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

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Plasma levels of interleukin 12 (IL-12), a cytokine consisting of two different polypeptide subunits (p40 and p35), were measured together with interferon γ (IFN- γ) and other cytokines in 46 children with septic shock and purpura. The median (range) plasma IL-12 p40 level on admission was 457 (244–2677) pg/ml in non-survivors vs 189 (<40–521) pg/ml in survivors ($P = <0.001$). IL-12 p70 levels were elevated in only nine patients. IL-12 p40 plasma levels were positively correlated with tumour necrosis factor α (TNF- α), IL-6, IL-8, IL-10 and PRISM-score, whereas they were negatively correlated with C-reactive protein (CRP), whole blood cell (WBC) and serum glucose levels. Twelve (29%) of the patients had detectable levels of IFN- γ . Thus, circulating levels of IL-12 p40 and to a lesser extent those of IL-12 p70, are elevated in children with septic shock and purpura, and correlate with severity of disease and outcome.

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Septic shock with purpura is a clinical syndrome predominantly caused by *Neisseria 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)-1,¹ IL-6,^{1,2} IL-8,³ and IL-10,^{4,5} are increased in

children with septic shock and purpura. Severity of disease is related to the initial plasma levels of LPS⁶ and of these cytokines.

Interleukin 12 (IL-12),^{7,8} initially called natural killer cell stimulatory factor or cytotoxic lymphocyte maturation factor, is unique in that it is a heterodimeric protein composed of two different polypeptide subunits, p40 and p35 (for a review see Refs 8–14). The precise role of IL-12 in vivo is not known, although it seems to play a key role in the differentiation of Th1 cells,^{10,15} and in the host defense against bacterial, parasitic and viral infections.⁸ IL-12 also induces the production of interferon (IFN)- γ by T cells and natural killer (NK)-cells.^{16,17} The plasma levels of IFN- γ are increased in experimental models for sepsis^{18–20} as well as in human sepsis,^{21,22} although not consistently.¹ Recently, IL-12 was characterized as a major cytokine in the pathogenesis of gram-negative endotoxaemia in mice¹⁶ and in primates.²³ We therefore questioned whether IL-12 and IFN- γ play a role in the pathogenesis of septic shock and purpura in humans. To this purpose initial plasma levels of IL-12 and IFN- γ were measured in children with this disease and their relation with outcome and severity of disease were studied. In addition, plasma levels of TNF- α , IL-6, IL-8, and IL-10 were determined and the possible correlation between these cytokines and IL-12 and IFN- γ , respectively, was studied.

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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 ($\times 10^9/l$)	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)	ns
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-survivors (Mann-Whitney U-test).

RESULTS

Patients

Forty-six patients admitted to the paediatric intensive care unit (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 *N. meningitidis* in 40 patients and *Haemophilus influenzae* in one patient. Cultures were sterile in five 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), in meningococcal septic shock. HA-1-A or placebo was 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%), seven patients died (29%). The results of only these 24 patients were used for outcome analysis.

Clinical and laboratory parameters

Clinical and laboratory parameters obtained on admission [PRISM Paediatric Risk of Mortality-score, arterial lactate, whole blood cell (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.

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 vs 189 (40–521) pg/ml in survivors ($P < 0.001$). In contrast, IL-12 p70 was elevated in only nine 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 nine patients with detectable IL-12 p70 levels was 117 (26–203) (Fig. 2).

Relation between IL-12 and other cytokines on admission

IL-12 p40 plasma levels on admission were positively correlated with tumour necrosis factor α (TNF- α), IL-6, IL-8, and IL-10 (Table 2). The association between IL-12 p70 and the other cytokines was different in comparison with that between IL-12 p40 and the other cytokines. Patients with detectable IL-12 p70 levels had significantly higher levels of IL-8 ($P = 0.042$) and IL-12 p40 ($P = 0.007$) levels than patients with undetectable levels of IL-12 p70.

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. Dashed lines indicate the range of normal values.

Figure 2. Scattergram of IL-12 p40 vs IL-12 p70.

Data represent the levels on admission. Dashed lines represent the lower detection limits.

Relation of IL-12 to clinical and laboratory parameters

A negative correlation was found between CRP levels or WBC vs 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 ($P = 0.019$).

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 nine patients with a detectable level of IL-12 p70, five had a detectable level of IFN- γ (56%), while of the 30 patients without detectable levels of IL-12 p70, only six had a detectable level of IFN- γ (20%). Due to small numbers this difference did not reach statistical significance ($P = 0.08$).

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

	IL-12 p40 ($n = 41$)	
	r^*	P 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.

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.

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 heterodimeric cytokine,⁷ which appears to play an important role as a functional bridge between natural resistance and adaptive immune response.¹² During endotoxaemia in mice, IL-12, in both p40 and p70 forms, was detected shortly after injection of LPS. Bioactive IL-12 circulated in serum before the appearance of IFN- γ . Pretreatment with anti-IL-12 antibodies blocked the production of IFN- γ ,¹⁶ thus protecting against lethality.¹⁷ Similar findings were reported in a model of a generalized Schwartzman reaction in mice.²⁴ In baboons challenged with *Escherichia coli*²³ the systemic release of IL-12 p40 and p70 was also reported. Our study confirms that circulating levels of IL-12 are also increased in human sepsis.

