determined with RIAs (252). The concentration in normal pooled plasma of fC1-Inh and iC1-Inh was 2.5 µM and 0.08 µM, respectively; levels in patients were expressed as % of this plasma pool. Type II secretory-phospholipase A2 ($sPLA_2$) was determined by ELISA (379). $SPLA_2$ levels in healthy volunteers are below 5 µg/l. Lactoferrin and elastase-α_1-antitrypsin (α_1AT) complexes were determined with RIAs (254). IL-6 and IL-8 were measured with ELISAs obtained from the Department of Immune Reagents (CLB, Amsterdam). These ELISA´s were performed according to manufacturers' instructions. Normal levels are <10 pg/ml for IL-6, and <20 pg/ml for IL-8. C4BP concentrations were measured by electro-immunodiffusion. Normal levels are 68-140% of normal.

2.4 Statistical analysis

Results are expressed as medians (range) unless otherwise specified. Mann-Whitney U-test and Wilcoxon signed rank test were used to evaluate between and within groups differences. Relationship between parameters were tested by assessing Spearman's rank correlation. Multiple regression analysis, taking account of duration of skin lesions, was used to compare survivors and non-survivors regarding the logarithmically transformed levels of inflammatory parameters. Logistic regression was performed to evaluate the relation of the various parameters to mortality. This analysis proceeded on two steps. In the first step parameters were grouped into categories (cytokines parameters, neutrophil degranulation, complement activation products, and complement regulation). For each category separately the variables most predictive for mortality were determined using backward elimination. In the second step, the remaining variables after the first step were combined and again the backward elimination procedure was applied. The same

procedure was used with multiple regression to assess the relation between the various parameters and capillary leakage and lactate respectively. Two-tailed p values ≤ 0.05 were considered statistically significant in each test.

3 RESULTS

3.1 Demographics

Fifty-two patients fulfilling the entry criteria and admitted to the PICU were enrolled in the study. Thirty-two were males (62%), 20 females (38%) The median age was 3.3 years (range 0.4-17.9). Thirty-eight of the 52 patients (73%) survived, the other 14 (27%) died. Cultures of blood, cerebrospinal fluid or skin biopsies revealed *Neisseria meningitidis* in 46 (89%) patients and *Haemophilus influenzae* in 1 patient. Cultures were sterile in 5 patients. Thirty-three (64%) patients needed mechanical ventilation. Due to the lack of sufficient plasma, complement parameters were not determined on admission in 7 patients. Forty-nine of the children participated in a randomized, placebo-controlled trial to evaluate the efficacy of a human monoclonal antibody, HA-1-A (Centoxin®, Centocor, Malvern, PA, USA), in meningococcal septic shock. On admission, HA-1-A or placebo were administered after blood was collected. There was no significant difference in mortality between HA-1-A-treated and placebo-treated patients.

3.2 Differences between survivors and non-survivors

In table 1 and 2 clinical and laboratory parameters related to severity of disease, and to the extent of cytokine release, neutrophil degranulation and complement activation, are compared between survivors and non-survivors. As expected clinical and biochemical parameters related to the severity of disease were significantly different between survivors and non-survivors. Regarding parameters related to cytokines, IL-6 and IL-8 levels were substantially elevated and significantly higher in non-survivors than in survivors. Levels of the two acute phase proteins CRP and $sPLA_2$, of which the synthesis is induced by cytokines and which hence reflect the release of cytokines, were both elevated in either patient group but in contrast to cytokines, the highest levels occurred in survivors. All patients had evidence of increased degranulation of neutrophils: elastase levels were elevated in all patients, although the difference between levels in survivors and non-survivors did not reach statistical significance (p=0.08). Also circulating levels of lactoferrin were increased in all but one child, levels in non-survivors were significantly higher.
Regarding activation of complement, C3a and C3b/c levels were increased in all but four the patients, whereas C4b/c was elevated in all but one. Levels of either complementactivation product were higher in non-survivors. All patients had increased

Table 1. Clinical and laboratory parameters related to severity of disease on the time of admission in children with septic shock and purpura

Parameter	survival		normal values
	survivors (N=38)	non-survivors (N=14)	
PRISM	10 (1-38)	20 (8-25)[&]	
lactate (mmol/l)	4.3 (1.1-15.5)	7.3 (2.9-20.0)[&]	< 2.0
duration petechiae (h)	5.3 (1.0-11.5)	3.6 (0.5-8.5)[&]	
WBC (x 10^9/l)	13.2 (1.4-44.4)	5.8 (1.3-12.6)[&]	35894
plasma infusion (ml/kg)	65 (12-307)	120 (59-182)[&]	

Values are medians (range); [&] $p < 0.05$; for the difference between groups (Mann-Whitney U-test), PRISM, pediatric risk of mortality score; WBC, white blood cell count.

complement-CRP complexes (C3 and C4) indicating that part of the complement activation had occurred via CRP. However, levels of these complexes were lower in non-survivors, the difference of C3-CRP complexes between survivors and non-survivors was significant. Functional C1-Inh levels varied widely. In survivors levels were decreased in some patients, whereas they were normal or elevated in others. In the non-survivors functional C1-Inh was decreased or normal, but never elevated. However, differences between survivors and non-survivors were not significant. Levels of proteolytically inactivated C1-Inh were normal in 9 of the patients. In the other patients the levels, though substantially elevated, varied widely. The difference between survivors and non-survivors just did not reach statistical significance (p=0.07).

3.3 Relation between inflammatory and clinical parameters

Multiple regression analysis for the relation between levels of inflammatory parameters and survival and duration of skin lesions showed that time adjusted concentrations of IL-6, IL-8, C3b/c, C3-CRP complexes, C4BP, and WBC were still significantly different between survivors and non-survivors: IL-8 levels were on the average 11.8 times higher in non-survivors (p=0.003) and were also negatively related to the duration of petechiae (p=0.005). Time adjusted levels of C3b/c on admission were on the average 2.2 times higher (p=0.004) in non-survivors than in survivors (Fig: 1). C3-CRP levels were on the average 1.9 times higher in survivors than in non-survivors (p=0.035), and not related to the duration of petechiae. Logistic regression with backward elimination showed that mortality was independently related to levels of C3b/c (p=0.03) and C3-CRP complexes (p=0.03). Fig: 2 shows the dichotomous distribution between these two parameters.
IL-6, IL-8 and CRP were closely correlated to all parameters for severity of disease (table 3). This was also observed for the complement activation products. The strongest

Table 2. Parameters related to cytokines, neutrophil degranulation, complement activation and complement regulation on day 0, 1 and 3 after admission in children with septic shock and purpura

Parameter	admission		d 1		d 3	normal
	survivors (N=38)	non-survivors (N=14)	survivors (N=34)	non-survivors (N=5)	survivors (N=31)	values
IL-6 (ng/ml) [49]	15.3 (0.036-1507)	280.6 (0.087-2002)[&]	0.1 (0.01-186)[§]	20.7 (0.7-50.0)[&]	0.02 (0.002-7.4)[§]	< 0.001
IL-8 (ng/ml) [49]	1.1 (0.013-381)	8.8 (0.023-369)[&]	0.03 (0.001-23.7)[§]	0.8 (0.15-2.1)[&]	0.03 (0.001-0.8)[§]	< 0.002
CRP (mg/l) [51]	128 (34-258)	70 (38-162)[&]	255 (145-444)[§]	210 (141-378)[§]	152 (42-401)	< 2
$sPLA_2$ (ng/ml) [48]	131 (2-1461)	97 (9-1907)	167 (15-847)	102 (12-573)	18 (1-384)[§]	< 5
elastase (ng/ml) [49]	512 (181-3376)	641 (251-3132)	317 (94-572)	410 (277-699)[&]	177 (67-908)[§]	< 100
lactoferrin (ng/ml) [49]	2584 (124-32285)	4275 (1757-31717)[&]	385 (146-3518)[§]	662 (307-1896)[§]	206 (88-920)[§]	< 400
C3a (nmol/l) [46]	12 (2-52)	30 (17-58)[&]	12 (3-49)	20 (6-30)	5.8 (<2-67)[§]	< 5
C3b/c (nmol/l) [49]	199 (26-826)	478 (196-1087) [&]	33 (14–604)[§]	67 (19-92)[§]	25 (9-305)[§]	< 50
C3-CRP (pmol/l) [31]	291 (68-695)	156 (59-286)[&]	425 (136-1221)[§]	266 (210-426)[&]	374 (79-1562)[§]	< 4
C4b/c (nmol/l) [49]	56 (22-1340)	110 (33-562)[&]	52 (13-255)	181 (33-310)	47 (20-175)	< 25
C4-CRP (pmol/l) [30]	67 (30-164)	53 (26-77)	99 (33-842)[§]	85 (52-106)	74 (15-341)[§]	< 4
fC1-Inh (%) [46]	79 (43-156)	72 (34-99)	100 (38-179)[§]	92 (76-103)	132 (52-192)[§]	80-120
iC1-Inh (%) [48]	378 (53-1141)	863 (96-2100)	168 (56-514)[§]	166 (128-476)	128 (75-181)[§]	< 160
C4-BP (%) [50]	72 (33-108)	53 (24-89)[&]	92 (58-139)[§]	99 (94-107)[§]	113 (73-224)[§]	80-120

Values are medians (range); [&] $p < 0.05$; for the difference between groups (Mann-Whitney U-test), [§] $p < 0.05$ for the differences within groups (Wilcoxon signed rank test); [number of measurements when deviant from 52]

Table 3. Correlation coefficients between clinical and inflammatory parameters.

	PRISM	lact	time	WBC	inf
IL-6	0.41	0.43	-0.52	-0.7	0.62
IL-8	0.36	0.45	-0.46	-0.7	0.59
CRP	-0.34	-0.3	0.42	0.43	-0.41
sPLA				0.36	
elast		0.59			
lactof	0.29	0.68	-0.33		0.37
C3a	0.69	0.46	-0.51	-0.4	0.75
C3bc	0.53	0.53	-0.39	-0.4	0.57
C3crp				0.39	
C4bc	0.46	0.33	-0.29		0.38
C4crp					
fC1in					
iC1in					
C4BP	-0.32			0.36	-0.43

Values are Spearman rank correlation coefficients; only significant ($p < 0.05$) values are presented; lact, arterial lactate; time, duration of petechiae; WBC, white blood cell counts; inf, total amount of plasma infused; IL-6, interleukin 6; IL-8, interleukin 8; CRP, C-reactive protein; sPLA, secretory phospholipase A2; elast, elastase; lactof, lactoferrin; C3a, C3b/c, C4b/c, C3 and C4 activation products; C3crp and C4crp, complexes between CRP and activated C3 or C4 respectively; fC1in, functional C1-Inh; iC1in, proteolytically inactivated C1-Inh; C4BP, C4 binding protein.

correlation appeared to be that between the complement activation products and the PRISM score (Fig: 3) or the total amount of plasma infused. Of the neutrophil degranulation products lactoferrin correlated with 4 out of 5 severity of disease parameters; there was a strong correlation between elastase and the arterial lactate levels ($r=0.59$, $p < 0.001$). Multiple regression analysis of the various categories of variables showed that the levels of C3a, ($p < 0.001$) and the C4BP levels ($p < 0.001$) were independently related with the total amount of plasma infused; Fig: 4 represents the relation ($r=0.77$; $p < 0.001$) between the total amount of plasma infused, and the weighted sum (using the regression coefficients as weight) of C3a and C4BP ($73x(\log_{10}C3a)-1.0xC4BP$). Similar statistical analysis showed that levels of C3a ($p < 0.05$), elastase ($p=0.007$), and lactoferrin ($p=0.001$) were independently related with the arterial lactate levels. Fig: 5 represents the relation (0.78; $p < 0.001$) between lactate levels and the weighted sum of C3a, elastase and lactoferrin ($0.142x(\log_{10}C3a) + 0.280x(\log_{10}elastase) + 0.257x(\log_{10}$ lactoferrin)).

Table 4. Mutual relation between inflammatory mediators

	CRP	sPLA	elast	lactof	C3a	C3b/c	C3crp	C4b/c	C4crp	fC1in	iC1in	C4BP
IL-6	-0.68		0.4	0.56	0.58	0.59	-0.56		-0.53			-0.33
IL-8	-0.61		0.41	0.59	0.56	0.65	-0.57		-0.52			-0.3
CRP		0.32		-0.3	-0.36	-0.3	0.64		0.55			0.36
sPLA	0.32				0.3						0.41	
elast				0.66		0.5		0.3				
lactof	0.3		0.66		0.39	0.53	-0.47		-0.37			
C3a	-0.36	-0.3		0.39		0.72		0.58			0.35	
C3b/c	-0.3		0.5	0.53	0.72			0.5				
C3crp	0.64			-0.5					0.91			0.67
C4b/c			0.3		0.58	0.5			0.38			
C4crp	0.55			-0.4			0.91	0.38				0.6
fC1in												0.52
iC1in		0.4			0.39					0.32		
C4BP	0.36						0.67		0.6	0.52		

Values are Spearman rank correlation coefficients; only significant ($p < 0.05$)values are presented. See also legends table 2 for explanation of the abbreviations.

3.4 Mutual relation between the inflammatory parameters

Complement activation products levels C3a and C3b/c correlated positively with IL-6 and IL-8, and negatively with CRP (table 4). The levels of complement-CRP complexes showed an inverse correlation pattern: the correlation of these markers for CRP-dependent complement activation with interleukins was negative, whereas that with CRP was positive. Levels of C4b/c, representing the activation of the classical pathway of complement, did not correlate with IL-6, IL-8 or CRP. However, these levels showed a strong correlation with C3a (Fig: 6) and C3b/c levels, as well as with C4-CRP complexes, indicating a substantial part of the activation of the complement system had occurred through the classical pathway, probably via CRP. C3- and C4-CRP complexes both closely correlated with IL-6 and IL-8, CRP, lactoferrin and C4BP. The functional C1-Inh levels correlated with levels of the other complement regulation protein C4-BP. The levels of inactivated C1-Inh correlated with levels of $sPLA_2$. Finally, levels of elastase and lactoferrin correlated well with those of IL-6 and IL-8 (table 4).

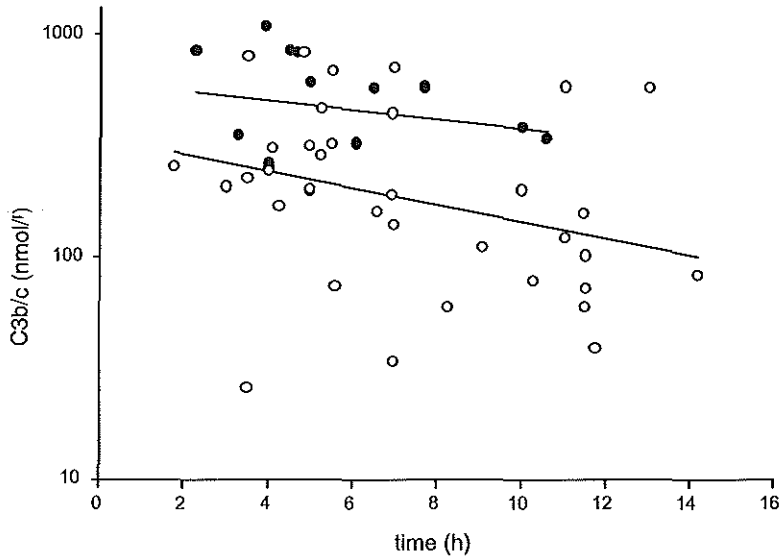

Figure 1. C3b/c levels on admission versus the time between the onset of petechiae and the moment of blood-sampling in surviving (open circles) and non-surviving (closed circles) patients. Solid and dashed lines represent least of squares regression lines of the two groups. The two angles of inclination of the regression lines do not significantly differ. Time-adjusted C3b/c-values were on the average 2.2 times higher in non-survivors (p=0.004).

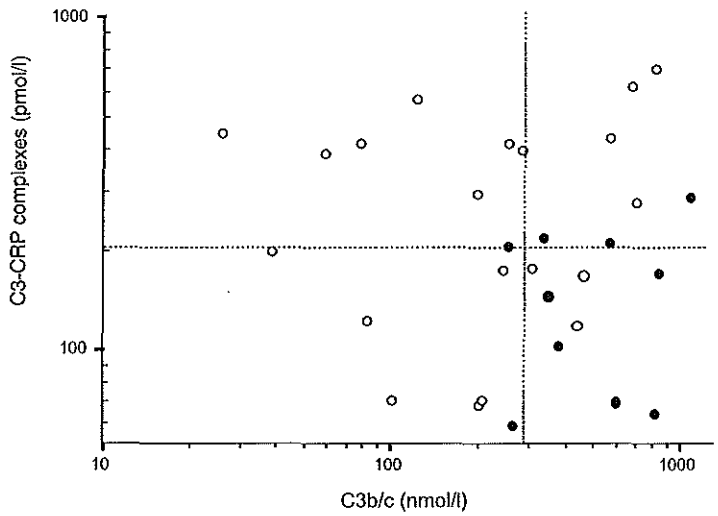

Figure 2. C3-CRP complex versus C3b/c levels on admission in surviving (open circles) or non-surviving (closed circles) patients show a dichotomous distribution (dotted lines represent medians). Both variables were independently related to survival.

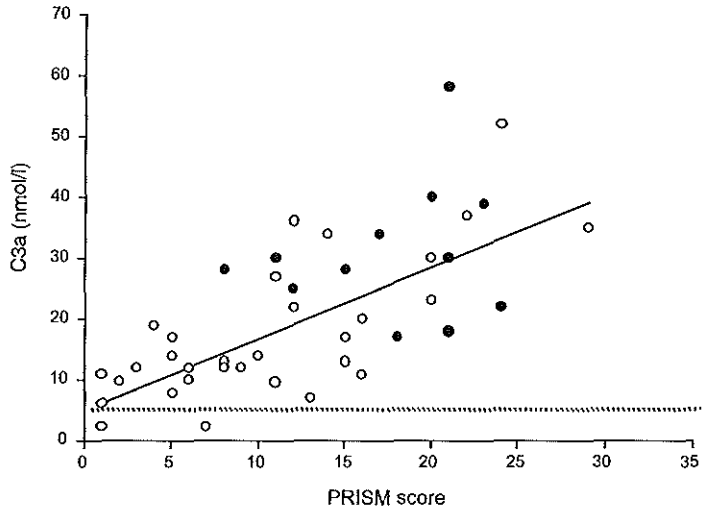

Figure 3. C3a levels on admission versus PRISM score in surviving (open circles) or non-surviving (closed circles) patients. Dotted line represents upper level of normal. Solid line represents the least squares regression line for all data points ($r=0.69$, $p < 0.001$).

