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Plasma levels of interleukin-6 and interleukin-10 in preterm neonates evaluated for sepsis

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Abstract In a prospective study, plasma interleukin-6 (IL-6) and interleukin-10 (IL-10) levels were measured by enzyme-linked immunosorbent assay in 45 premature neonates (25–34 weeks gestational age) with signs and symptoms of suspected sepsis at 0, 12 and 24 h; C-reactive protein (CRP) was measured at 0–24 h after enrolment. Six subjects were excluded due to insufficient blood sampling. The remaining 39 neonates were assigned to one of three groups: 25 newborns with sepsis (blood culture positive), seven with pneumonia (positive results on broncho-alveolar lavage fluid culture and characteristic chest radiography) and seven with necrotising enterocolitis (NEC) (characteristic intestinal and radiological signs according to the criteria of Bell et al.). A group of 20 healthy preterm neonates represented control subjects. On admission, higher levels of IL-6, IL-10 and CRP were observed in neonates with sepsis: IL-6 (median 1500 pg/ml, range 487–10000 pg/ml), IL-10 (median 113 pg/ml, range 70–196 pg/ml), CRP (median 22 mg/l, range 4–80 mg/l); pneumonia: IL-6 (median 1500 pg/ml, range 747–8000 pg/ml, IL-10 (median 84 pg/ml, range 76–92 pg/ml), CRP (median 10 mg/l, range 8–33 mg/l) and NEC: IL-6 (median 6650 pg/ml, range 1595–7950 pg/ml), IL-10 (median 80 pg/ml, range 61–147 pg/ml), CRP (median 3 mg/l, range 2.8–8 mg/l) as compared to controls (IL-6 median 208 pg/ml, range 198–349 pg/ml; IL-10 median 36 pg/ml, range 19–50 pg/ml; CRP median <2 mg/l) ($P < 0.05$). In neonates with sepsis, IL-6 levels were significantly correlated with IL-10 levels ($r = 0.65$; $P = 0.04$) at the time of the second sample. The highest IL-6 levels were observed at onset, while IL-10 was predominant 12 h later. On admission, IL-10 and CRP levels were significantly higher in non-survivors (IL-10 median 507 pg/ml, range 422–753 pg/ml; CRP median 123 mg/l, range 20–219 mg/l) than in survivors (IL-10 median 76 pg/ml, range 61–143 pg/ml; CRP median 8 mg/l range 3–46 mg/l), while IL-10 levels were significantly higher ($P < 0.05$) also 12 h after admission (non-survivors: IL-10 median 600 pg/ml, range 538–800 pg/ml; survivors: IL-10 median 74 pg/ml, range 53–161 pg/ml). IL-6 and IL-10 levels were significantly correlated with CRP levels on admission ($r = 0.45$; $P = 0.05$).

Conclusion Preterm neonates with sepsis, pneumonia or necrotising enterocolitis showed increased interleukin-6, interleukin-10 and C-reactive protein levels. High interleukin-10 concentration was associated with mortality and could be an early indicator of prognosis.

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Key words Interleukin-6 · Interleukin-10 · Necrotising enterocolitis · Preterm neonates · Sepsis

Abbreviations *BALF* broncho-alveolar lavage fluid · *CRP* C-reactive protein · *INFγ* γ-interferon · *IL-1* interleukin-1 · *IL-6* interleukin-6 · *IL-8* interleukin-8 · *IL-10* interleukin-10 · *NEC* necrotising enterocolitis · *NICU* neonatal intensive care unit · *TNF-α* tumour necrosis factor-α

Introduction

The under-developed immune system predisposes preterm neonates to infection, which is a major cause of neonatal morbidity and mortality [3, 11]. Sepsis and endotoxin activate monocytes, macrophages, lymphocytes, fibroblasts and endothelial cells that produce and secrete interleukin-1 (IL-1), tumour necrosis factor-α (TNF-α), γ-interferon(INFγ), interleukin-6 (IL-6), interleukin-8 (IL-8) and other pro-inflammatory cytokines. IL-6, stimulated by TNF-α, IL-1, endotoxin, viral and bacterial infections, acts as a T-cell activation indicator, induces antibody secretion by human B-cells, causes differentiation of cytotoxic T-cells, and also has the ability to inhibit TNF-α production. Moreover, IL-6 is the major stimulant in hepatic protein synthesis, i.e., C-reactive protein (CRP) and fibrinogen during acute phase responses [15]. Previous studies have shown that determinations of IL-6 in neonatal blood are of diagnostic value in sepsis and necrotising enterocolitis (NEC) [2, 6, 14, 21].