IL-12 p70 was detected in only nine of the patients and these levels were only slightly increased. In these nine 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 excessive production of IL-12 p40 was also found in in vitro experiments with human peripheral mononuclear^{10,25} and polymorphonuclear cells²⁶ as well as in septic baboons.²³ The physiological significance of this excessive 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.²⁷ Studies by Ling *et al.*²⁸ 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.²⁹ Nevertheless, in vitro and in vivo studies have clearly shown that the production of p40 is linked to that of IL-12,^{8,23} and hence elevated levels of p40 subunit in our patients probably reflected the production of bioactive IL-12. Consistent herewith 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 pro-inflammatory 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.^{26,30,31} Moreover, a negative correlation between IL-10 and IL-12 was found in baboons with sepsis, suggesting that IL-10 downregulates the release of IL-12 in this sepsis model.²³ Thus, the positive correlation between IL-12 and IL-10 in our patients was in contrast to the findings in baboons. We propose that the synthesis of pro- and anti-inflammatory cytokines is so strongly and continuously stimulated^{5,32} in patients with meningococcal sepsis, that counter-regulatory mechanisms are insufficient to suppress excessive production.

IL-12 can induce IFN- γ production by T and NK cells in the presence of cofactors as TNF- α or IL-1 β .⁷ The role of IFN- γ during in vitro LPS-challenge,³⁰ in vivo endotoxemia in mice,^{16–20} or the generalized Shwartzman reaction in mice,^{24,33,34} has been well established. Disseminated intravascular coagulation and shock associated with meningococcal sepsis are considered to be the clinical counterparts of the “classical” generalized Shwartzman 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 shock²² and in children with meningococcal septic shock.¹ In contrast, Girardin *et al.* reported high levels of IFN- γ in children with severe meningococcal septic shock. Their plasma concentrations of IFN- γ were related to severity of the disease and correlated with serum levels of TNF- α .²¹ In our study, only 12 patients had plasma IFN- γ levels above the detection limit. Those children also had significantly higher levels

of other cytokines. The proportion of children that had elevated IL-12 p70 levels was higher, although just not significantly, in the group with detectable IFN- γ , compared to the group with undetectable IFN- γ . A possible explanation for the absence of a relation between IFN- γ and IL-12 p40 is that these cytokines were not released simultaneously, as was observed in animal models for sepsis.²³

The clinical and laboratory parameters in this study are commonly used to assess the severity of disease in patients with meningococcal septic shock. PRISM score is a scoring system to calculate the risk of mortality in pediatric intensive care patients.³⁵ Serum lactate is related to the degree of circulatory failure.² WBC and CRP are negatively correlated with the fulminant evolution of meningococcal septic shock.^{36,37} Low serum glucose levels are reported by some authors,³⁸ 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.

MATERIALS AND METHODS

Study protocol

Children above 3 months and below 18 years of age with septic shock and petechiae/purpura were enrolled in this study. Primary or secondary referrals were admitted to the paediatric 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 h; (2) presence of shock defined as sustained hypotension (systolic blood pressure <75 mmHg for children between 3–12 months, <80 mmHg for 1–5 years, <85 mmHg for 6–12 years, <100 mmHg 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 $\leq$ 7.3 or base excess $\leq$ -5 mmol/l or plasma lactate levels >2 mmol/l); (b) arterial hypoxia (PaO₂ $\leq$ 75 mmHg, a PaO₂/FiO₂ ratio <250, or a transcutaneous SaO₂ $\leq$ 0.96) in patients without overt cardiopulmonary disease; (c) acute renal failure (diuresis <0.5 ml/kg/h for at least 1 h despite acute volume-loading or evidence of adequate intravascular volume) without preexisting renal disease; or (d) sudden deterioration of the baseline mental status. The paediatric risk of mortality (PRISM) score³⁵ was calculated using the most abnormal value of each variable recorded during the

first 4 h after admission at the PICU. All patients received maximal supportive therapy: antibiotics, volume suppletion, inotropic support, and mechanical ventilation. Informed consent was obtained from the parents or legal representatives. The Medical Ethics Committee of the University Hospital Rotterdam approved the study protocol.

Collection of blood

On admission arterial blood was collected within 2 h. Blood for cytokine analysis was collected in vials containing 3.8% trisodium citrate, immediately chilled on ice, and centrifuged at $2800 \times g$ for 15 min and then at $45\,000 \times g$ for 30 min at $+4^{\circ}\text{C}$. Plasma was stored at -70°C until tests were performed.

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.³⁹

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

Assays of IL-12

IL-12 p40 antigen was measured with an ELISA.⁴⁰ Briefly, mAbC11.79 and biotinylated mAbC8.6, both directed against the IL-12 p40 subunit,²⁵ 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 five normal children age: 43 months (36–48), the median value was: 28 pg/ml. As levels of IL-12 p40 were similar in children and adults (and healthy children of young age are difficult to obtain blood from), we used both groups together as control for the septic children.

IL-12 p70 antigen was measured using a newly developed ELISA.²³ Briefly, mAb20C2, which has relative specificity for the IL-12 p70 heterodimer,²⁵ and mAbC8.6 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 analysed at least at a ten-fold dilution. Therefore, the actual detection limit was 2.5 pg/ml. Normal values are below this detection limit.

Statistical analysis

Results are expressed as medians (range) unless otherwise specified. Differences between groups were tested with the Mann-Whitney 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.

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