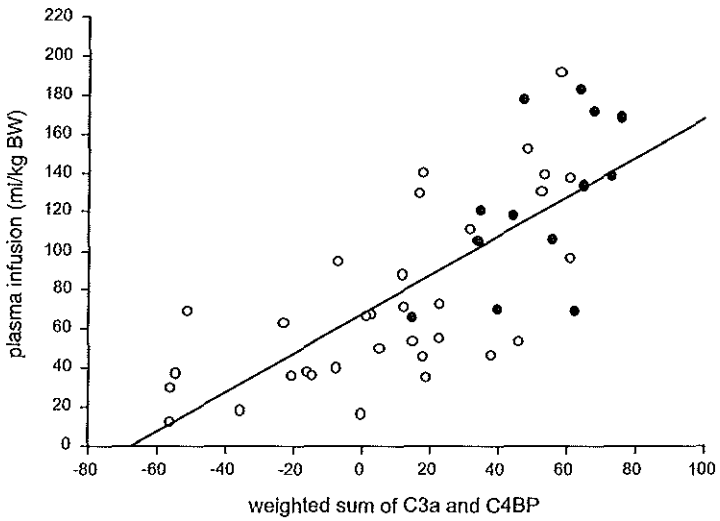

Figure 4. Total amount of plasma infused (ml/kg body weight) versus the weighted sum of the initial levels of C3a and C4BP ($73.1x(\log_{10}C3a) - 1.0xC4BP$) in surviving (open circles) or non-surviving (closed circles) patients. The solid line represents the least squares regression line for all data points ($r=0.77$; $p < 0.001$).

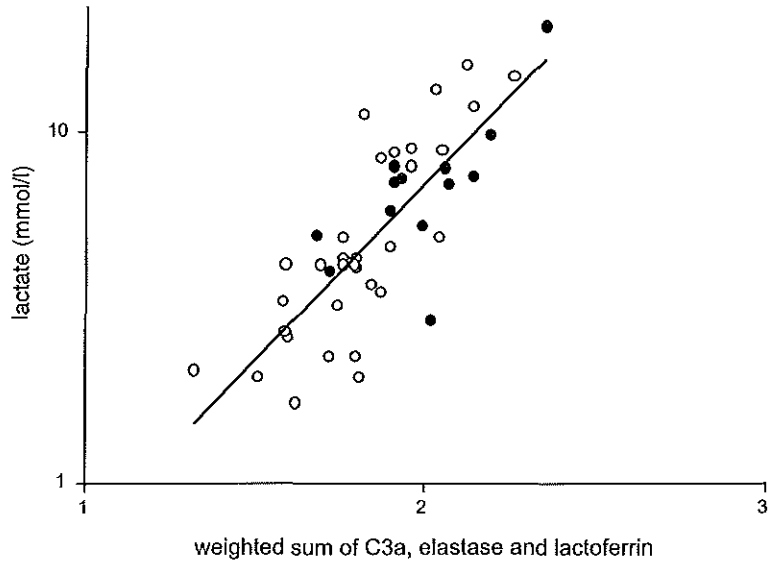

Figure 5. Arterial lactate (mmol/l) on admission versus the weighted sum of the initial levels of C3a, elastase and lactoferrin ($0.142x(\log_{10}C3a) + 0.280x(\log_{10}elastase) + 0.257x(\log_{10}lactoferrin)$) in surviving (open circles) or non-surviving (closed circles) patients. The solid line represents the least squares regression line for all data points ($r=0.78$; $p < 0.001$).

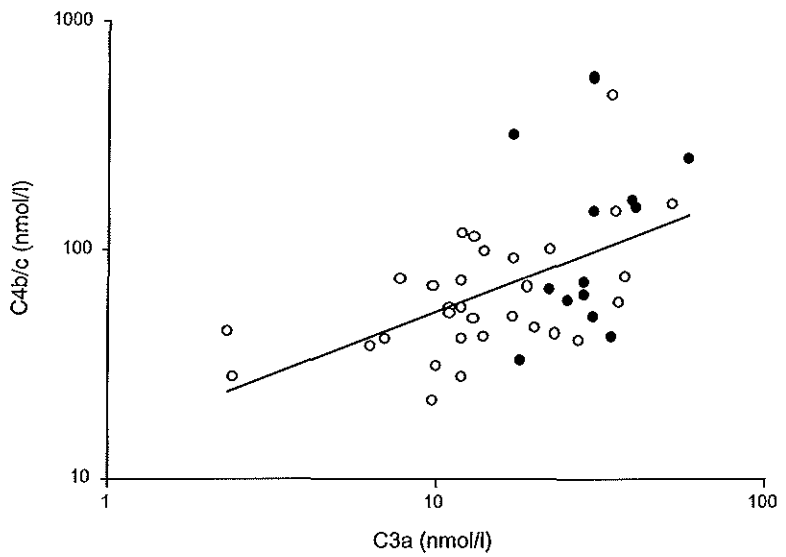

Fig 6: Initial C4b/c values versus C3a levels in surviving (open circles) or non-surviving (closed circles) patients (all patients: $r=0.58$, $p < 0.001$).

3.5 Time course of laboratory parameters

Table 2 presents the time-course of the different inflammatory variables. Levels of CRP and $sPLA_2$ remained increased until 72 h, and were not different between survivors and non-survivors except for CRP levels on admission. The levels of complement activation products C3a and C4b/c were still elevated at 72 h, while those of C3b/c were already normalized at 24 h. In 50 % of the patients C3a and C4bc were higher at 24 h than on admission. The course of complement-CRP complexes was remarkable in that levels were higher in survivors, and that higher levels were observed on days 1 and 3 compared to those on admission. Elastase levels decreased until 72 h, but remained elevated. At 24 h levels were different between survivors and non-survivors. At 72 h levels in surviving patients were still elevated except in 2 patients. Levels of lactoferrin decreased more rapidly: at 24 h levels were within the normal range in 50 % of the survivors. The levels of functional C1-Inh increased gradually until 72 h. At that time 50 % of the survivors had levels above the upper level of normal. There was a more rapid decrease in levels of iC1-Inh, at 24 h 50 % of both survivors and non-survivors had levels within normal limits. C4BP levels increased gradually in time, although at 72 h there was a wide range (73-224) of plasma levels.

4 DISCUSSION

In this study of children with septic shock and purpura a high degree of complement activation was observed, which closely correlated with mortality, severity of disease and degree of capillary leakage, as estimated by the amount of plasma infused. A substantial part of this complement activation was through the classical pathway as can be deduced from the close relation between C4 and C3 activation products (C4b/c and C3a or C3b/c). This activation persisted for days and in some patients even increased after admission. The complement system can be activated through the classical pathway by antigen-antibody complexes, through the alternative pathway by bacteria or LPS, and through the mannan binding protein pathway. As the more severe cases of SMD occur in patients with low or no titers of antibodies, the prevailing view is that the alternative pathway mainly contributes to complement activation in this disease. Recently Brandtzaeg and colleagues measured activation products of both the alternative as well as the classical pathway in 20 patients with systemic meningococcal disease (41). Activation of C4 was not different between patients with or without shock, nor did they find a correlation between C4 and C3 activation products. They concluded that the classical pathway only little contributed to total complement activation in patients with shock. In our patients C4b/c significantly correlated

with C3b/c or C3a, indicating that at least part of the C3 was activated via the classical pathway. Since we included only patients with shock, the difference with the results of Brandtzaeg et al. may have been caused by the larger number of children with severe disease in our study. Accordingly levels of activated C3 correlated better with severity indices than those of activated C4 (see for example table 2), implicating that the latter correlation may have been missed with a smaller number of patients.

Assuming that levels of antibodies against meningococci are low in patients with SMD, one can raise the question through which mechanism the classical pathway was activated. CRP can activate the classical pathway upon binding to the phospholipid phosphatidylcholine, and it has been suggested that this in particular may occur in the presence of the enzyme secretory phospholipase A2, another acute phase protein (137). We measured CRP-complement complexes as an indicator for CRP-mediated complement activation (378), and also assessed the relationship between CRP levels and complement activation parameters to address the question whether CRP was involved in the observed complement activation. CRP levels were negatively correlated with complement activation products C3a, and C3b/c and there was a negative relation between C3-CRP levels and survival (Fig: 2). Hence, the contribution of CRP-mediated activation to the total complement activation was probably minor. On the other hand the correlation between C4b/c levels and C4-CRP complexes was significant, indicating that at least a substantial part of the classical pathway activation had occurred via CRP (see table 4). CRP levels also positively correlated with the duration of petechiae suggesting a shorter disease course in the patients with lower CRP levels. CRP levels were lower in non-survivors than in survivors, at 24 h the levels had further increased in both groups, but there was no difference between the groups anymore. Thus, very likely the difference between surviving and non-surviving patients regarding CRP levels on admission, reflected that the non-survivors were admitted earlier in their disease course. Accordingly levels of $sPLA_2$, of which levels increase somewhat earlier during an acute phase reaction (283), were not different between survivors and non-survivors.

Activation of the classical pathway of the complement system is amongst others regulated by the plasma proteins C1-inhibitor (C1-Inh) and C4b binding protein (C4BP). C1-Inh inhibits activated C1, whereas C4BP inactivates C4b by acting as a co-factor for the cleavage of C4b by factor I. Both C4BP and C1-Inh are acute phase proteins and during severe infections plasma levels are increased. In our patients the levels of C4BP were decreased and negatively correlated to outcome and severity of disease (PRISM and total plasma infused), and positively to C1-Inh. The decreased levels of C4BP may be explained by suppression of its synthesis by TNF-α (269), binding to serum amyloid protein P (313), or binding to bacterial surface proteins (183). To what extent these or other mechanisms accounted for the decreased levels of C4BP in our patients is difficult to say. Yet, this decrease is remarkable considering the acute phase behavior of C4BP and together with

the relatively low levels of C1-Inh may indicate a poor inhibition of the classical pathway. Furthermore, decreased levels of C4BP may have implications for the coagulation system: low levels of C4BP lead to relatively higher levels of free protein S, which may be of benefit during septic shock (167, 324).

C1-Inh is the only known inhibitor in plasma of activated C1, and the major inhibitor of activated factor XII and kallikrein of the contact pathway of coagulation. Inhibition of these so-called target proteinases by C1-Inh leads to the formation of proteinase-C1-Inh complexes. The formation of these complexes is accompanied by the generation of proteolytically inactived C1-Inh species. The latter species may also result from inactivation by other endogenous (e.g., neutrophilic elastase) or exogenous, i.e. bacterial, proteinases. C1-Inh in plasma may thus exist in three forms: functional C1-Inh, inactive C1-Inh and C1-Inh complexed to a proteinase (252). In our patients the levels of functional C1-Inh were decreased or normal, which considering its acute phase behavior suggests a relative deficiency of this inhibitor, and hence a diminished regulation of the complement and contact system, with subsequent release of biologically active peptides. The time course of the levels of functional C1-Inh suggested consumption of this inhibitor, in particular during the early phase of the disease. The high levels of inactivated C1-Inh have been observed in experimental septic shock in baboons (29) as well as in patients with vascular leakage syndrome during IL-2 therapy (142). Our data do not allow conclusions regarding the mechanism of the generation of iC1-Inh in our patients.

Neutrophils have been implicated as important mediators of vascular injury (35, 310, 352) by the release of toxic oxygen species and lysosomal proteinases, such as elastase, upon stimulation by a large variety of agonists. In addition, elastase may facilitate activation of complement, coagulation and fibrinolytic systems by inactivating the major inhibitors of these cascade systems (31, 254). The levels of elastase and lactoferrin in our patients were similar to those found in adult patients with sepsis (254). The levels correlated with outcome, as well as with lactate (Fig: 6). Hence, activation and degranulation of neutrophils may contribute to tissue hypoxia and capillary leakage, for example by plugging of capillaries. Elastase and lactoferrin were also both correlated to complement activation products and IL-6 and IL-8, suggesting a cooperative effect of cytokines and complement in the process of neutrophil adherence and degranulation.

During sepsis capillary leakage is caused by an increase in vascular permeability. Vascular permeability is dependent on a number of factors and can be affected both by direct damage to the endothelium, i.e. by activated neutrophils, and by mediators which alter its barrier function. These mediators include TNF, platelet activating factor, complement, kinins (such as bradykinin), and leukotriens, though the specific mechanisms for most of these remain to be elucidated. In our study the degree of capillary leakage was closely related to the extent of complement activation. Individuals with inherited component deficiencies have a markedly increased risk for acquiring systemic meningococcal

infections and may experience recurrent episodes of these. A striking finding in individuals with late complement component deficiencies compared with normal persons is the low mortality rate associated with meningococcal disease (83). It is therefore tempting to speculate that complement plays a dual role in the pathogenesis of meningococcal sepsis: on the one hand it contributes to the defense against meningococci, on the other hand in patients suffering from SMD too excessive activation in particular via mechanisms not 'triggered' by the micro-organisms themselves, may contribute to tissue damage and a complicated course.

In conclusion, excessive activation of the complement system was demonstrated in children with septic shock and purpura, which activation in part had occurred via the classical pathway, and was related to outcome, severity of disease and extent of capillary leakage. Further studies, for example focusing on the effects of the therapeutical administration of C1-esterase inhibitor, may reveal whether this activation contributes to a detrimental course.

CHAPTER 4.2

CARDIOVASCULAR ASPECTS OF EXPERIMENTAL MENINGOCOCCAL SEPSIS IN YOUNG AND OLDER AWAKE PIGLETS: AGE RELATED DIFFERENCES

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1 INTRODUCTION

Septic shock with purpura, mainly caused by *Neisseria meningitidis*, is a life threatening entity in previously healthy children. Despite the use of antibiotics and intensive care treatment meningococcal sepsis (MS) is still associated with mortality rates up to 40% in the youngest children (0-3 years) and 13% in the older children (3-18 years) (158). While numerous studies have defined the hemodynamic and metabolic responses to sepsis in the adult, these responses have been poorly documented in young subjects and provide no clear answer to the finding of a greater morbidity/mortality in our younger patients. There is substantial, but controversial, experimental and clinical evidence for age related differences between neonatal, pediatric and adult sepsis (71, 91, 171, 172, 215, 266, 284). Consequently, information obtained from adult animals or humans cannot simply be extrapolated to the pediatric situation. Also, in many experimental sepsis studies chemically purified endotoxin from gram negative bacteria was used. However, in contrast to other gram negative bacteria, during their growth the *N. meningitidis* bacteria release endotoxin together with membrane material in the form of vesicle-like structures (blebbing). It is probable that in this way rough meningococcal endotoxin bears more antigenic potency than chemically purified endotoxin. In those studies where purified meningococcal endotoxin was used, small laboratory animals were studied, which do not allow as detailed an analysis of global and regional hemodynamic changes during sepsis, compared to larger animals such as pigs.

Therefore, the purpose of the present study was to investigate whether significant differences exist between the global hemodynamic and regional blood flow responses to different dosages of rough meningococcal endotoxin challenge in young (8 kg), corresponding to a child of 1 year of age, and older piglets (40 kg), corresponding to a child of 10 years of age. Animals were chronically instrumented and studied in the awake state, to avoid the effects of anesthesia and acute surgical trauma. Since the major component of first therapeutical intervention in the clinical situation of sepsis is intravascular volume expansion, the effect of this treatment during placebo and endotoxin infusion was studied.

2 MATERIALS AND METHODS

2.1 Experimental groups

In the present study Landrace x Yorkshire domestic piglets of 4 months of age, 40.5±0.8 kg (n=19) and of 3 weeks of age, 7.2±0.3 kg (mean±SEM) (n=16) were studied. The animals were randomly assigned to a group receiving either a low (1 µg per kg body weight) or a high (10 µg per kg body weight) dose of endotoxin, yielding a total of 4 groups of animals: old, low dose (OL) and old, high dose (OH), and young, low dose (YL), and young, high

dose (YH).

All experiments were performed in accordance with the "Guiding Principles in the Care and Use of Laboratory Animals" as approved by the Council of the American Physiological Society and with the approval of the Animal Care Committee of the Erasmus University Rotterdam.

2.2 Surgical procedure

The animals were fasted for 15 h prior to the surgical procedure. The older animals were sedated with intramuscular (im) injection of 25 mg/kg ketamine (Ketalin®, Apharmo, Arnhem, The Netherlands), followed by induction of anesthesia with an intravenous (iv) injection of 10 mg/kg methomidate (Hypnodil®, Janssen Pharmaceutical, Tilburg, The Netherlands) through an ear-vein cannula. The younger animals were sedated by mask inhalation of a mixture of O_2/N_2O mixture (1:2) and isoflurane (3%), followed by induction of anesthesia with iv methomidate.

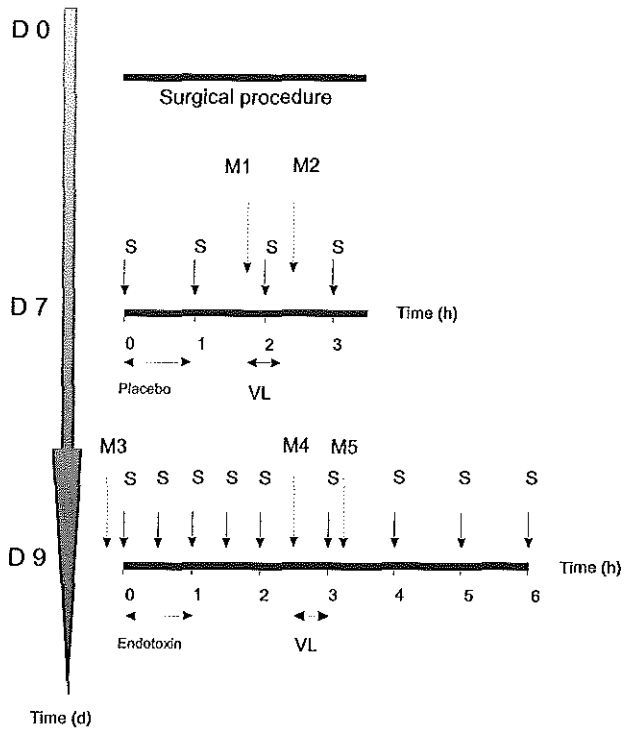

Figure 1. Schematic description of surgical and experimental procedure. D0-D9: days, h: hours, M1-M5: microspheres injections, S: blood sample, VL: volume loading.

All animals were intubated and ventilated with a Servo 900-B ventilator (Siemens-Elema, Solna, Sweden). Anesthesia was maintained with an O_2/N_2O mixture (1:2) and isoflurane (0.25-3%). The respiratory rate was set at 25/15 min^{-1} (young/old), while minute volume and FiO_2 were adjusted to keep the arterial $PaCO_2$ and PaO_2 within physiological limits. To maintain fluid and metabolic balance, 5 ml/kg/h glucose 5% in NaCl 0.225% was administered iv continuously. Under sterile conditions both external jugular veins and common carotid arteries were surgically exposed through a ventral paramedian incision. On the right side a 7/8 (young/old) F catheter (Cordis®, Roden, The Netherlands) was inserted and positioned in the descending aorta for blood sampling and measurement of arterial blood pressure (MAP); while through the jugular vein a thermodilution catheter 6/7.5 (Y/O) F (Arrow®, Reading, USA/Spectramed, Bilthoven, The Netherlands) was introduced and advanced into the pulmonary artery to monitor the mean pulmonary artery pressure (MPAP), cardiac output (CO), pulmonary artery occlusion pressure (PAOP), and central venous pressure (CVP), and to sample mixed venous blood. The left carotid artery was cannulated with a 7F pressure transducer-tipped catheter (Sensodyn MTC®, Braun Medical, Uden, The Netherlands), which was positioned in the left ventricle to measure the left ventricular pressure, and via a separate lumen to inject radio-active labeled micro spheres. A 6/7 (Y/O) F catheter (Cordis®) was inserted into the external jugular vein for drugs and fluid administration. Once the proper position of the catheters was verified, using fluoroscopy, all catheters were tunneled subcutaneously to the dorsal side of the neck and secured with sutures. Both the subcutaneous layers and the skin edges were approximated with single sutures. After weaning from anesthesia and ventilation, the animals were extubated and allowed to recover from surgery. Catheters were protected with a soft jacket (317).

2.3 Post surgical period

Catheters were flushed with heparin-solution (500 IU/ml) once a day. Prophylactically, the animals received 25 mg/kg amoxicilline (Clamoxyl®, SB-Farma, Rijswijk, The Netherlands) and 5 mg/kg gentamicin (A.U.V. Cuyk, The Netherlands) iv once daily starting on the day of the surgical procedure. Every day, the animals were adapted to the laboratory facilities (7-10 days) to ensure hemodynamic stability. The experimental protocols were executed when the animals could rest unrestrained and quietly for up to 4 h, and systemic hemodynamic parameters therefore remained stable.

2.4 Endotoxin preparation

Outer membrane (OM) blebs were prepared from *Neisseria meningitidis* group B strain H44/76 using a previously described method (275). Briefly, bacteria were treated with 0.2

M LiCl and 0.01 M EDTA at pH 7.0 during 2 h at 45°C. The mixture was centrifuged at 15,000 rpm for 10 min to remove bacteria and the supernatant ultracentrifuged to peel off the outer membranes. The pellet was resuspended in saline with 2% sucrose and 0.02% merthiolate and stored in vials at 4-8°C. Blebs were analyzed for KDO (2-keto-3-deoxy-D-manno-octonate) content (186), and quantified by electrophoresis (SDS-PAGE) following silver staining (338). The concentration of protein was 50µg/ml. On the day of the endotoxin protocol the needed amount of endotoxin was diluted with saline to a total volume of 10/20 ml (Y/O).

2.5 Experimental setup

2.5.1 hemodynamic measurements

The fluidfilled catheters were connected to pressure transducers (DT-XX, Viggo-Spectramed, Bilthoven, The Netherlands), which drove an amplifier with a digital readout. CO was measured in duplicate by the thermodilution technique, using ice cold saline, 3/10 (Y/O) ml, and a cardiac output computer (Adquipment Medical, Rotterdam, The Netherlands). When CO readings differed by more than 10 %, a third measurement was made. The first derivative of the left ventricular pressure (LV dP/dt) was obtained via electrical differentiation.

2.5.2 clinical chemistry

Blood gas, hemoglobin and oxygen saturation were determined by running 0.3 ml of arterial and pulmonary arterial blood through a hemoximeter (OSM-2, Radiometer, Copenhagen, Denmark) and a blood gas analyzer (ABL 505®, Radiometer, Copenhagen, Denmark). Serum lactate (Sigma Diagnostics, St Louis, USA) and glucose (Merck Diagnostics, Darmstadt, Germany) were both measured by enzymatic end-point determination. Arterial hemoglobin levels were determined using a cell counter (OSM-2, Sysmix, Kobe, Japan).

2.5.3 regional organ blood flows

Regional organ blood flows were determined at two time points in the placebo protocol and at 3 time points in the endotoxin protocol (M1-M5 in Fig. 1) by injecting a batch of 0.5/2 x10^6 (Y/O) carbonized plastic microspheres (15±1µm in diameter) labeled with either ^{46}Sc, ^{95}Nb, ^{103}Ru, ^{113}Sn or ^{141}Ce into the left ventricle. To calculate regional blood flows a reference blood sample was withdrawn from the catheter in the descending aorta at a rate of 5/10 ml/min (Y/O), starting 15 s before the injection of microspheres, until 90 s after completion of the injection of the micro-spheres. At the end of the experiments animals were sacrificed

with an overdose of sodium pentobarbital (Euthesate, Apharmo, Arnhem, The Netherlands), and various organs (adrenals, liver, spleen, small intestine, brain and kidneys) and representative aliquots of abdominal skin and skeletal muscle (*M. sternocleidomastoideus* and *M. iliopsoas*) were excised, weighed and put into vials. The hearts were fixated in formaldehyde (3.6%v/v) and 48 h later the atria and right ventricle were dissected from the left ventricle and the total myocardium of the left ventricular free wall was divided into three layers of equal thickness, i.e. subepicardium, mesocardium, and subendocardium. Radioactivity was counted and the amount of blood flow to the various tissues (Φ_{tis}) calculated as:

$$\Phi_{tis}(ml/min)=(I_{tis}/I_{art})x_{art}$$

where I_{tis} and I_{art} are the radioactivity (cpm) in a particular tissue and that of the arterial blood sample, while Φ_{art} is the rate of withdrawal of the blood sample, respectively. Blood flows were expressed as ml.min^{-1} per 100 g of tissue. Regional vascular conductances were calculated by dividing the respective regional blood flows by the mean arterial blood pressure. Full details of the procedures and the calculation of flow data using this technique have been reported earlier (376).

2.6 Experimental protocols

In each of the four experimental groups, animals underwent a placebo protocol and an endotoxin protocol performed on separate days (Fig. 1).

2.6.1 placebo

The placebo protocol consisted of infusion of saline with the same sucrose (2%) and merthiolate (0.02%) concentration as the endotoxin solution. The volume of placebo solution was identical to the volume of endotoxin solution and also diluted with saline to a total volume of 10/20 (Y/O) ml. This volume was infused in one hour. To study the hemodynamic effect of volume loading, after a washout phase of 1 hour a volume infusion was started consisting of 30 ml/kg plasma substitute (Haemaccel®, Behring Pharma, Amsterdam, The Netherlands) infused over 30 min. After this volume loading there was a washout phase of one hour. The time points of blood sampling (S) for blood gas, glucose and lactate determination, and microsphere injections (M1 and M2) are indicated in Fig.1. The endotoxin protocol was performed at least 48 h later. The two day period between the two protocols was chosen to allow wash out of the plasma administered during volume loading.

2.6.2 endotoxin

The dose of endotoxin was estimated from experience obtained in pilot experiments. The low dose (1 µg/kg) ought to be non-lethal, and the high dose sub-lethal. Since it is known that there is a rapid increase in pulmonary vascular resistance in the early phase of endotoxin infusion (259), we supplemented oxygen (3-5 l/min) to prevent hypoxia in the initial phase. When the MPAP increased threefold (usually at 10 min), the endotoxin infusion was interrupted until MPAP had decreased to below 2 times the baseline value (usually at 30 min). At that time the infusion was restarted at a higher rate to accomplish the infusion within 1 h. During the complete protocol 5 ml/kg/h fluid was iv administered (glucose 5% in NaCl 0.225%). After the endotoxin infusion a period of 1-2 h was allowed to establish a stable hemodynamic situation before starting the volume loading, which was also 30 ml/kg plasma substitute in 30 minutes. After completion of the volume loading a washout period of 2 h was taken, before termination of the protocol. Moribund animals during the protocol were humanely euthanized.

2.7 Data acquisition and analysis

Data were recorded and digitized at a sample rate of 200 samples/sec/channel using an eight channel data-acquisition program ATCODAS (Dataq Instruments, Inc., Akron Ohio, USA) and stored on a computer for later post-acquisition analysis with a curve analysis software program written in Matlab (Matlab®. The MathWorks, Mass, USA) to calculate average values of 20-30 heart beats at 30 minute intervals. The cardiac index (CI), stroke index (SI), stroke work index (SWI), systemic vascular resistance index (SVRI), pulmonary vascular resistance index (PVRI), O2 content (C_{O2}), oxygen delivery (DO_2), and oxygen consumption (VO_2) were calculated with standard formulas: CI=CO body weight^{-1} [L.min^{-1}.kg^{-1}], SI=CI/HR [mL.kg^{-1}], SWI=SIxMAP [L.kg^{-1}.mmHg], SVRI=(MAP-CVP)xCI^{-1} [mmHg.L^{-1}.min.kg], PVRI=(MPAP-PAOP)xCI^{-1}[mmHg.L^{-1}.min.kg], C_{O2}=0.6xHbxSaO_2 +0.00131xpO_2 [ml.l^{-1}], DO_2=CIxC_{O2}art, VO_2=CIx(C_{O2}art-C_{O2}mix ven), respectively.
Statistical analysis was performed using (SPSS®, Chicago, USA) Wilcoxon signed rank test or paired Student *t*-tests to compare values at baseline with subsequent time points within groups. Differences between groups regarding longitudinal measurements, were tested with repeated measurements ANOVA on the relative change from baseline values (BMDP5V - unbalanced repeated measures models with structured covariance matrices, 1990 by BMDP Statistical Software, Inc. Los Angeles, CA 90025 USA). When appropriate, logarithmically transformed values were used. The values at the start of the separate interventions (endotoxin and volume loading) were taken as baseline for the evaluation of the particular interventions. Evaluation of dose and age regarding regional blood flow measurements were performed using two-way ANOVA. As there were no differences

between low dose placebo and high dose placebo, the groups of YL and YH were combined to evaluate the effects of placebo. The same applied to OL and OH. In view of the number of comparisons made, significance was accepted for p-values of <0.01 (two-tailed). Data are presented as mean±SEM, or median (range).

3 RESULTS

3.1 Survival and general manifestations

Infusion of placebo was uneventful in all animals, but during infusion of endotoxin both the old and young animals became restless and even agitated when pulmonary vascular resistance started to increase suddenly between 10-30 min after the start of infusion. This was accompanied by tachypnea (breathing frequencies >80/min), vomiting, shivering, reduced reaction to stimuli and finally lethargy. Core temperature increased approximately 2°C during infusion of endotoxin, which was substantially more than during infusion of placebo (0.5°C, p< 0.01). Mortality was 0, 3, 1 and 4 animals in group YL (0%), YH (37.5 %), OL (12.5 %) and OH (44 %), respectively. The time to death was 149 ± 13 min after the start of the endotoxin infusion.

3.2 Hemodynamic responses

3.2.1 baseline values (table 1 and 2)

As expected, the baseline values of most hemodynamic parameters were different for the old and young animals, with the most pronounced differences in CI, HR, SI, MAP, pulse pressure, SVRI, SWI and PVRI. These differences are related to developmental changes in cardiac output and vascular resistance (150).

3.2.2 placebo infusion

Besides a significant increase in SVRI in the young animals, there were no changes in hemodynamic variables during the placebo infusion or the period immediately after placebo infusion (table 1). Volume loading resulted in an increase in: CI, SI, SWI, filling pressures, mean pulmonary and systemic pressures, and a decrease in SVRI. There was no change in PVRI. Although the variables recovered slightly towards baseline values, most parameters were still significantly increased 60 min after completion of the volume loading.

Table 1. Hemodynamic variables during and after placebo infusion and volume loading in old and young piglets

	Group	N	Placebo (min) 0	Placebo (min) 60		Volume Loading (30 min) begin		Volume Loading (30 min) end		Wash out (min) 60	
CI	Old	15	0.13±0.00	0.13±0.00		0.13±0.00		0.17±0.00	§	0.15±0.01	§
(l/min/kg)	Young	14	0.1:±0.01	0.19±0.01		0.19±0.01		0.25±0.01	§	0.22±0.01	§
HR	Old	17	100±4	97±4		98±4		107±5		104±4	
(beats/min)	Young	14	124±6	125±5		124±5		141±6	§	129±7	
SI	Old	15	1.3±0.1	1.3±0.1		1.3±0.0		1.6±0.1	§	1.5±0.1	§
(ml/beat/kg)	Young	14	1.6±0.1	1.5±0.0		1.5±0.0		1.8±0.1	§	1.7±0.1	
LVEDP	Old	14	7±1	8±1		10±2		23±2ß	§	16±2	
(mm Hg)	Young	11	8±1	10±1		10±1		19±1ß	§	14±1	
LV dP/dt	Old	14	2718±17:	2646±178		2548±165		2980±7:	§	2655±115	
(mm Hg/s)	Young	10	2668±171	2548±179		2446±141		2983±222		272:±216	
MAP	Old	17	98±2	97±2		102±2		110±3	§	112±3	§
(mm Hg)	Young	14	83±	287±2		89±2		97±4ß	§	96±3	§
SAP-DA	Old	17	3:±2	40±2		40±2		45±3		40±2	
(mm Hg)	Young	14	41±3	43±3		43±3		43±3		43±3	
CVP	Old	13	1±2	2±2		1±1		9±2ß	§	7±2	
(mm Hg)	Young	12	3±1	3±1		3±2		8±1		6±2	
SVRI	Old	12	776±33	769±37		838±4:		614±23	§	673±14	
(mm Hg.min.kg/l)	Young	12	403±1:	436±19	#	457±23	#	330±1:	§	415±17	
SWI	Old	15	125±7	127±7		132±5		178±12	§	167±8	§
(mm Hg.ml/b/kg)	Young	14	134±7	133±6		135±5		173±:	§	15:±8	§
MPAP	Old	17	16±1	17±1		18±2		29±2ß	§	26±2	§
(mm Hg)	Young	13	17±1	18±1		16±1		23±2ß	§	18±2	
PVRI	Old	9	91±10	104±12		109±9		10:±13		104±8	
(mm Hg.min.kg/l)	Young	9	67±6	63±7		61±8		63±11		43±7	

CI, cardiac index; HR, heart rate; SI, stroke index; LVEDP, left ventricular end diastolic pressure; LV dP/dt, first derivative to time of LV pressure; MAP, mean arterial pressure; SAP-DAP, pulse pressure; CVP, central venous pressure; SVRI, systemic vascular resistance index; SWI, stroke work index; MPAP, mean pulmonary arterial pressure; PVRI, pulmonary vascular resistance index; $p < 0.01$, # vs baseline, § vs prevolume load, & young vs old; VLb, e, begin and end volume load.

3.2.3 endotoxin infusion and volume loading OL (table 2)

During the endotoxin infusion and the subsequent 1-2 hours, the CI remained stable, despite a nearly 50% decrease of in stroke index ($p<0.005$). This was possible because an increase in heart rate of 100 ± 3 b min^{-1} at baseline to 178 ± 11 b min^{-1} at 60 min after the end of infusion. The MAP decreased significantly at t=120. There were no significant changes in LVEDP and CVP, a trend towards increase of the LV dP/dt, and towards an increase in the pulse pressure (SAP-DAP). The SWI decreased more than 50%. An increase in MPAP was usually the first hemodynamic sign after the start of the endotoxin infusion. At about 10 min, there was an abrupt rise in MPAP to 2-3 fold the baseline level. The PVRI, measured at 1 hour intervals, increased significantly to 2 fold the baseline levels. The volume loading did not result in a significant increase of CI and SI, the HR decreased significantly and this decrease persisted in the hours after the VL. There was a trend to an increase in LVEDP, while LV dP/dt and SVRI did not change significantly. The SWI and the MAP increased significantly by the VL, an increase which persisted in the hours after the VL. The MPAP and PVRI decreased to baseline levels in the hours after the VL.

3.2.4 endotoxin infusion and volume loading YL (table 2)

Except for the CI, LVEDP and pulse pressure, the hemodynamic pattern in the young animals was merely the same as the old animals. The CI did not remain stable during the ET infusion, it decreased significantly despite an early increase in HR. There was a decrease in LVEDP, this decrease was significant at the beginning of volume loading compared to baseline. The pulse pressure decreased significantly in YL, while in OL the pulse pressure increased.
After the volume loading (VL) the LVEDP was significantly higher when compared to pre-VL levels, an increase which did not persist to the direct post-VL level. The significant increase in CVP however, persisted for a longer period.

3.2.5 endotoxin infusion OH (table 3)

In the non-survivors (N-Surv) the CI increased while in the survivors (Surv) the CI did not change (table 3). Due to the small numbers, the differences were not significant. The pattern of change in HR, LVEDP and MAP, was not different between N-Surv and Surv. The LV dP/dt increased more in the N-Surv than in the Surv. At 2h and just before volume loading (VL) this difference was significant. The pulse pressure tended to increase more in the N-Surv than in the Surv as the SVRI, which tended to decrease in the N-Surv. There was no difference in the pattern of pulmonary pressure or resistance. Only 1 non-surviving

Table 2. Hemodynamic variables during and after low dose endotoxin infusion and volume loading in old and young piglets (see table 1 for legends)

Hemodyn			endotoxin			volume loading		wash out (min)	
parameter	group	N	0	60 (min)	120 (min)	begin	end	60	120
CI	Old	6	0.13±0.01	0.13±0.01	012±0.02	011.±0.02	0.14±0.01	0.12±0.01	0.13±0.01
	Young	8	0.20±0.01	0.17±0.01	0.17±0.01	0.16±0.01#	0.21±0.02	0.18±0.02	0.19±0.02
HR	Old	6	101±4	133±5#	170±9#	154±8#	130±6§	129±10§	137±6
	Young	8	110±7	169±14#	183±9#	185±9#	166±9§	142±7§	153±9§
SI	Old	6	1.30±0.08	0.97±0.06#	0.73±0.08#	0.72±0.11#	1.05±0.07	0.95±0.08	0.95±0.05
	Young	8	1.8±0.07	1.05±0.11#	0.94±0.10#	0.89±0.06#	1.29±0.11§	1.31±0.12§	1.26±0.12
LVEDP	Old	3	6±2	7±2	3±3	4±2#	22±6§	19±7	
	Young	5	8±2	2±3	-1±2	2±1	19±2§	15±2§	14±0.3§
LV dP/dt	Old	3	2378±616	2752±379	2574±621	2817±837	2465±373	1957±369	
	Young	5	2425±330	3042±542	2665±417	3231±647	3112±714	1736±94	1874±151
MAP	Old	6	96±6	77±5	67±6#	86±6	104±13§	115±9§	109±6§
	Young	7	89±4	77±5	74±4#	87±5	95±7§	98±6§	101±5§
SAP-DAP	Old	6	33±3	33±1	56±8	45±6	43±3	37±3	36±2
	Young	7	45±4	30±3#&	35±4&	38±3	42±3	41±5	47±8
CVP	Old	5	4±2	8±2	6±5	3±3	12±4§	11±4	8±4
	Young	7	3±1	4±3	6±2	3±3	8±3	7±2	9±2
SVR1	Old	5	740±81	569±58#	534±79	797±154	814±216	934±147	789±48
	Young	7	447±35	424±33	440±43	525±49	448±56	540±76	503±59
SWI	Old	6	123±8	75±5#	50±8#	62±11#	109±9§	109±10§	101±12
	Young	7	161±11	81±13#	70±10#	77±8#	105±20§	128±14§	129±18
MPAP	Old	6	21±2	45±4#	38±2#	36±3#	37±1	32±2	30±2
	Young	8	17±2	37±3#	32±3#	32±3	31±3	19±3§&	19±1§&
PVRI	Old	6	136±13	267±30#	255±43	277±40	166±17	162±24	114±59
	Young	4	76±8	174±15#	194±40	178±23#	130±32	79±25	80±27

animal survived long enough to have a VL, this animal remained to have a high CI despitea high PVRI.

3.2.6 endotoxin infusion YH (table 3)

Also in these animals the hemodynamic pattern was comparable with the older ones. The CI remained stable in the N-Surv, while in the Surv the CI decreased. This was different compared to the older animals. The decrease in SI was sooner in the younger animals which did not survive: at 1 h the relative decrease was already 40 %, while in the older N-Surv it was 3 % at that time point. The increase in HR, the LV dP/dt and the pulse pressure tended to be higher in the N-Surv than in the Surv.

3.2.7 comparison between the groups

Because the baseline values of many of the hemodynamic parameters were different between the two age groups, for comparison between the groups the relative change (%) from baseline in the time interval until the volume loading was compared. Repeated measurements ANOVA (RmANOVA) showed that there was a significant difference ($p<0.001$) in the relative change of CI after start of infusion between old (average increase 5.7%) and young (average decrease 14.7%). This 20% difference was not significantly affected by the dose. In Fig: 2 the absolute values for CI are depicted for the young and old animals. The same pattern emerged for the SI, a dose independent decrease of on the average 40% was found. This decrease was at all time points about 10% higher for the younger ones, however, this difference was not statistically significant ($p=0.024$) according to our criteria. The HR increased on average with 67%, and was not affected by age and dose. There were no significant changes in LVEDP and CVP. The LVdP/dt increased with 39%, the MAP decreased with 16%, and the SWI decreased with 49% all irrespective of dose and age. RmANOVA showed that both age and dose affected pulse pressure (SAP-DAP), in the low dose groups, the older animals showed an increase (43%) in pulse pressure, while in the younger a decrease 22% ($p=0.01$) could be observed at all time points. For SVRI, there was a 20% difference ($p=0.007$) in response between younger and older animals (younger +3%, older -17%), not significantly affected by dose. The MPAP increase was on the average 126% independent of dose and age. Independent of age, the PVRI increased in the low dose groups with 134%, in the high dose group 218%, a difference which did not reach statistical significance ($p=0.02$).

3.3 Blood gases and oxygen transport

The calculated values for oxygen delivery (DO_2), oxygen consumption (VO_2), and oxygen

Table 3. Hemodynamic and end organ oximetric variables during and after high dose endotoxin infusion

	Old endotoxin infusion						Young endotoxin infusion					
			absolute	relative change from baseline (%)					absolute	relative change from baseline (%)		
	Surv	N	0	60	120	150 (VLB)	Surv	N	0	60	120	150 (VLB)
CI	y	4	0.12±0.01	12±10	2±14	-5±13	y	4	0.19±0.01	-14±8	-16±13	-14±18
	n	3	0.13±0.01	27±14	23±23	19±27	n	3	0.19±0.01	-24±8	15±8	2±10
HR	y	5	89±4	68±14#	9±10#	70±14#	y	4	115±7	55±23	78±28	69±24
	n	4	96±3	28±10	87±8#	90±8#	n	3	128±14	36±8#	106±18#	97±15
LV dP/dt	y	5	2568±193	16±7	7±18	-8±9	y	2	2569±388	5±17	46±10	43±17
	n	4	2627±177	31±26	95±16#$	63±9$	n	2	2216±263	-7±6	179±78	140±39
MAP	y	5	99±5	-32±5#	-29±6#	-28±7#	y	4	78±4	-15±8	-22±8	-11±9
	n	4	92±4	5±18	-26±8	-32±5	n	3	69±2	-31±1#	1±5	-1±5
SAP-DAP	y	5	43±4	28±18	13±13	-6±9	y	3	36±5	17±15	24±9	36±16
	n	3	43±2	84±24	65±23	65±18$	n	3	51±3	-16±9	71±26	84±31
SVRI	y	4	842±93	-31±9	-17±14	-15±12	y	4	381±27	2±15	13±14	27±29
	n	3	717±68	-16±17	-31±17	-33±16	n	3	359±17	-9±9	-19±6	-6±3
PVRI	y	3	88±7	143±37	162±36#	193±42	y	3	45±26	438±278	332±189#	329±103
	n	3	99±16	133±96	142±153		n	3	40±5	515±102#	270±157	179±3
lactate	y	4	0.59±0.06	357±179	379±80#	394±73#	y	4	0.59±0.07	527±642#	775±300	720±266
	n	4	0.46±0.14	1520±714	3077±650$		n	3	0.45±0.05	755±54#	4066±1447	
VO_2	y	3	0.23±0.06	31±33	26±30	45±33	y	4	0.34±0.06	34±3#	36±16	32±19
	n	4	0.22±0.03	46±1697±22			n	3	0.34±0.04	17±28	94±7#	
ER-O_2	y	5	0.38±0.05	10±16	22±14	21±10	y	4	0.41±0.03	32±12	36±23	38±28
	n	4	0.39±0.10	11±1239±10			n	3	0.36±0.04	64±27	102±23	

Values are mean±sem; Surv, survival; CI, cardiac index; HR, heart rate; LV dP/dt, first derivative to time left ventricular pressure; MAP, mean arterial blood pressure; SAP-DAP, systolic-diastolic arterial blood pressure; SVRI, PVRI, systemic and pulmonary vascular resistance; VO_2, oxygen consumption; ER-O_2, oxygen extraction ratio. # $p < 0.001$, different from baseline; $ $p < 0.001$, different between survivors and non-survivors.

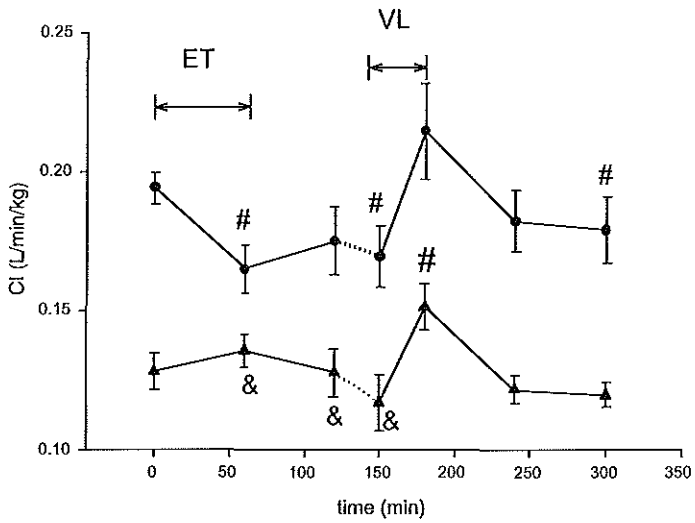

Figure 2. Course of the cardiac index (CI) in time of the young (closed circles) and old animals (closed triangles), low and high doses pooled. ET, endotoxin infusion; VL, volume loading. # $p < 0.01$ vs baseline (t=0); & $p < 0.01$ between groups.

extraction ratio (ER-O_2) for the placebo and the low dose endotoxin groups are depicted in table 4. During the placebo infusion there were no significant changes in these parameters. RmANOVA considering the change from baseline values of all age an dose groups during the endotoxin infusion for the time interval until the volume loading, showed that there were on the average 18% lower DO_2 values at all time points for the younger animals (p=0.01), and a trend towards a 22 % higher ERO_2 (p=0.03). The mean of the highest relative increase (%) in VO2 was significantly different between N-Surv and Surv: 97 ± 11% versus 48 ± 7% (p=0.002).

The volume loading (VL) after the placebo infusion resulted in an increase in DO_2, which was only significant in the young animals and an increase in VO_2, which was significant in the older animals. The ER-O_2 did not change significantly in both groups. After the endotoxin infusion (table: 4), in both OL and YL the DO_2 had a tendency to decrease; this decrease was not influenced by the VL. So, in the placebo situation the volume loading (VL) resulted in an increase in DO_2 and in the endotoxin situation the VL resulted in a decrease in DO_2.

In Fig: 3 the course of the arterial lactate levels is represented for the low dose groups. There were no differences between OL and YL. In the high dose groups (table: 4), there was a trend towards higher lactate levels in YH in comparison with OH (p=0.03). The highest peak lactate levels were found in the N-Surv: 14.8 (8.9-24.7) mmol/l versus 4.3 (2.0-19.5) in the Surv (p=0.001).

Table 4. Effect of low dose endotoxin infusion and subsequent volume loading on total oxygen delivery, consumption and extraction in old and young pigs

			time (min)						
			infusion			volume loading			
	treatment	group	0	60	120	150 (begin)	180 (end)	240	300
DO_2	placebo	O(L+H)	0.67±0.04	0.67±0.03		0.66±0.03	0.74±0.02	0.70±0.04	
	endotoxin	OL	0.59±0.05	0.67±0.03	0.64±0.07	0.56±0.08	0.52±0.04	0.54±0.02	0.55±0.04
	placebo	Y(L+H)	0.98±0.05	0.89±0.04		0.87±0.05	1.02±0.06§	0.98±0.04	
	endotoxin	YL	0.90±0.04	0.93±0.08	0.84±0.08	0.83±0.06	0.77±0.05	0.74±0.04	0.80±0.06
VO_2	placebo	O(L+H)	0.26±0.02	0.28±0.02		0.28±0.02	0.32±0.02	0.31±0.03	
	endotoxin	OL	0.23±0.03	0.31±0.02	0.30±0.06	0.32±0.04	0.28±0.03	0.27±0.02	0.26±0.05
	placebo	Y(L+H)	0.36±0.01	0.36±0.03		0.32±0.02	0.40±0.03	0.43±0.03§	
	endotoxin	YL	0.35±0.02	0.47±0.05#	0.44±0.03#	0.45±0.02#	0.48±0.04	0.38±0.02	0.37±0.09§
$ER\text{-}O_2$	placebo	O(L+H)	0.38±0.01	0.41±0.02		0.41±0.02	0.43±0.02	0.43±0.02	
	endotoxin	OL	0.39±0.03	0.46±0.02#	0.56±0.02#	0.59±0.03#	0.54±0.03	0.51±0.03	0.47±0.05
	placebo	Y(L+H)	0.37±0.02	0.40±0.03		0.38±0.02	0.42±0.04	0.44±0.02	
	endotoxin	YL	0.38±0.02	0.51±0.03#	0.55±0.04#	0.56±0.04#	0.63±0.03	0.52±0.03	0.48±0.04

Values are mean ± SEM; DO_2= oxygen delivery (ml/min/kg); VO_2= oxygen consumption (ml/min/kg); $ER\text{-}O_2$= arteriovenous oxygen extraction ratio; # $p<0.01$ vs baseline (t=0); §$p<0.01$ vs begin volume load.

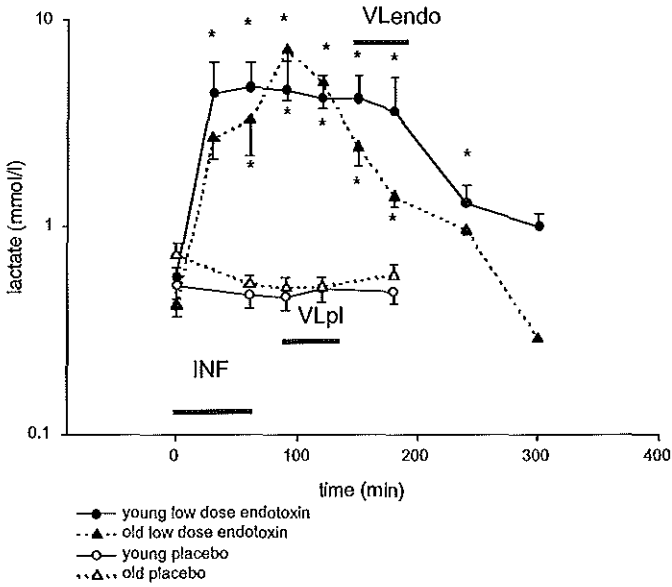

Figure 3. Arterial lactate level versus time in minutes. INF: infusion of placebo Y (L+H) and O (L+H); or endotoxin YL and OL, VL: volume loading. #, $p < 0.01$ vs baseline (t=0), Wilcoxon signed rank test.

Because of hemodynamic instability and/or agitation during the microspheres injection (procedure takes at least 2 minutes), not all measurements could be included. In Fig: 4 the

3.4 Regional blood flow changes

conductance of several organs are presented from old and young, low dose, 5 animals each. Since there were no differences between the left and right kidney, the results of both kidneys were averaged in the further analysis.

3.4.1 effect of endotoxin infusion

Since the baseline values of the conductance and flow values were different for the two age groups, the relative change from baseline was analyzed for each organ for all data available, including the high dose groups using two-way ANOVA. There was a significant decrease in conductance of the kidney, which appeared to be age dependent (p=0.013), while there was a trend towards a dose dependency (p=0.04): the changes were -21%

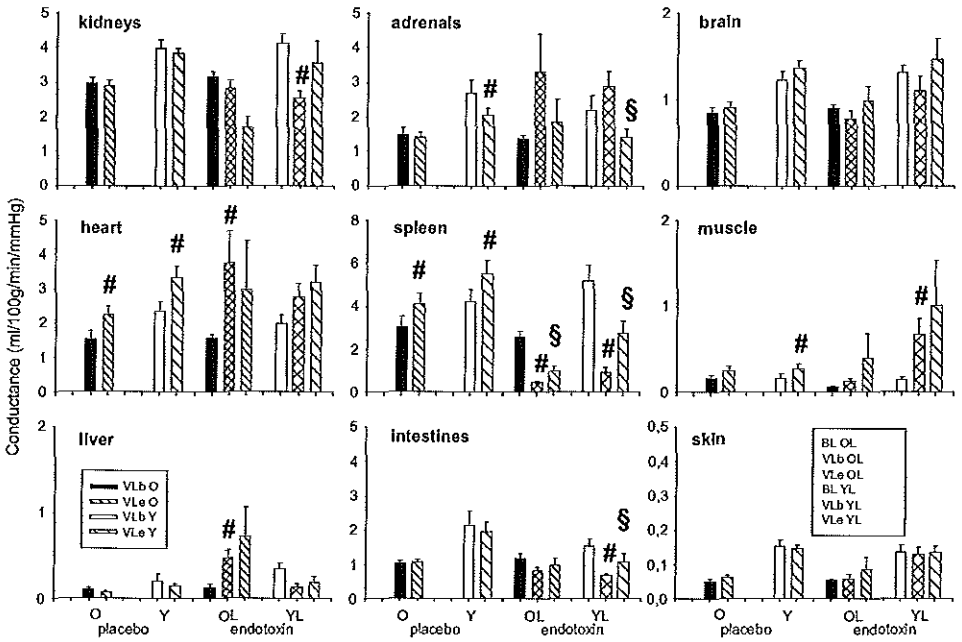

Figure 4. The conductance of various organs (ml.100g^{-1}.min^{-1}.mmHg^{-1}) in the placebo situation, O (L+H) and Y (L+H); and endotoxin situation, OL and YL. Values are means ± sem. #, $p < 0.01$ vs baseline (BL, t=0); §, $p < 0.01$ vs begin volume load (Vlb); Vle= end volume load.

(n=5), -66% (n=6), -8% (n=5), and -16% (n=6) for YL, YH, OL, and OH respectively. There was an age and dose independent decrease in cerebral blood flow of the total brain of 21% (p=0.017). The decrease in conductance was not significant. The flow of the muscles after the endotoxin-infusion increased 341 ± 130% in the younger animals and 59 ± 23% in the older, this difference did not reach statistical significance (p=0.049). In the adrenals both flow and conductance increased, the increase in conductance tended to be dose dependent, 134 ± 43% and 282 ± 49% for the low and high dose respectively (p=0.043). In the liver there was an age (p<0.001) and dose (p=0.001) dependent change in flow and conductance in response to endotoxin, YL: -30 ± 17%; YH: 120 ± 64%; OL: 233 ± 90%; and OH: 1190 ± 410%. The perfusion of the spleen showed remarkable changes: after endotoxin there was a decrease in conductance, which tended to be age dependent: -82 ± 4% for the young animals versus -71 ± 3% for the old animals (p=0.046). The skin perfusion did not change. In the intestines there was an age dependent decrease in conductance: -46 ± 6% for the young animals and 7 ± 13% for the old animals (p=0.012). The cardiac blood flow and conductance increased dose and age independent, right ventricle more than the left ventricle, epicardium more than the endocardium. The change

in endo/epicardium ratio tended to be dose dependent: -10 ± 4% for the low dose and -20 ± 2% for the high dose groups (p=0.047). The highest increase in conductance was achieved in the non-survivors, in the right ventricle more than left , in the OL more than in the YL. In table 5 the conductances of the right and left ventricle from the low dose groups are represented

3.4.2 effect of volume loading (Fig: 4)

Since two-way ANOVA showed no age or dose dependent differences, the response to volume loading was analyzed using the relative changes (%) of all groups combined. Volume loading after placebo infusion showed no significant change in flow or conductance of the kidneys. Cerebral blood flow increased significantly, both after infusion of endotoxin and of placebo with the increase being higher after the endotoxin infusion (p=0.008); the conductance increased only in the endotoxin situation. The muscles flow and conductance increased, only in the placebo and not in the endotoxin situation. There was a significant decrease in flow and conductance to the adrenals especially after endotoxin infusion. Flow and conductance to the liver did not change after volume loading. There was a substantial increase in flow to the spleen in the placebo and endotoxin situation in young and old animals. In the placebo situation the flow to the intestines did not change, after endotoxin infusion the flow increased significantly. The total cardiac blood flow increased due to the volume loading both in the placebo situation and in the endotoxin situation. The conductance of the right and left ventricle as well as its distribution over the sub epi-, meso- , and endocardium showed a significant increase in conductance after placebo infusion and a trend towards increase in the endotoxin situation (p-values between 0.01 and 0.05). The change in LV endo/epi ratio after volume loading was -2 ± 3% in the placebo situation versus 7 ± 4% in the endotoxin situation (p=0.015).

4 DISCUSSION

The present study was designed to examine the differences in circulatory responses in awake young and near adult piglets to a meningococcal endotoxin challenge as well as the response to volume loading after this challenge. Most important findings are that (i) there is a dose-related, but not an age-related difference in mortality, (ii) whereas CI after meningococcal endotoxin decreased in the young animals, it was well preserved in the older animals, this difference was probably related to a different vascular response: in the older animals the SVRI dropped 20%, while in the younger ones there was no change in resistance; there was a trend towards a higher decrease in SI, a higher decrease in DO_2, a higher ERO_2, (iii) there were significant differences in perfusion of the abdominal organs (a higher decrease in flow and conductance to kidneys, intestines and spleen, and a lower

Table 5. Effect of low dose endotoxin infusion and volume loading on conductance of different regions of the heart in old and young pigs

		placebo		endotoxin		
				infusion		
		volumeloading		volume loading		
	group	M1	M2	M3	M4	M5
RV	OL	1.12±0.16	1.65±0.21§	1.15±0.16	3.83±0.85	3.20±1.58
	YL	1.74±0.21	2.44±0.25	1.70±0.17	3.44±0.23#	3.71±0.80
LV	OL	2.09±0.33	2.97±0.34§	2.06±0.32	4.25±1.19	3.39±1.56
	YL	2.79±0.33	4.06±0.37§	2.62±0.34	2.91±0.55	4.5±0.86
LV endo/epi	OL	1.16±0.04	1.18±0.04	1.19±0.04	0.99±0.03	1.10±0.12
	YL	1.25±0.05	1.14±0.06	1.22±0.07	1.15±0.09	1.18±0.07

Values are mean ± SEM in $ml.min^{-1}.100g^{-1}$. RV, right ventricle; LV, left ventricle;, endo/epi, ratio between endo-and epicardium. M1-5 moment of microsphere injection (Fig: 1). M1: before volumeloading after placebo infusion, M2: after volumeloading, M3: before endotoxin infusion, M4: after endotoxin infusion before volume loading, M5: after volume and loading and endotoin infusion. M1, M2 from O (L+H) and Y (L+H) each 10 animals; M3,4,5 from OL and YL each 5 animals. # $p<0.01$, vs baseline; § $p<0.01$, vs begin volume loading.

increase to the liver) of young and old animals, (iiii) subsequent volume loading resulted only partly in a recovery of the hemodynamic parameters, but failed to improve oxygen delivery (DO_2).

4.1 Methodological considerations

Meningococcal sepsis is a particular form of gram negative sepsis. Its special pathophysiological features are the very high serum levels of endotoxin located on bacterial membrane fragments (blebs), the very rapid evolvement of the disease with its high mortality in previously healthy children, extreme disseminated intravascular coagulation (DIC) with microvascular thrombosis and skin bleeding, capillary leakage, and myocardial failure (38, 229, 238, 242). Based mainly on experimental data in rats (71, 266, 306, 367, 384, 387), rabbits (312), dogs (172, 284, 286), or piglets (215) it has been proposed that the circulatory response during sepsis is different between neonates and adults. It is likely that there are also, probably to a lesser extent, differences between pediatric and adult individuals, although these differences in response have hardly been studied. The results of these studies are difficult to compare because of the different sensitivity to endotoxin of the different species, the dosages of endotoxin used, the different ages of the animals, and the difference in methodology. However, the differences between young and old animals described in these studies, are most likely related to maturity suggesting that, in the young subject, decreased cardio-circulatory and metabolic reserves

might contribute to differences in mortality and morbidity. In the clinical situation we are dealing with pediatric patients (median age in our patient population is 3 years), which implies that the use of invasive techniques to study the circulatory alterations is limited. There is therefore a need for a "pediatric" animal model to study in detail the pathophysiology, specific treatments, or preventive measures like vaccines, which implicates a large laboratory animal. The pig has been shown to resemble humans quite closely in its cardiovascular anatomy and physiology also during sepsis (259), and a young pig can be instrumented easily. In order to study possible age related differences, two ages were compared: 3 weeks (8 kg) representing a pediatric age, and 4 months (40 kg) representing the (near) adult. We chose a chronically instrumented, awake animal model in order to avoid interference of anesthesia and acute surgical trauma with cardiovascular responses and to prevent anesthesia from interfering with host LPS-clearance mechanisms (305). For the induction of experimental sepsis in pigs viable bacteria or LPS are used. However, to challenge the animals with viable meningococci would be complicated and dangerous for the environment. Previously, purified meningococcal LPS has been used in experimental settings, mostly in small laboratory animals (297). However, this LPS is not the form which is found in vivo in humans. During their growth, the bacteria are continuously spreading membrane material (blebs) containing endotoxin (40). This rough material can be separated from the bacteria and standardized to a LPS concentration (275). Two different dosages of this endotoxin preparation were used to challenge the animals.

4.2 Cardiovascular responses

The pattern of response to low to moderate dose of endotoxin was comparable to that described in literature (184, 215, 259, 370). However, the older animals in our study were able to maintain their CI, despite a 40 % decrease in stroke volume; the LV dP/dt and MAP remained stable or increased instead of decreasing (184, 259); in the older animals the SVRI decreased instead of increasing (184, 259, 270). This disagreement with earlier observations can be explained by the absence of anesthesia in the present study and by the difference in type of endotoxin used. Striking was the finding that both in the older and the younger animals there was a trend towards a higher CI, LV dP/dt, and pulse pressure in the non-survivors in comparison with the survivors, suggesting a difference in vascular response. In humans with sepsis, CI is usually normal to elevated and SVRI is typically low, at least in adults. In our study the young animals were unable to preserve their CI, nor did their SVRI or systolic-diastolic arterial pressure difference change. So the older animals show a more normodynamic picture, and younger animals initially show a hypodynamic picture. This would be in accordance with the study of Mercier et al. who reported in pediatric meningococcal patients, that CI was low to normal in survivors, but was

decreased in non-survivors, and that SVRI was normal in survivors but increased in the non-survivors (238). This difference between survivors and non-survivors was opposite to the finding in our study that the CI was higher in non-survivors than in survivors, both in older and younger animals.

The changes in organ flow and conductance after endotoxin infusion show a pattern of redistribution which matches with heavy exercise and high levels of catecholamines: decrease of flow to kidneys, spleen and intestines, little or no change in flow to brain, and skin, increase in flow to liver, muscle, adrenals and heart and these changes were comparable with those described in literature: (73, 165, 172, 196, 255, 256, 259, 369). However, the design of these sepsis studies was not always completely the same (difference in kind of animals, ages, endotoxin, bolus or intermittent infusion, rescue therapy). In the low endotoxin dosage, the flow and conductance of different regions of the heart of the older animals showed an increase after challenge, which was not apparent in the young ones; in the high dosage this increase was present in both groups and most extreme in the non-survivors. This finding together with the high lactate levels and the high total oxygen extraction ratio might lead to the hypothesis that the increase in cardiac flow is secondary to an increased total body oxygen demand. We did not determine the cardiac oxygen consumption. However, Herbertson described an awake porcine endotoxin model in which the myocardial oxygen extraction ratio was dramatically decreased (166). This decrease in myocardial oxygen extraction ratio was due to an increase in overall myocardial flow and an increase in heterogeneity of this flow. These changes were explained to be induced by components of the inflammatory response of sepsis.

4.3 Differences between young and old

In our clinical setting the mortality was significantly different (40 vs 13 %) between younger patients (< 3 years) and older (3-18 years); 91% of the non-surviving patients died of irreversible shock (158). In our experimental study the hemodynamic response to the endotoxin was overall comparable in the older and young animals, but there were some findings suggesting a difference in cardiac performance, possibly related to preload; and a difference in vascular response (more vasodilatation in the older animals). The organ flow and conductance showed a significant decrease in renal conductance in the young animals and not in the old ones; the flow to the muscles increased more in the young animals; there was much less increase in flow to the liver and a greater decrease in intestinal and spleen flow in the young animals. With these findings of a more depressed splanchnic perfusion in the young animals, one may speculate that the risk of developing organ failure is increased in the young animals. However, the mortality was not different between the young and the old animals.

4.4 Volume resuscitation

Volume resuscitation using colloid or crystalloid solutions, is a major component of management of the acute phase of sepsis. Its aim is to compensate for the extravasated fluid and to restore the decreased preload caused by the capillary leakage. In our experiments the volume loading (VL) changed all of the hemodynamic variables towards baseline values, some of them above (CI), except for the SV and SWI which still remained below baseline. Remarkable was that the VL in the placebo situation resulted in an increase in DO_2, while in the endotoxin situation the DO_2 either did not change (older) or decreased (younger). So, from this finding we could suggest that in the clinical situation a volume expansion during sepsis should be accompanied by a blood transfusion to improve the DO_2 and not only the CI. Most of the organs in which there was a decrease in flow after endotoxin showed an improvement after VL. Striking was after an initial rise caused by the endotoxin infusion, the flow to the adrenals decreased after the VL; these changes in flow together with the injured endothelial cell lining present during sepsis, might explain the high frequency of adrenal bleeding reported in meningococcal sepsis.

4.5 Conclusions

To our knowledge this is the first report in which the effect of rough meningococcal endotoxin was studied in a large, chronically instrumented awake laboratory animal showing a hemodynamic picture comparable with adult animals, but also showing age related differences. Our study shows that a model consisting of a chronically instrumented piglet of 8 kg is feasible and that a rough meningococcal endotoxin preparation is potent in a low dosage and can be used to give a reproducible endotoxin model. On the other hand it is possible that many of the circulatory changes can be contributed to the rapid increase in PVRI, which is not so extreme in the human situation. This is probably caused by the fact that the endotoxin is infused in 1 hour and reaches levels, which are reached only after several hours in the human situation. Moreover this study had an observation period of about 6 hours, which means that the prolonged or delayed effects of host response to endotoxin like coagulation disturbances, are to be missed [Haberstroh, 1995 #537]. This problem might be overcome by gradually infusing the endotoxin over time and extend the observation period to 24 h.

This "pediatric" experimental meningococcal endotoxin model in piglets can be used to study therapies like vaccines and anti-inflammatory agents specific for meningococcal sepsis.

CHAPTER 4.3

MENINGOCOCCAL SEPTIC SHOCK IN CHILDREN: CLINICAL AND LABORATORY FEATURES, OUTCOME, AND DEVELOPMENT OF A PROGNOSTIC SCORE

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1 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 (MD) still remains major health problem in both developing and industrialized countries. Group B is the predominant serogroup among strains causing MD followed by group C (304).

From 1970 to 1980 the annual incidence of MD in the Netherlands varied between 0.7 and 2.0 cases per 100,000 population. The incidence of MD 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) (229. 304). In addition, the percentage of patients with meningococcal sepsis without clinical meningitis increased in the same period (1, 291). 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% (129, 131, 205). 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 (86, 93, 120, 131, 185, 205, 206, 258, 308, 316, 328, 331, 339). 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 features 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 features and outcome of meningococcal septic shock in children admitted to the Sophia Children's Hospital between 1988 and 1995. Additionally, the prognostic significance of several clinical and laboratory features was evaluated and a new prognostic score was developed.

2 PATIENTS AND METHODS

The records of all patients 18 years and younger admitted from October 1988 through June 1995 with meningococcal septic shock 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 (9) and/or the presence of poor end-organ perfusion defined by at least two of the following criteria: a.) unexplained metabolic acidosis (pH ≤ 7.3), base excess ≤ -5 mmol/L or arterial plasma lactate levels > 2.0 mmol/L; b.) arterial hypoxia defined as a PaO_2 ≤ 75 mm Hg, a PaO_2 to FiO_2 ratio < 250 or TcO_2 ≤ 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 (Centoxin, Centocor, Malvern, PA) in meningococcal septic shock.

Medical records were analyzed for demographic, clinical, and laboratory features 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.

2.1 Definitions

The severity of illness on admission at the PICU was assessed using the pediatric risk of mortality (PRISM) score (274). 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 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) (207). Patients were divided in different groups for statistical analyses. Survivors were compared with non-survivors.

2.2 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 (179). 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 (309). Serotyping and subtyping were performed by means of a whole cell ELISA (207) (3).
Clinical hematology and chemistry: Laboratory studies including a complete blood count and serum chemistry analysis were routinely performed on admission. Blood samples for analysis of hematologic characteristics 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.
Characteristics 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 (69) (Behringwerke AG, Marburg, Germany). A semi-quantification of FDP in plasma was performed by latex agglutination (Diagnostica Stago, Asnières-sur-Seine, France).

2.3 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 variables between survivors and non-survivors. Logistic regression analysis with backward elimination was performed to develop a prognostic score for mortality based on variables obtained on admission (7). Two-tailed P values ≤ .05 were considered statistically significant.

3 RESULTS

3.1 Patient characteristics

Seventy-five patients with meningococcal septic shock were evaluated. Forty-two were males and 33 were females. The children had a median age of 3.2 years (range 3 weeks - 17.9 years). Twenty-four (32%) children were less than 2 years, 35 (47%) between 2 and 10 years, 16 (21%) were older than 10 years. Forty-nine of the children participated in the clinical trial to study the efficacy of HA-1A human monoclonal antibody (23 HA-1A and 26 placebo recipients). The PRISM score at admission in the PICU ranged from 0 to 38 (median 11). Twelve patients were directly admitted to our hospital, and 63 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 95% of the patients. In 10 (13%) patients 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 55 of the 63 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 19.2 ± 7.3 and 7.1 ± 5.8 hours respectively.

A lumbar puncture was performed in 53 cases at the time of admission. Meningitis was documented in 33 (62%) cases. A positive CSF culture was obtained in 9 patients. All 75 patients needed inotropic and vasopressor support. Forty-four of the 75 (59%) patients needed mechanical ventilation.

3.2 Bacteriological findings

Cultures of blood, CSF or skin biopsies grew N. meningitidis in 75 children. 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 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 vs. 7.7 ± 5.3 years: P = .04).

3.3 Outcome

Survivors vs non-survivors: The mortality was 21% (95% confidence interval [CI]: 12% - 32%). We did not observe a difference in mortality rate between HA-1A and placebo recipients (5 [22%] of 23 vs. 7 [27%] of 26; P = .75). Fourteen children died as a consequence of irreversible septic shock. Two patients died as a result of central nervous

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

	Serogroup	
	B	C
	58 (82%)	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%)

The number of patients with a specific serogroup, serotype or subtype is indicated followed by the percentage of the total number of patients between parentheses.

system complications. Fifty percent of the deaths occurred within the first 24 hours and nearly 90 % occurred within 48 hours. The median (range) duration from the onset of symptoms until death was 40 hours (11-143 hours). The demographic and clinical characteristics of the 59 survivors and the 16 non-survivors at admission to the PICU are shown in Table 2. The mortality rate was higher in children younger than 4 years of age (13 [33%] of 40 vs. 3 [9%] of 35; P = .02). Patients admitted primarily to the Sophia Children's Hospital had a higher mortality rate in comparison with secondary referrals (5 [42%] of 12 vs. 11 [17%] of 63; P = .12]. The PRISM score of the primary referrals was worse than that of the secondary referrals (14.3 ± 5.2 vs. 11.7 ± 7.9; P = .21). The interval between the onset of petechiae and admission to the PICU was shorter in non-survivors.

Complications and sequelae of survivors: The median hospital stay was 13 days (range 10 - 207) among the survivors. Twenty-eight of the 59 survivors were mechanically ventilated for a median duration of 7 days (range 1 - 24 days). Most survivors recovered without sequelae. Two patients had serious neurologic sequelae. Dermatologic or orthopedic sequelae requiring skingrafts or amputations occurred in 9 of the 59 survivors. Two patients required hemofiltration because of renal failure, one patient developed osteomyelitis. Seizures occurred in 7 patients.

3.4 Laboratory findings

Demographic and laboratory features of survivors and non-survivors are depicted in

Table 2. Characteristics of 75 children with meningococcal septic shock on admission at the PICU.

characteristic	n	survivors (n = 59)	nonsurvivors (n = 16)	p-value
Age (y)	75	5.8 ± 5.0	3.6 ± 3.7	NS
Sex (% male)	75	33 (56)	9 (56)	NS
Transferred	75	52 (88)	11 (69)	NS
Interval (h) from				
- onset symptoms				
to admission PICU	72	19.5 ± 7.1	18.0 ± 8.3	NS
- appearance of petechiae				
to admission PICU	73	7.6 ± 5.9	5.5 ± 5.1	.06
PRISM score	75	10.1 ± 7.1	18.6 ± 5.1	<.001
Hematology				
Hb	75	6.6 ± 0.9	6.1 ± 1.2	NS
WBC (x10^9/L)	75	14.5 ± 10.0	9.0 ± 9.6	.02
platelets (x10^9/L)	74	110 ± 51	63 ± 37	.001
Chemistry				
sodium (mmol/L)	75	135 ± 5	137 ± 4	NS
potassium (mmol/L)	75	3.4 ± 0.6	4.1 ± 0.7	<.001
calcium (mmol/L)	70	1.92 ± 0.24	1.83 ± 0.26	NS
glucose (mmol/L)	65	6.6 ± 2.9	4.6 ± 2.8	.02
lactate (mmol /L)	70	5.1 ± 3.3	7.3 ± 4.1	.005
creatinine (µmol/L)	70	87 ± 55	118 ± 67	NS
CRP (mg/L)	66	135 ± 69	80 ± 53	.006
albumen (g/L)	67	33 ± 6	34 ± 10	NS
Acid Base balance				
pH	75	7.37 ± 0.08	7.27 ± 0.12	.003
BE (mmol/L)	74	-6.2 ± 3.9	-11.4 ± 4.4	<.001
bicarbonate (mmol/L)	75	17.4 ± 3.5	13.9 ± 3.1	.001

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.

Tables 2 and 3. Occasionally laboratory data were missing, but this never occurred in more than 11 patients for a given characteristic.

Initially, 16 (21%) patients 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 13 of 74 (18%) patients. The acid-base status and the arterial serum lactate levels showed striking abnormalities that were more severe in non-survivors. Serum glucose levels were significantly lower in the non-survivors but hypoglycemia (< 2.5 mmol/L) was observed in 7 children. Hypokalemia (< 3.5 mmol/L) was observed in 52%. Serum potassium levels were highly correlated with the arterial pH (r_s =

Table 3. Coagulation and fibrinolysis characteristics in 75 patients with meningococcal septic shock

characteristic	reference range	n	survivors (n = 59)	nonsurvivors (n = 16)	p-value
Coagulation					
APTT (sec)	28-40	66	54 (29->200)	104 (53-200)	<.001
Factor V* (%)	70-140	64	40 ± 21	21 ± 14	.002
Fibrinogen (g/L)	1.8-3.5	67	2.6 (<0.4-5.8)	1.1 (<0.4-5.4)	.001
Fibrinolysis					
FDP (mg/L)	<5	65	50 (<5->300)	110 (35->300)	.003

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.

-.46, P < .001). Analysis of covariance showed that serum potassium levels were significantly higher in non-survivors in comparison to survivors irrespective of the arterial pH (Figure 1). Serum calcium concentrations were measured in 70 cases (93%). Hypocalcemia (< 2.2 mmol/L) was detected in 62 patients (89%). 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 (1.69 ± 0.12 mmol/L vs. 1.92 ± 0.03 mmol/L; P = .03). The serum levels of CRP were significantly lower in non-survivors 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 = .62, P < .001 and r = .54, P < .001, respectively).
Coagulation studies were performed in most patients (Table 3). Fibrinogen levels were less than or equal to 1.5 g/L in 17 of 67 (25%) patients. DIC could be determined in 60 patients. DIC occurred significantly more often in non-survivors than in survivors (12 [92%] of 13 vs. 23 [49%] of 47; P = .005).

3.5 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. Two of these variables were significantly associated with the duration of petechiae (base excess: r = .32, P = .007; CRP: r = .54, P < .001). However, logistic regression analysis including the duration of petechiae did not improve the predictive value of the new prognostic scoring system, the Rotterdam score. The mathematical expression

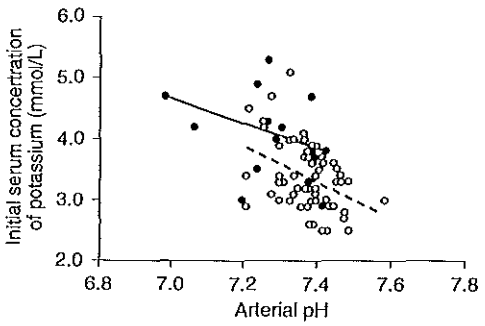

Figure 1. Relation between initial serum concentrations of potassium and arterial pH in 75 children with meningococcal septic shock. Dashed and solid lines indicate the regression lines through the values for survivors (o) and non-survivors (•) respectively. Slopes between the regression lines of survivors and non-survivors did not significantly deviate from parallelism.

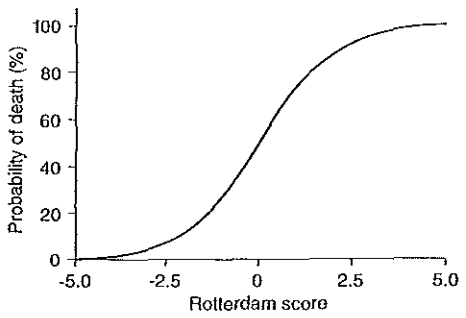

Figure 2. Probability of death according to the Rotterdam score that predicts the outcome of patients with meningococcal septic shock based on four laboratory variables (C-reactive protein, serum potassium, base excess, and platelet count).

of the probability of PICU death in this study was as follows:

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

in which the Rotterdam score (RS) = 1.01 + 1,21 x Potassium (mmol/L) - 0.29 x Base Excess (mmol/L) - 0.024 x Platelets (10^9/L) - 3.75 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 ($r = .58$, $P < .001$).

4 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 collapse, renal failure, adult respiratory distress syndrome and death.
In the present study we show that meningococcal septic shock is associated with a mortality rate of 21% and serious sequella in 17% 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

Table 4. Outcome prediction in patients with meningococcal septic shock based on different prognostic scoring systems.

Score	Number of patients	Predictive value for survival	Predictive value for death	Accuracy
Niklasson (250)	53	88%	25%	45%
Leclerc (205)	63	93%	61%	84%
CRP (206)	66	91%	39%	66%
Giraud (131)	67	90%	47%	78%
PRISM (274)	75	88%	72%	85%
Rotterdam	65	90%	71%	86%

The Rotterdam score predicts the outcome of patients with meningococcal septic shock based on four laboratory features (C-reactive protein, serum potassium, base excess, and platelet count).

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 (328). In our study, the mortality rate was also 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 (279). Long-term morbidity was observed in 17% of the survivors and was caused by deforming amputation or large areas of soft tissue destruction secondary to the coagulopathy and by neurological sequelae. A similar percentage was observed by Madden et al. (222, 245).

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) (304).

Striking differences were observed in clinical and laboratory characteristics between survivors and non-survivors. The shorter interval between the appearance of petechiae and admission and the lower level of CRP in non-survivors suggest a shorter disease course. These data indicate that non-survivors deteriorate more quickly because they accumulate more native LPS per time span, trigger all mediator systems more intensively or because they have a higher responsiveness to LPS or proinflammatory cytokines (195).

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 non-survivors 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 (188). The relatively higher serum potassium levels in non-survivors may be caused by metabolic derangements (188), the more severe renal impairment, or rhabdomyolysis. In addition, hypocalcaemia was also seen in a large number of patients as observed by others (50, 225, 299). 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 (93, 120, 185, 214, 250, 316). 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 (328). 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 (316, 331). We therefore developed a simple score for patients with meningococcal septic shock which only requires objective variables available at any emergency room or PICU soon after admission. Logistic regression analysis revealed four laboratory features 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 (195). The mortality risk predictor developed by us only needs simple laboratory features which are routinely performed. The prognostic value was higher than in previously developed scoring systems. However, since we validated this new score for patients with meningococcal septic shock on the same group of patients used to develop this score, a slight overestimate of the utility of the score in predicting mortality may have occurred.

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.

Beneficial effects of HA-1A on the outcome of children in the present study with meningococcal septic shock were not shown. This observation is in accordance with a

recent study that did not find a reduction in the 14-day mortality rate in patients with gram-negative bacteremia and septic shock (236).

We conclude that meningococcal septic shock in children is associated with a mortality of 21% (95% CI 12% - 32%). The mortality was even higher in children below 4 years of age. About 17% of the survivors had serious sequelae such as skin necrosis requiring skingrafts or amputation, osteomyelitis, neurologic sequelae. Logistic regression analysis identified four laboratory features which were used in a prognostic score to predict outcome. The predictive value for death and survival were 71% and 90% respectively. The overall outcome was predicted correctly in 86% of the cases.

CHAPTER 5

SUMMARY & FUTURE PERSPECTIVES

1. THE PROBLEM OF SEPSIS IN CHILDREN

The physiological changes that constitute the process of sepsis are induced by microbial agents during bloodstream infection or by the toxic products of pathogens that are released from sites of focal infection. This process involves changes generated by the immune system in which hormones, cytokines and enzymes are involved. Many host and microbial factors may unfavorably influence this complex immune-response. There are several differences between the host response of young children and adults with (meningococcal) sepsis. One of the major factors responsible for these differences is the immaturity of the immune system in young infants. Issues related to this topic are reviewed in **Chapter 1.2.** The severity of meningococcal disease ranges from sepsis to sepsis syndrome and finally septic shock. Septic shock with purpura is a rapidly evolving clinical picture characterized by sepsis (tachycardia, tachypnea, fever), shock (hypotension or signs of end-organ failure) and a spectrum of coagulation disorders (ranging from petechiae, purpura to ecchymoses). It is mainly (80 %) caused by *N. meningitidis* (group A, B or C). The disease is also called meningococcal sepsis or severe meningococcal disease (SMD). In The Netherlands the incidence of SMD is 3.5 per 100.000 inhabitants. Approximately 60 persons die yearly due to SMD. Two-third of these are below the age of 15 years. The growing interest in infections caused by meningococci has resulted in a large number of research projects in this field during the last decade. The application of molecular and immunological methods in animal experimental and human studies of meningococcal sepsis has contributed to our understanding of the disease. Recent progress in the understanding of the pathophysiology of meningococcal sepsis in children is discussed in **Chapter 1.3**

2. ENDOTOXIN AND INFLAMMATORY MEDIATORS

2.1 endotoxin clearance

Characteristic features of meningococcal sepsis are the specific nature of the endotoxin and the very high concentrations in plasma during the acute phase. It is not yet fully understood how endotoxin is cleared from the circulation. Certain specific and non-specific endotoxin binding proteins are involved in the clearance as discussed in **Chapter 1.3.** Lipopolysaccharide binding protein (LBP) is a specific endotoxin binding protein and acts as an acute phase protein. LBP forms a complex with lipopolysaccharides (LPS). Subsequently this complex is attached to the CD14-receptor of cells of the immune system such as monocytes. These cells are then activated and start to synthesize mediators such as TNF-α and IL-1ß. Binding of LPS to LBP leads to an amplification of the biological effect

of LPS.
In addition, during meningococcal sepsis neutrophils are destroyed and their content degranulates. One of the released proteins is bactericidal permeability increasing protein (BPI). BPI is also capable to bind LPS. However, binding to BPI leads in contrast to binding with LBP to neutralization of the biological effects of LPS. A recombinant form of BPI has been tested in a phase I trial in patients with meningococcal sepsis and recently a phase III trial in children was started. The natural course of these plasma proteins in children with septic shock still needs to be elucidated.
We studied experimental meningococcal sepsis in young (3 weeks) and old (4 months) pigs and the possible age-related differences in endotoxin levels and cytokine profiles after a standardized challenge of low and high dose of crude meningococcal endotoxin. This study is described in **Chapter 2.1.** We assessed endotoxin levels, as well as the balance between pro- and counter-inflammatory cytokines (TNF-α, IL-6, and IL-10 levels) in the 4 hours following an one-hour infusion of endotoxin. The clinical picture and the mortality were similar in all groups. In young animals the median of the maximal endotoxin levels was 4.4 times higher than in the older animals, whereas endotoxin was cleared at a lower rate compared to the older pigs. Despite higher levels of endotoxin in young pigs, the levels of TNF-α, IL-6, and IL-10 were similar or even slightly higher in the older animals. Age related differences therefore seem to play a role in the endotoxin binding and clearance.
HDL and lipoproteins are non-specific endogenous LPS-binding proteins. Binding of LPS to these proteins leads to neutralization, at least in the presence of a normal liver function. In adults with sepsis it has been shown that levels of these proteins are much lower than in control subjects. In children with meningococcal sepsis the course of these plasma lipoprotein levels has not been studied, but probably shows a comparable picture: a relative deficiency of these proteins. Therefore lipoproteins and HDL have a potential therapeutical value. As a consequence a phase II study using reconstituted HDL in children with meningococcal sepsis has been initiated.

2.2 cytokines

Once activated, monocytes, macrophages and endothelial cells start to produce cytokines, which play a crucial role in the immune response as discussed in **Chapters 1.2 and 1.3.** In a limited number of patients (n=17) with sepsis or septic shock with purpura, we analyzed the levels of these cytokines (TNF-α, IL-1-ß and IL-6) and correlated these values with routine laboratory values. This study is described in **Chapter 2.2.** All cytokine levels were significantly increased, but only IL-6 levels had a good predictive value for severity of disease. Il-6 has previously been shown to be a reliable indicator for severity of disease and outcome in other pediatric and adult patients with sepsis. From the laboratory parameters

(fibrinogen, CRP, and lactate), only arterial lactate was positively correlated to outcome and severity of disease.

In a subsequent study, described in **Chapter 3.3**, we estimated the levels of TNF-α, IL-6, IL-8, IL-10, and the soluble TNF receptors p55 and p75 in 30 patients with septic shock and purpura. All cytokine levels were higher in non-survivors. However, when these levels were corrected for the time between the moment the petechiae were first noticed and the moment of blood sampling, these differences disappeared. Another interesting finding was that both levels of pro-inflammatory (TNF-α, IL-6, IL-8) as well as counter-inflammatory cytokines (IL-10 and s-TNF-R) were significantly increased in non-survivors. It is not yet clear whether this represents a situation of excess-production of pro-inflammatory cytokines, or an insufficient production of counter-inflammatory cytokines. Perhaps sequential analysis of the immune status during the disease e.g. using whole blood culture systems looking at cytokine profiles and leucocyte activation markers after a standardized stimulus, can provide an answer to this question.

In the same group of 48 patients, we measured plasma levels of IL-12 and IFN-γ. Interleukin 12 (IL-12), a recently described cytokine composed of two different polypeptide subunits (p40 and p35), which plays a role in the host defense against microbial infections and has been shown to induce the production of interferon (IFN)-γ. Levels of IFN-γ are increased in experimental as well as clinical sepsis. We hypothesized that IL-12 and IFN-γ play a role in the pathogenesis of septic shock and purpura **(Chapter 2.3).** The median plasma IL-12 p40 level on admission was related to outcome, severity of disease and the levels of other cytokines even when corrected for time of petechiae, while IL-12 p70 (complete cytokine) levels were only increased in 9 patients (19%). Patients with detectable IL-12 p70 levels had higher IL-12 p40 levels than those without detectable IL-12 p70 levels. Twelve (29 %) of the patients had detectable levels of IFN-γ. No relation with outcome could be detected. In those 12 patients, other cytokine levels, among which IL-12 p70, although not significantly, were increased. However, this was not true for the IL-12 p40 levels. In our patients a positive correlation was found between IL-10 and IL-12, which was in contrast to findings in sepsis in baboons where a negative correlation was found. In vitro, IL-10 is a strong inhibitor of LPS-dependent IL-12 production. We propose that the synthesis of pro- and counter-inflammatory cytokines is so strong and continuously stimulated in patients with meningococcal sepsis, that counter-regulatory mechanisms are insufficient to suppress excessive production.

3. COAGULATION DISORDERS

3.1 link between coagulation and inflammation

Inflammation and coagulation can not be considered as two separate processes, since there are several links making them part of the host response during not only infectious, but also traumatic, hypoxic and hypovolemic incidents. The endothelium represents the interface between inflammation and coagulation: it is a surface where coagulation is activated, but under cytokine stimulation it provides a site of attachment for inflammatory effector cells. During inflammation coagulation is initiated through activation of factor XII and tissue factor mainly by TNF-α, IL-1-ß, and IL-6. Activation of factor XII leads to the production of bradykinin, kallikrein, factor XIa and plasmin, all forming part of the contact system. These factors are related to the complement system through one regulating protein: C1-esterase inhibitor. The formation of thrombin following the activation of the coagulation cascades is an essential issue in the development of hemostasis. Thrombin stimulates fibrin formation and leads to platelet aggregation and degranulation by which a number of vasoactive mediators are released. In addition, thrombin affects a series of endothelial functions and stimulates cells involved in inflammation. Thrombomodulin (TM) is a high affinity endothelial receptor for thrombin. The thrombin-TM complex activates protein C. However, TNF-α downregulates the expression of thrombomodulin in this way interacting with protein C. Activated protein C complexes with a co-factor protein S. Protein S is bound in plasma to C4 binding protein (C4bp), which is a complement system regulating protein. This is another indication of the interaction between coagulation and complement system. Therefore, in the study of the host defense during a severe infection, the involvement of the coagulation pathway has to be taken into account.

3.2 protein C

Protein C inactivates factors Va and VIIIa, and, at the same time stimulating the fibrinolytic pathway, by inactivating plasminogen activator inhibitor (PAI-1), an acute phase protein from which the production is promoted by cytokines such as TNF-α.
Since the initial report in 1991 of decreased levels of protein C levels in meningococcal disease, we became interested in the protein C system in our patients. In a study with the University Hospital of Lille, France, we analyzed protein C and S levels in 40 children with septic shock with purpura **(Chapter 3.1).** Protein C levels (both antigen and activity) were strongly decreased and the decrease was related to the presence of DIC and outcome. For this reason protein C supplementation should be evaluated in children with severe septic shock and purpura.

3.3 relation with age

Many studies have reported coagulation parameters in patients with meningococcal sepsis. Most of these studies encompassed small numbers of patients, with a

heterogenous population (mild and severe cases), different age groups (children and adults), or analyzed a limited number of parameters. We prospectively measured in a large number (79) of children with septic shock and purpura several parameters of coagulation and fibrinolysis on admission and after 24 and 72 hours **(Chapter 3.2).** The results were consistent with a heavily activated coagulation system, inhibition of natural anticoagulants such as antithrombin and protein C, depletion of coagulation factors, and a strong inhibition of the fibrinolytic system mainly by the presence in plasma of very high concentrations of PAI-1. After classifying the patient group with respect to the median age (3.1 years), it became clear that the severity of coagulation disorders (presence of DIC, depletion of coagulation factors and inhibitors) was more intense in the younger age group. However, the differences between the two age groups with respect to cytokines were not significant. Therefore, one may hypothesize that these age related differences in coagulation parameters are partly responsible for the difference in mortality between younger and older patients.

3.4 relation PAI, cytokines and protein C

Since PAI-1 levels were clearly increased and PAI-1 behaves as an acute phase protein, we became interested in the relation between PAI-1 and cytokines such as TNF-α **(Chapter 3.3).** There was a significant correlation between PAI-1 and TNF-α. To our surprise we found that this relation was different for survivors and non-survivors. With the same level of TNF-α the PAI-1 levels were 1.9 higher in the non-survivors. The same was true for other cytokines. This indicates that the individual PAI-1 production is directly related to outcome. In this study PAI-1 antigen was measured and not PAI-1 activity. New studies should direct attention to measure PAI-1 activity, since this may not be similar to PAI-1 antigen level. One of the possible explanations for the extreme low protein C levels in our patients, reported in **Chapter 3.1 and 3.2,** is the fact that activated protein C forms a complex with PAI-1 and in this way is consumed. Since protein C is important in the regulation of coagulation and fibrinolysis, it is worthwhile assessing the therapeutic value of restoring these extreme low levels of protein C to normal, or even supranormal values. In 1997 a phase II dose finding study has started with reconstituted protein C. Aim of this trial, which is coordinated by the Department of Pediatrics of the Sophia Children's Hospital, is to establish an appropriate dosing regimen and to study the course of these PAI-1/protein C complexes as well as activated protein C and soluble protein C receptors.

3.5 PAI polymorphism

The significantly increased levels of PAI-1 in non-survivors from SMD raised the question,

whether a polymorphic immune response was involved in the outcome of these patients. When studying the literature we indeed found a description of a common functional polymorphism in the PAI-1 gene. A single base pair insertion (5G) / deletion (4G) polymorphism 675 base pairs upstream from the start of transcription is functionally important in the regulation of the expression of the PAI-1 gene. Subjects homozygous for the 4G allele have higher basal and inducible levels of PAI-1 than those with 1 or 2 copies of the 5G allele. A reduced fibrinolytic capacity due to increased plasma levels of PAI-1 may have pathogenetic importance in myocardial infarction, particularly in young patients with hypertriglyceridemia or with diabetes. We postulated that children possessing the high responding 4G/4G genotype would produce higher levels of PAI-1, develop more severe coagulopathy, and be at greater risk of death during meningococcal sepsis **(Chapter 3.4)**. The relationship between outcome, PAI-1 levels and genotype were investigated in a Rotterdam cohort of 37 children with sepsis and a London cohort of 138 children with sepsis or meningitis. PAI-1 levels were measured by ELISA and the 4G/5G PAI-1 polymorphism detected by PCR and hybridization. Plasma levels on admission correlated with presentation (sepsis or meningitis) and outcome. The median PAI-1 level in fatal cases was 6.6 fold higher than in survivors. Patients possessing the 4G/4G genotype had significantly higher PAI-1 levels than those with the 4G/5G or 5G/5G genotype and had a 2 fold increased risk of death for the 2 cohorts combined. This re-emphasizes the important role of fibrinolysis in the pathophysiology of meningococcal sepsis.

It is becoming more and more obvious that the role of genetic factors determining susceptibility and severity of disease are of crucial importance. In cardiovascular disease genetically determined polymorphisms in the genes for factor V, factor VII, homocysteinemia, and PAI-1 play an equally important role as conventional risk factors like smoking and hypertension. The genetic constitution probably plays an equally important role in the pathogenesis of infectious disease as environmental and microbial factors. At least 2 clinically relevant polymorphisms have been reported in meningococcal sepsis: TNF-α polymorphism (an association between a polymorphic variation in the TNF-α gene promotor region, leading to a higher TNF-α production, and death) and PAI-1 polymorphism. These two are strongly related to outcome, but are probably not the only determinants for outcome. A better understanding of the influence of genetic factors will enable us to explain why in one individual a bacteremia leads only to a non-fatal meningitis and in another to a possibly fatal sepsis, and to explain the variability in severity of disease between individuals.

3.6 other therapeutic options

Since the inhibition of fibrinolysis is a hallmark of meningococcal disease, it would be

logical from a therapeutical point of view to consider administration of endogenous plasminogen activators like tissue type plasminogen activator (t-PA). Recently, a number of case reports on this issue have been published. However, the development of cerebral bleeding is a considerable risk in patients in whom t-PA is administered. The dose of this drug needs to be titrated individually since the ratio of PAI-1/t-PA is high on admission, but in many cases already normalized at 24 hours. Another recently developed drug is an endogenous regulator of the coagulation called tissue factor pathway inhibitor, which is a regulator of the extrinsic pathway. Not much is known about the natural course of this protein during meningococcal sepsis. Therefore serum levels of this protein should be studied in patients before therapeutic use may be considered.
Prostacyclin has been used in individual patients to inhibit platelet aggregation and produce peripheral vasodilatation. However, it has not been tested in prospective randomized clinical trials, nor has the importance of the platelet (adherence, aggregation, platelet activating factor, serotonin etc) been evaluated in this disease.

4. EXPERIMENTAL AND CLINICAL ASPECTS OF CIRCULATORY FAILURE

4.1 capillary leakage

The enormous capillary leakage early in meningococcal disease is an important pathophysiological and clinical finding in patients with meningococcal sepsis. This feature, caused by increased vascular permeability and necessitating high amounts of volume resuscitation, is related to the extent of activation of the complement system. To assess the relation between capillary leakage and inflammatory mediators, blood samples were taken on admission, and 24 and 72 hours after admission in 52 children with meningococcal sepsis. Parameters related to cytokine production, neutrophil degranulation, complement activation and complement regulation were determined. The degree of capillary leakage was derived from the amount of plasma infused **(Chapter 4.1)**. Levels of IL-6, IL-8, C3b/c, C3-CRP complexes and C4bp on admission adjusted for the duration of skin lesions, were significantly different between survivors and non-survivors. A substantial part of the complement activation was through the classical route, probably by CRP. Mortality was independently related to levels of C3b/c and C3-CRP complexes. In agreement herewith, levels of complement activation products correlated well with the PRISM score or capillary leakage. Therefore, excessive complement activation is associated with a poor outcome and a more severe disease state. Further studies should reveal whether complement activation may be a target for therapeutical intervention, e.g. with C1-inhibitor.
Since the capillary leakage is such an important clinical feature, it would be of great value to possess a tool useful in the clinical situation to assess vascular permeability. Indirect

quantification is obtained from the amount of plasma infused, total body water measurements like bioimpedance, or the measurement of extravascular lungwater. Efforts should be directed into either improvement of these techniques or development of new ones like micro-dialysis or infrared spectroscopy.

4.2 circulatory failure

Not all hemodynamic disturbances can be easily studied in humans. Because of our observation that major differences exist in the outcome of young (< 3 years) and older patients, we decided to develop an experimental model for meningococcal sepsis in young (8 kg) and older (40 kg) pigs. We studied **(Chapter 4.2)** the possible age related differences in global hemodynamic and regional blood flow responses to different dosages (1 and 10 µg/kg body weight) of crude meningococcal endotoxin in younger and older piglets. Animals were chronically instrumented and studied in the awake state after a period (5-7 d) of adaptation. The response to plasma infusion (30 ml/kg in 30 minutes) was evaluated after infusion of placebo and endotoxin. The clinical picture was similar in all groups and the mortality was 0/8 in young, low dose group; 3/8 in young, high dose; 1/8 in old, low dose; and 4/9 in old, high dose. Major findings were that cardiac index (CI) decreased in the young animals after endotoxin infusion, while it was well preserved in the older animals. This difference was probably related to a different vascular response: in the older animals the systemic vascular resistance (SVRI) decreased 20%, while in the younger ones there was no change in resistance. Conductance to the kidneys, intestines and spleen decreased significantly more in the younger animals, while the increase in conductance and flow to the liver was higher in the older animals. Subsequent volume loading resulted only partly in a recovery of the hemodynamic parameters, but failed to improve oxygen delivery. Our study shows that this "pediatric" experimental meningococcal sepsis model in piglets, a model consisting of a chronically instrumented piglet of 8 kg, can be used to study the pathophysiology and the effects of vaccines and anti-inflammatory agents against meningococcal sepsis. Administration of a crude meningococcal endotoxin preparation was potent in a low dosage and could be used to analyze the effects of endotoxin. However, it is possible, that many of the circulatory changes may be contributed to the rapid increase in pulmonary vascular resistance (PVRI), which is less extreme in humans. This is likely to be caused by the fact that the endotoxin is infused in one hour and reaches levels, which in the human situation are only approached after several hours. Moreover, this study had an observation period of about 6 hours, which means that the prolonged or delayed effects of host response to endotoxin like coagulation disturbances, could have been overlooked. This problem might be overcome by gradually infusing the endotoxin over time starting at a very low dose and extending the observation period to 24

h. A new study with this design is under preparation.
Clinical assessment of the hemodynamic situation of the patient is usually done using clinical parameters as well as simple to measure hemodynamic variables like heart rate, arterial and central venous blood pressure. Our hemodynamic study in pigs showed that the central venous pressure is of limited value. It is not routine clinical practice in pediatric patients to insert pulmonary artery catheters and use parameters like cardiac output and vascular resistance, despite the use of various inotropic drugs in high dosages. To be more adequately informed about the hemodynamic condition of the patient and to tune the medication, we should either encourage the use of a pulmonary artery catheter, or improve less invasive techniques like echo doppler cardiography, or thermo-dilution with a thermistor at the arterial site instead of the pulmonary site.

4.3 prognostic score

It is often difficult on arrival to assess the clinical condition immediately of a child with meningococcal sepsis. We evaluated a group of 75 patients for their clinical and laboratory parameters and analyzed these parameters statistically to produce a mortality prediction score consisting of simple laboratory parameters, this study is described in **Chapter 4.3.** Logistic regression analysis identified four laboratory parameters, which were independently associated with mortality: serum potassium, CRP, platelet number and base deficit. Predictive value for death and survival was 71 % and 90 %, respectively. The outcome was predicted correctly in 86 % of the patients, which is higher than rates previously predicted for scoring systems. Our score was developed on the basis of a retrospective analysis and is currently validated in a prospective way together with the group of St Mary's Hospital in London.

5. ENDOCRINOLOGICAL ASPECTS

From a therapeutical point of view it still is not clear whether a patient with a meningococcal sepsis is optimally stressed or is nearly exhausted. Especially with respect to the adrenal function and the post-mortem findings of adrenal bleeding (described by Waterhouse and Friederichsen). The few studies evaluating the adrenal function showed conflicting results. More efforts should be directed to provide a better understanding of the adrenal function of the individual patient. When the patient has survived the first 48 hours, the mortality depends not any longer on the original severity of the disease, but more on the supportive treatment or possible complications. In this respect it is important, that adequate attention is given to the provision of optimal nutritional support.

6. LONG TERM OUTCOME

There are only limited data on the long-term follow up of pediatric patients after meningitis or sepsis. Especially in meningococcal sepsis it is generally assumed that when a patient survives the acute phase, there is good chance for complete recovery, besides the necrotic sequella. However, this has never been evaluated in an adequate number of patients. Studies aiming at the psycho-social and medical long term outcome of children after a meningococcal sepsis should therefore, be seriously considered.

SAMENVATTING EN TOEKOMST

1. Het probleem sepsis op de kinderleeftijd

De fysiologische veranderingen welke plaatsvinden tijdens het doormaken van een sepsis worden geïnduceerd door micro-organismen op het moment dat deze zich in de bloedbaan bevinden, of door de toxische producten van deze pathogenen die vrijkomen vanuit plaatsen met een focale infectie. Dit sepsis proces omvat veranderingen ingezet door het immuunsysteem, waarbij diverse hormonen, cytokines en enzymen betrokken zijn. Vele gast- en microbiële factoren kunnen deze complexe afweerreactie ongunstig beïnvloeden. Er zijn diverse verschillen tussen de gastheerrespons van een jong kind en die van een volwassene met een (meningococcen) sepsis). Een van de belangrijke factoren verantwoordelijk voor deze verschillen is de onrijpheid van het afweersysteem bij jonge kinderen. Een aantal van deze leeftijdsspecifieke factoren wordt besproken in **Hoofdstuk 1.2.**

De ernst van meningococcenziekte kan variëren van een meningitis met bacteriëmie, via sepsis, sepsis syndroom tot een septische shock. Een fulminante meningococcensepsis, of zoals het formeel genoemd wordt een septische shock met purpura, is een zich zeer snel ontwikkelend klinisch beeld gekarakteriseerd door een sepsis (tachycardie, tachypneu, koorts), shock (hypotensie, of aanwijzingen van orgaan falen) en een spectrum van stollingsstoornissen (petechiae, purpura en ecchymosen).

Het wordt vooral (> 80%) veroorzaakt door de *N. Meningitidis* (groep A, B of C). In Nederland is de incidentie van ernstige meningococcenziekte (SMD) ca 3.5 per 100.000 inwoners. Ongeveer 60 personen overlijden jaarlijks aan een SMD, waarvan zeker twee/ derde kinderen onder de 15 jaar zijn. De toenemende interesse in meningococcen-infecties heeft gedurende het laatste decennium geresulteerd in een groot aantal onderzoeksprojecten op dit gebied . De toepassing van moleculaire en immunologische methoden bij dier-experimentele en humane studies van meningococcensepsis heeft geleid tot een beter begrip van deze ziekte. Recente vorderingen op het gebied van de pathofysiologie van meningococcensepsis bij kinderen worden besproken in **Hoofdstuk 1.3.**

2. Endotoxine en ontstekings-mediatoren

2.1 Endotoxine klaring

Karakteristieke kenmerken van meningococcen sepsis zijn de bijzondere aard van het endotoxine en de zeer hoge concentraties ervan in plasma gedurende de acute fase van de ziekte. Het is nog niet volledig begrepen hoe endotoxine verwijderd wordt uit de circulatie. Bepaalde specifieke en niet-specifieke endotoxine bindende eiwitten zijn betrokken bij deze klaring, zoals besproken in **Hoofdstuk 1.3.** Lipopolysaccharide binding

protein (LBP) is een specifiek endotoxine bindend eiwit, welke zich gedraagt als een acuut fase eiwit. LBP vormt een complex met lipopolysaccharide (LPS). Vervolgens wordt dit complex gebonden aan de CD14-receptor van imuuncellen zoals monocyten. Deze cellen worden dan geactiveerd en gaan mediatoren synthetiseren waaronder TNFα en IL-1ß. Binding van LPS aan LBP leidt op die manier tot een versterking van het biologische effect van LPS.

Tevens worden gedurende sepsis neutrofiele granulocyten afgebroken, waarvan de inhoud vervolgens vrijkomt. Een van de uit de granulae vrijkomende eiwitten is het bactericidal permeability increasing protein (BPI). BPI is ook in staat om LPS te binden, maar in tegenstelling tot LBP leidt binding met BPI tot neutralisatie van de biologische effecten van LPS. Een recombinante vorm van dit BPI is onderzocht in een fase 1 studie bij kinderen met een meningococcen sepsis en recent is een fase 3 studie gestart. Het natuurlijke beloop van deze plasma eiwitten bij kinderen met sepsis is nog niet goed vastgelegd.

Wij bestudeerden in een experimentele meningococcen sepsis bij jonge (3 weken) en oude (4 maanden) biggen de mogelijke leeftijds gerelateerde verschillen in endotoxine concentraties alsmede de cytokine profielen na een gestandaardiseerde test met een lage en hoge dosering van ruw meningococcen endotoxine. Deze studie is beschreven in **Hoofdstuk 2.1.** Wij onderzochten de endotoxine concentraties, alsmede de balans tussen de pro- en anti-inflammatoire cytokines (TNFα , IL-6 en IL-10 concentraties) in de 4 uren volgend op een 1 uurs infusie van endotoxine. Het klinisch beeld en de mortaliteit waren overeenkomstig in alle groepen. Bij de jongere dieren was de mediaan van de maximale endotoxine concentratie 4.4 x hoger dan die van de oudere dieren, terwijl de endotoxine langzamer geklaard werd dan bij de oudere biggen. Ondanks hogere endotoxine concentraties in serum bij de jonge biggen, waren er vergelijkbare of zelfs enigszins hogere concentraties van TNFα , IL-6 en IL-10 in serum bij de oudere biggen. Leeftijds gerelateerde verschillen spelen klaarblijkelijk een rol in de endotoxine binding en klaring. High density lipoproteines (HDL) en andere lipoproteines zijn niet-specifieke endogene LPS-bindende eiwitten. Binding van LPS aan deze eiwitten leidt tot neutralisatie, althans bij een normale leverfunctie. Bij volwassenen met sepsis is aangetoond dat de plasma concentraties van deze eiwitten in de acute fase veel lager zijn dan bij controle personen. Bij kinderen met meningococcen sepsis is het verloop van deze plasma lipoproteine concentraties nog niet bestudeerd, maar zal mogelijk een vergelijkbaar beeld vertonen als bij volwassenen: een relatief tekort in de acute fase. Daarom hebben lipoproteines en HDL een therapeutische betekenis. Als gevolg daarvan is er recent een fase 2 studie gestart met een humaan geconcentreerd HDL preparaat bij kinderen met een meningococcen sepsis.

2.2 Cytokines

Eenmaal geactiveerd, zullen monocyten, macrofagen en endotheelcellen cytokines gaan produceren, die een zeer belangrijke rol spelen in de immuunrespons zoals reeds besproken in **Hoofdstuk 1.2 en 1.3.** In een beperkt aantal patiënten (n=17) met sepsis of septische shock met purpura, analyseerden wij de plasma oncentraties van deze cytokines (TNFα , IL-1ß en IL-6) en correleerden deze waarden met de resultaten van routinematig bepaalde laboratorium parameters. Deze studie is beschreven in **Hoofdstuk 2.2.** Alle cytokine concentraties waren duidelijk verhoogd, maar alleen de IL-6 concentraties hadden een goede predictieve waarde voor de ernst van de ziekte. IL-6 was eerder al aangetoond een betrouwbare indicator voor de ernst van de ziekte en mortaliteit te zijn bij pediatrische en volwassen patiënten met sepsis. Van de laboratorium parameters (fibrinogeen, C-reactive protein en lactaat) was alleen het arteriële lactaat positief gecorreleerd aan mortaliteit en ernst van de ziekte.
In een vervolg studie, beschreven in **Hoofdstuk 3.3,** bepaalden wij de concentraties van TNFα , IL-6, IL-8, IL-10 en de oplosbare TNF receptoren p55 en p75 in 38 patiënten met septische shock en purpura. Alle cytokine concentraties waren hoger in de niet-overlevers. Echter wanneer de concentraties gecorrigeerd werden voor de tijd tussen het moment waarop de petechiae voor het eerst ondekt werden en de tijd van bloedafname, waren de verschillen verdwenen. Een andere interessante bevinding was dat zowel de concentraties van de pro-inflammatoire (TNFα , IL-6, IL-8) als die van de anti-inflammatoire cytokines (IL-10 en s-TNF-R) significant verhoogd waren in de niet-overlevers. Het is niet geheel duidelijk of er hier nu sprake is van een overproductie van pro-inflammatoire cytokines, dan wel van een te lage productie van anti-inflammatoire cytokines. Mogelijk dat opeenvolgende analyses van de immuun staus gedurende de ziekte, b.v door volbloed kweek systemen, waarbij gekeken wordt naar cytokine profielen en leukocyten activatie markers na een standaard stimulus, een antwoord op deze vraag kunnen verschaffen.
In een groep van 48 patiënten, werden plasma concentraties van IL-12 en interferonγ (IFNγ) bepaald. IL-12, een recentelijk voor het eerst beschreven cytokine bestaande uit 2 verschillende polypeptide subunits (p40 en p35), speelt een rol in de gastheeerrespons tegen microbiële infecties en waarvan is aangetoond dat het de produktie van IFNγ induceert. Plasma concentraties van IFNγ zijn verhoogd in zowel experimentele als klinische sepsis. Wij veronderstelden dat IL-12 en IFNγ een rol zouden spelen in de pathogenese van septische shock en purpura **(Hoofdstuk 2.3).** De mediane plasma IL-12 concentratie bij opname was gerelateerd aan mortaliteit, ernst van de ziekte en de concentraties van andere cytokines, zelfs wanneer deze gecorrigeerd waren voor de duur van de petechiae, terwijl de IL-12 p70 (het complete cytokine) concentraties slechts verhoogd waren in 9 patiënten (19%). Daarentegen hadden patiënten met aantoonbare IL-12 p70 concentraties hogere IL-12 p40 plasma waardes dan die zonder aantoonbare IL-12

p70 concentraties. Twaalf patiënten (29%) hadden aantoonbare concentraties IFNγ , zonder dat er een relatie met mortaliteit kon worden vastgesteld. Bij deze 12 patiënten waren de andere cytokine concentraties, waaronder IL-12 p70, ook verhoogd. Echter dit was niet het geval voor IL-12 p40. Bij onze patiënten kon een positieve correlatie aangetoond worden tussen IL-10 en IL-12. Dit was in tegenstelling met de bevindingen bij de sepsis in bavianen, waar een negatieve correlatie gevonden werd. In vitro is IL-10 een sterke inhibitor van de LPS-afhankelijke IL-12 productie. Wij veronderstellen dat de synthese van pro- en anti-inflammatoire cytokines zo sterk en continu gestimuleerd is in patiënten met een meningococcensepsis, dat de anti-inflammatoire mechanismen tekort schieten om deze overproductie te compenseren.

3 Stollingsstoornissen

3.1 Verband tussen stolling en ontsteking

Ontsteking en stolling kunnen niet als 2 aparte processen gezien worden daar er verschillende schakels zijn die hen gezamenlijk onderdeel maken van de gastheerrespons niet alleen tijdens een ernstige infectie, en ook na trauma, hypoxie en hypovolemie. Het endotheel kan beschouwd worden als een contactplaats tussen ontsteking en stolling: het is een oppervlak waar stolling geactiveerd wordt, maar na cytokine stimulatie ontstaat er een hechtingsplaats voor ontstekingscellen. Gedurende ontsteking wordt de stolling geinitieerd via activering van factor XII en weefselfactor door TNFα , IL-1ß en IL-6. Activering van factor XII leidt tot de productie van bradykinine, kallikreine, factor XIa en plasmine, welke allen onderdeel zijn van het contactsysteem. Deze factoren zijn gerelateerd aan het complementsysteem door een gemeenschappelijk regeleiwit: C1-esterase inhibitor. De vorming van trombine als gevolg van de activering van stollingscascades is een essentieel onderdeel van de hemostase. Trombine stimuleert de vorming van fibrine en leidt tot plaatjes-aggregatie en degranulatie waardoor een aantal vasoactieve mediatoren vrijkomen. Bovendien beïnvloedt trombine een aantal endotheelfuncties en stimuleert cellen betrokken bij de ontstekingsreactie. Trombomoduline is een endotheelreceptor voor trombine. Het trombine-trombomoduline complex activeert proteine C, een belangrijke inhibitor van de stollingscascade. Echter, TNFα downreguleert de expressie van trombomoduline en beïnvloedt op die manier proteïne C. Geactiveerd proteïne C complexeert met een co-factor proteïne S. Proteïne S is in plasma gebonden aan C4 binding proteïne (C4bp), wat een regulatie eiwit is van het complement systeem. Dit is weer een voorbeeld van de interacties die er bestaan tussen stolling en het complement systeem. Daarom is het noodzakelijk dat in de studie van de gastheerrespons gedurende een ernstige infectie, het stollingssysteem mede betrokken wordt.

3.2 Proteïne C

Proteïne C inactiveert de factoren Va en VIIIa, en stimuleert op hetzelfde moment de fibrinolyse, door plasminogeen activator inhibitor 1 (PAI-1) te inactiveren. PAI-1 is een acuut fase eiwit waarvan de productie gestimuleerd wordt door cytokines zoals TNFα . Sedert de eerste vermelding in de literatuur in 1991 van de sterk verlaagde plasma concentraties van proteïne C, zijn wij geïnteresseerd geraakt in deze materie. In een studie gezamenlijk met het Universiteitsziekenhuis van Lille in Frankrijk, analyseerden wij de proteïne C en S concentraties van 40 kinderen met septische shock en purpura **(Hoofdstuk 3.1).** Proteïne C niveau's (zowel antigeen als activiteit) waren sterk verminderd en het niveau was gerelateerd aan de aanwezigheid van diffuse intravasale stolling (DIC) en mortaliteit. Om die reden werd als conclusie getrokken dat proteïne C suppletie overwogen zou moeten worden bij kinderen met septische shock en purpura.

3.3 Relatie met leeftijd

Er zijn meerdere studies geweest die stollingsparameters gerapporteerd hebben van patiënten met een meningococcensepsis. De meeste van deze studies omvatten kleine aantallen patiënten, met een heterogene populatie (milde en ernstige gevallen), verschillende leeftijdsgroepen, of analyseerden een beperkt aantal parameters. Wij onderzochten prospectief bij een groot aantal (79) kinderen met septische shock en purpura diverse parameters van stolling en fibrinolyse, bij opname, en na 24 uur en 72 uur. **(Hoofdstuk 3.2).** De resultaten vertoonden een beeld overeenkomend met een sterk geactiveerd stollingssysteem, een inhibitie van natuurlijke anticoagulanten (proteïne C, antitrombine), een tekort aan stollingsfactoren, en sterke inhibitie van het fibrinolytisch systeem vooral door de aanwezigheid in serum van zeer hoge concentraties PAI-1. Na stratificatie van de patiënten naar de mediane leeftijd (3.1 jaar), werd het duidelijk dat de ernst van de stollingsafwijkingen (aanwezigheid van DIC, tekort van stollingsfactoren en inhibitors) ernstiger was in de jonge leeftijdsgroep. Echter, de verschillen tussen de 2 leeftijdsgroepen met betrekking tot cytokines waren niet significant. Daarom veronderstelden wij dat deze leeftijd gerelateerde verschillen in coagulopathie ten dele verantwoordelijk zijn voor het verschil in mortaliteit tussen jongere en oudere patiënten.

3.4 Relatie PAI, cytokines en proteïne C

Omdat de PAI-1 concentraties zo duidelijk verhoogd waren en PAI-1 zich gedraagt als een acuut fase eiwit, raakten wij geïnteresseerd in de relatie tussen PAI-1 en cytokines als

TNFα **(Hoofdstuk 3.3).** Er bleek een significante relatie te bestaan tussen PAI-1 en TNFα. Tot onze verassing constateerden wij dat deze relatie verschillend was voor de overlevers en de niet-overlevers. Bij een zelfde concentratie aan TNFα was de concentratie van PAI-1,1.9x hoger in de niet-overlevers. Het zelfde was het geval voor de relatie met de andere cytokines. Dit betekent dat de individuele PAI-1 productie direct gerelateerd is aan mortaliteit. Bij deze studie is het PAI-1 antigeen bepaald en niet de activiteit. Bij nieuwe studies moet aandacht gericht worden op het bepalen van de activiteit daar dit niet perse hetzelfde hoeft te zijn. Een van de mogelijke verklaringen van de extreem verlaagde concentraties proteïne C gerapporteerd in **Hoofdstuk 3.1 en 3.2**, is het feit dat geactiveerd proteïne C een complex vormt met PAI-1 en op die manier verbruikt wordt. Aangezien proteïne C belangrijk is in de regulatie van stolling en fibrinolyse, is het van belang de therapeutische waarde te analyseren van het herstel van deze extreem lage concentraties tot normale of zelfs supra-normale waarden. In maart 1997 is er een fase 2 dosis evaluerende studie gestart met geconcentreerd proteïne C. Doel van deze studie, die gecoördineerd wordt door de afdeling Kindergeneeskunde van het Sophia Kinderziekenhuis, is een zinvolle dosering te vinden en het verloop te bestuderen van PAI-1/proteïne C complexen, het geactiveerde proteïne C en de recent ontdekte oplosbare proteïne C receptoren.

3.5 PAI-1 polymorfisme

De significant verhoogde concentraties PAI-1 m.n in de niet-overlevers, brachten de vraag op of er mogelijk een polymorfe immuunrespons betrokken zou kunnen zijn bij de mortaliteit van deze patiënten. Bij het bestuderen van de literatuur, vonden wij inderdaad een beschrijving van een functioneel polymorfisme in het PAI-1 gen. Een enkele base paar insertie (5G) / deletie (4G) polymorfisme 675 base paar stroomopwaarts van de start van de transcriptie is functioneel van betekenis in de regulatie van de expressie van het PAI-1 gen. Personen homozygoot voor het 4G allel hebben een hogere basale en te induceren PAI-1 concentratie dan personen met 1 of 2 kopieën van het 5 G allel. Een verminderde fibrinolytische capaciteit door een verhoogde plasma concentratie van PAI-1 kan van pathogenetisch belang zijn bij myocard infarct, m.n bij jonge patiënten met hypertriglyceridemie of met diabetes. Wij veronderstelden dat kinderen met het hoog responderende 4G/4G genotype meer PAI-1 zouden produceren, een ernstiger coagulopathie zouden ontwikkelen, en een groter risico op een slechte afloop zouden hebben bij het doormaken van een meningococcensepsis **(Hoofdstuk 3.4).** De relatie tussen mortaliteit, PAI-1 concentraties en genotype werd onderzocht in een cohort van 37 Rotterdamse kinderen met sepsis en een cohort van 138 Londense kinderen met sepsis of meningitis. PAI-1 concentraties werden bepaald met een ELISA methode en het 4G/5G

PAI-1 polymorfisme met een polymerase ketting reactie (PCR) en hybridisatie. Plasma concentraties bij opname correleerden met het klinisch beeld (sepsis of meningitis) en mortaliteit. De mediane PAI-1 concentratie in de fatale gevallen was 6.6x hoger dan bij de overlevers. Patiënten die in het bezit waren van het 4G/4G genotype hadden significant hogere PAI-1 concentraties dan die met het 4G/5G of 5G/5G genotype en hadden een 2x zo grote kans om te overlijden. Dit benadrukt opnieuw het belang van fibrinolyse in de pathofysiologie van meningococcensepsis.
Het wordt meer en meer duidelijk dat genetische factoren een belangrijke rol spelen in het bepalen van de gevoeligheid voor en ernst van ziekten. In hart- en vaatziekten spelen genetisch bepaalde polymorfismen in b.v. het gen voor factor V, factor VII, homocysteinemie en PAI-1 een even belangrijke rol als klassieke risico factoren als roken en hypertensie. Voor de pathogenese van een infectieziekte is de genetische constitutie zeker zo belangrijk als de omgeving- of microbiële factoren. Zeker 2 relevante polymorfismen zijn nu gerapporteerd in meningococcensepsis: TNFα polymorfisme (een associatie tussen een polymorfe variant in het TNFα promotor gen, welke tot een hogere TNFα productie en hogere mortaliteit aanleiding geeft) en het PAI-1 polymorfisme. Deze 2 zijn sterk gerelateerd aan mortaliteit, maar zijn waarschijnlijk niet de enige bepalende factoren. Een beter begrip van de invloed van genetische factoren zal het ons mogelijk maken te verklaren waarom bij het ene individu een bacteriëmie aanleiding geeft tot een niet fatale meningitis en in een ander geval er een mogelijk fatale sepsis ontstaat, en waarom er zo'n variatie in ernst van de ziekte bestaat tussen de verschillende individuen.

3.6 Andere therapeutische opties

Daar de remming van de fibrinolyse zo'n belangrijk kenmerk is van de meningococcensepsis, zou het vanuit therapeutisch perspectief logisch zijn toediening van endogene plasminogeen activatoren zoals weefsel type plasminogeen activator (t-PA) te overwegen. Recentelijk zijn er een aantal case reporten m.b.t. deze therapie gepubliceerd. Echter, de mogelijkheid van hersenbloedingen vormt een belangrijk risico bij patiënten aan wie t-PA wordt toegediend. De dosering van dit medicijn moet individueel getitreerd worden daar de verhouding PAI-1/t-PA hoog is bij opname, maar in vele gevallen genormaliseerd is na 24 uur. Een ander recent ontwikkeld medicijn is ook een endogene regulator van de stollingscascade, nl de weefsel factor pathway inhibitor. Niet veel is er bekend over dit eiwit tijdens meningococcensepsis. Daarom dienen de plasma concentraties bij patiënten eerst onderzocht te worden alvorens het therapeutisch gebruik overwogen kan worden.
Prostacycline wordt gebruikt in individuele patiënten om de plaatjes aggregatie te verminderen en perifere vasodilatatie te produceren. Echter, prostacycline is nog niet in prospectieve gerandomiseerde klinische trials onderzocht, noch is het belang van de

bloedplaatjes op zich (adherentie, aggregatie, platelet activating factor, serotonine) geëvalueerd bij deze ziekte.

4 Experimentele en klinische aspecten van circulatoir falen

4.1 Capillair lek

Het enorme capillair lek vroeg in de ziekte is een belangrijk pathofysiologisch en klinische bevinding bij patiënten met een meningococcensepsis. Dit verschijnsel veroorzaakt door een verhoogde permeabiliteit van de vaatwand en welke grote hoeveelheden volume resuscitatie noodzaakt, is gerelateerd aan activatie van het complement systeem. Om de relatie tussen capillair lek en ontstekings-mediatoren te analyseren, werd bloed afgenomen bij opname, en 24 en 72 uur na opname bij 52 kinderen met meningococcen sepsis. Parameters gerelateerd aan cytokine productie, granulocyten degranulatie, complement activatie en complement regulatie werden bepaald. De mate van capillair lek werd bepaald aan de hand van de hoeveelheid geïnfundeerd plasma **(Hoofdstuk 4.1).** Plasma concentraties van IL-6, IL-8, C3b/c, C3-CRP complexen en C4bp bij opname gecorrigeerd voor de duur van de huidlesies, waren significant verschillend tussen overlevers en niet-overlevers. Een belangrijk deel van de complement activatie was ontstaan door de klassieke route, vermoedelijk door CRP. De mortaliteit was onafhankelijk gerelateerd aan de concentraties van C3b/c en C3-CRP complexen. De concentraties van de complement activatie producten correleerden uitstekend met de PRISM score en met de capillaire lek. Dus excessieve complement activatie is geassocieerd met een hoge mortaliteit en een meer ernstige ziekte. Aanvullende studie moet aantonen of complement activatie een doel moet zijn voor therapeutische benadering, b.v met C1-esterase inhibitor. Daar het capillaire lek zo'n belangrijk klinisch verschijnsel is, zou het van groot belang zijn in de klinische situatie te kunnen beschikken over een meetinstrument om de vaat permeabiliteit te kunnen kwantificeren. Indirecte kwantificering wordt verkregen vanuit de hoeveelheid geïnfundeerd plasma, totaal lichaamswater metingen, of de meting van extravasculair longwater. Richting zal er gegeven moeten worden aan het onderzoek om deze technieken te verbeteren, dan wel nieuwe te ontwikkelen zoals micro-dialyse dan wel infrarood spectroscopie.

4.2 Circulatoir falen

Niet alle hemodynamische verstoringen kunnen makkelijk bestudeerd worden bij de mens. Mede omdat wij observeerden dat de mortaliteit van jonge (< 3 jaar) en oudere kinderen zoveel van elkaar verschilden, hebben wij daarom besloten een experimenteel

meningococcen sepsis model in jonge (3 weken) en oude (4 maanden) op te gaan zetten. In dit model bestudeerden wij **(Hoofdstuk 4.2)** de mogelijke leeftijd gerelateerde verschillen in totale hemodynamische en regionale bloed flow veranderingen na verschillende doseringen van ruw meningococcen endotoxine in jonge en oude biggen. De dieren waren chronisch geïnstrumenteerd en werden bestudeerd in wakkere toestand na een periode van ca 5-7 dagen adaptatie. De respons op plasma infusie (30 ml/kg in 30 minuten) werd geëvalueerd zowel na een placebo als na de endotoxine infusie. Het klinisch beeld was overeenkomstig in alle groepen en de mortaliteit was 0/8 in de jonge, lage dosis groep; 3/8 in de jonge, hoge dosering groep; 1/8 in de oude, lage dosering groep; en 4/9 in de oude, hoge dosering groep. De belangrijkste bevindingen waren dat de cardiac index (CI) verminderde in de jonge dieren na de endotoxine infusie, terwijl deze redelijk stabiel bleef in de oudere dieren. Dit verschil was waarschijnlijk gerelateerd aan een verschillende vasculaire respons: in de oudere dieren daalde de systemische vaatweerstand (SVRI) met ca 20 %, terwijl in de jongere dieren er geen verandering in weerstand was. Conductantie naar de nieren, darmen en milt nam duidelijk meer af bij de jongere dieren terwijl de toename in conductantie en flow naar de lever duidelijk meer was bij de oudere dieren. De na de endotoxine volgende volume belasting resulteerde maar ten dele in een verbetering van de hemodynamische parameters, maar had geen toename van het O_2-transport naar de weefsels ten gevolg. Onze studie toont aan dat dit "pediatrisch" experimenteel meningococcen sepsis model in biggen, een model bestaande uit een chronisch geïnstrumenteerde big van 8 kg, gebruikt kan worden om de pathofysiologie te bestuderen, alsmede de effecten van vaccins en anti-inflammatoire therapie te evalueren. Toediening van een ruw meningococcen endotoxine preparaat was potent in een lage dosering en hiermee kan het effect van endotoxine bestudeerd worden. Echter, het is mogelijk dat veel van de circulatoire veranderingen toegerekend moeten worden aan de snelle stijging in pulmonale vaatweerstand (PVRI) die bij de mens minder extreem is. Mogelijk heeft dit te maken met het feit dat de endotoxine geïnfundeerd wordt in 1 uur en dan niveau's bereikt die bij de mens pas na uren bereikt zal worden. Tevens was er bij deze studie sprake van een observatie periode van "slechts" 6 uur, wat betekent dat late effecten van respons op endotoxine zoals stollingsstoornissen gemist kunnen worden. Dit probleem kan verholpen worden door het endotoxine langzamer te infunderen, met een zeer lage start dosis en de observatie periode uit te breiden tot 24 uur. Een nieuwe studie met deze opzet is in voorbereiding.

Klinische evaluatie van de hemodynamische conditie van de patiënt vindt meestal plaats gebruikmakend van klinische parameters en eenvoudig te meten hemodynamische variabelen zoals hartfrequentie, arteriële en centraal veneuze drukken. Onze hemodynamische studie in biggen toonde aan dat de centraal veneuze druk van beperkte waarde is. Het is geen klinische routine in pediatrische patiënten pulmonaal katheters te

plaatsen en parameters als cardiac output en vaatweerstanden te gebruiken. Om adequater geïnformeerd te zijn omtrent de hemodynamische conditie van de patiënt en de medicatie beter af te stellen, moeten we of het gebruik van de pulmonaal katheter stimuleren, of minder invasieve technieken verbeteren als echo Doppler cardiografie, of thermodilutie met een thermistor op een arteriële locatie i.p.v. pulmonaal.

4.3 Prognostische score

Het is vaak moeilijk om onmiddellijk bij opname de klinische conditie en mogelijke uitkomst van een kind met een meningococcen sepsis in te schatten. Met die achtergrond evalueerden wij van een groep van 75 patiënten de klinische en laboratorium parameters en analyseerden wij deze parameters statistisch om een mortaliteitspredictie score te bepalen bestaande uit simpele laboratorium parameters, deze studie wordt beschreven in **Hoofdstuk 4.3.** Logistische regressie analyse identificeerde 4 laboratorium parameters, welke onafhankelijk geassocieerd bleken te zijn met mortaliteit: serum kalium, CRP, aantal plaatjes en base deficit. De predictieve waarde voor dood en overleving was 71 % en 90 % respectievelijk. De uitkomst werd correct voorspeld in 86 % van de patiënten, wat hoger is dan de uitkomsten van een aantal bestaande scoring systemen. Onze score werd bepaald op basis van een retrospectieve studie en wordt op dit moment gevalideerd in een prospectieve, landelijke registratie studie en tevens met de onderzoeksgroep uit het St Mary Ziekenhuis in Londen.

5 Endocrinologische aspecten

Mede vanuit een therapeutisch oogpunt is het nog niet geheel duidelijk of een patiënt met een meningococcen sepsis optimaal gestresst is of nagenoeg uitgeput is. Met name met betrekking tot de bijnierfunctie en de post-mortem bevindingen van bijnierbloedingen (voor het eerst beschreven door Waterhouse en Friederichsen). De enkele studies die de bijnierfunctie in de acute fase evalueren tonen conflicterende resultaten. Meer aandacht moet er besteed worden aan een beter begrip van de bijnierfunctie van de individuele patiënt. Als de patiënt de eerste 48 uur overleeft heeft, hangt de mortaliteit niet meer af van de oorspronkelijke ernst van de ziekte, maar meer van de ondersteunende therapie of mogelijke complicaties. Wat dat betreft is het belangrijk dat er aandacht wordt besteed aan een adequate voeding.

6 Lange termijn uitkomst

Er zijn slechts weinig gegevens omtrent de lange termijn uitkomsten van pediatrische patiënten na een meningitis of sepsis. Met name bij de meningococcen sepsis wordt in het

algemeen aangenomen dat als een patiënt de acute fase overleeft, er een goede kans is op volledig herstel, behoudens eventuele huidlesies. Echter, dit is nooit geëvalueerd in een adequaat aantal patiënten. Studies gericht op de psycho-sociale en medische lange termijn uitkomst van kinderen na een meningococcen sepsis moeten daarom serieus overwogen worden.

CHAPTER 6

LITERATURE

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CHAPTER 8

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EDUCATION

1966-1971	Secondary School: MULO-B (Vlijmen)
1971-1976	Poly-Technical School (HTS), Dordrecht. Physical Engineering.
1978-1981	Nursing assistant, Pediatric Intensive Care ("Extra Zorg")
1976-1983	Medical School, Erasmus University, Rotterdam.
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1983-1987	Pediatric Residency, Sophia Children's Hospital, Erasmus University Rotterdam, (head Prof. Dr. H.K.A. Visser).
1987-1989	Fellowship Pediatric Intensive Care, Sophia Children's Hospital, Erasmus University, Rotterdam (head Drs. E. v.d. Voort).

POSITIONS

1989-	Staff member Pediatric Intensive Care, Sophia Children's Hospital, Rotterdam.
1989-1993	(Vice)-chairman Council of Employees, University Hospital Rotterdam.
1993-1998	Member (treasurer) of the Organizing Committee 2nd World Congress of Pediatric Intensive Care 1996.

RESEARCH ACTIVITIES

- Experimental meningococcal sepsis in piglets. Sponsored: Netherlands Heart Foundation
- Investigator HA-1A phase III study in children with meningococcal sepsis. Sponsored by Centocor
- Principal Investigator phase II study inhaled NO in neonatal and pediatric patients. Sponsored by AGA/Siemens. Intens Care Med (1997), 23: 773-9
- Principal Investigator Protein C phase II/III study in children with meningococcal sepsis. Sponsored by Immuno, Austria.

PUBLICATIONS (international)

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17 Hazelzet JA, deGroot R, van Mierlo G, et al
Complement factors in relation to capillary leakage in septic shock and purpura
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PUBLICATIONS (national)

1. Smit M.J.M, de Groot R., van Dongen J.J.M., Hazelzet J.A., Sluiters J.F., Neijens H.J. Pneumocystis carinii pneumonie bij patienten met een ernstige gecombineerde immunodeficientie. Ned. Tijdschr. Geneeskd. 1991,135:1.

2. Hazelzet J.A., Oudshoorn J.H., v.d. Voort E. Toxische-shocksyndroom bij 8 kinderen. Ned. Tijdschr. Geneeskd 1994;138:2001-2004.

CONTRIBUTIONS TO BOOKS (international)

1. Hazelzet J.A, van der Voort E, Blom W, Catsman-Berrevoets C. Hypotonia and acute respiratory insufficiency of unknown origin. In: Case book of pediatric intensive care. Eds: Rogers and Helfaer. 1993, pp. 29-33.

2. Hazelzet J.A, de Groot R. Sepsis related problems in pediatric patients. In: Sepsis, current perpectives in pathophysiology and therapy. Ed: Reinhart, Eyrich, Sprung. Springer 1994, p: 217-228.