Interleukin-10 (IL-10) is produced by monocytes, B-cells and TH-cells and is induced by endotoxin and its subsequent pro-inflammatory cytokine cascade. IL-10 has anti-inflammatory properties such as suppression of in vitro synthesis of multiple pro-inflammatory cytokines, i.e. TNF-α, IL-1, IL-6, INFγ, and IL-8 [8, 20] and in vivo suppression of cell-mediated immunity. Moreover, IL-10 could play an important role as a regulatory factor in the intestinal immune response. Kuhn et al. [16] showed that IL-10 deficient "knockout" mice were predisposed to inflammatory enterocolitis indicating that IL-10 counteracts the intestinal inflammatory response stimulated by enteric antigens and food. Edelson et al. [10] report that systemic concentration of IL-10 is elevated in preterm neonates with NEC, especially in those with stage 3. Animal studies have shown that IL-10 administered as a bolus reduces the antigen-presenting capacity of monocytes and pro-inflammatory cytokine response and protects against endotoxin-induced mortality in mice [12]. Marchant et al. [19] reported that IL-10 concentrations increased in adults with sepsis, particularly in cases of septic shock. A positive correlation between pro-inflammatory (TNF-α, IL-6) and anti-inflammatory cytokines (IL-10) in meningococcal disease was observed by Riordan et al. [22] who found higher IL-10 concentrations in non-surviving infants. The role of IL-10 in neonatal sepsis is still not clear [4] and the relationship between plasma IL-6 and IL-10 levels has not previously been investigated in premature neonates.

The principal aim of this study was to evaluate plasma IL-6 and IL-10 concentrations and to analyse the interaction between IL-6 and IL-10 and CRP in premature neonates investigated for sepsis.

Patients and methods

Study population

This study was carried out at the Neonatal Intensive Care Unit (NICU) of the Catholic University of Rome over a 1-year period ending in October 1999. During this period, 130 preterm neonates were admitted to the NICU. Neonates presenting with malformations, chromosomal abnormalities, severe brain damage, or who had undergone major surgery, were excluded from the study. A group of 45 infants were prospectively and consecutively evaluated when they showed the following signs and symptoms of suspected sepsis: (1) respiratory dysfunction, as demonstrated by a progressive increase in ventilation settings or oxygen intake in a previously stable baby and/or apnoea spells and/or tachypnoea, (2) bradycardia, tachycardia, poor peripheral circulation, arterial hypotension, (3) temperature instability, hypotonia, seizures, irritability, lethargy, (4) feeding intolerance, vomiting, abdominal distension, bloody stools, (5) persistent metabolic acidosis, hyperglycaemia.

In these neonates blood, CSF, stool and urine cultures were immediately carried out; broncho-alveolar lavage was also performed in ventilated neonates. Chest and abdominal X-rays were taken when clinically indicated. All these babies were then treated with antibiotics. A group of 20 healthy preterm neonates, who had no respiratory or infectious problems and were born consecutively on the NICU, were included in the study. In all study neonates, CRP was measured at the start of the study and at 24 h. IL-6 and IL-10 were determined upon enrolment and then 12 h and 24 h later. Only two blood samples at 12 hourly intervals were taken in control subjects. The study was approved by the Ethics Committee of the Department of Paediatrics. Informed consent was obtained from the parents of all studied babies before the start of the study.

The enrolled neonates were grouped as follows: Group 1. Confirmed sepsis: neonates with positive results on blood culture; Group 2. Pneumonia: neonates with characteristic chest radiography and positive broncho-alveolar lavage fluid (BALF) results; Group 3. NEC: neonates with characteristic intestinal and radiological signs according to criteria of Bell et al. [1]; Group 4. Controls: healthy preterm neonates. For the purposes of our study, neonates were considered non-survivors if death was infection-related.

Methods

CRP was determined by the nephelometric method using 500 μl of heparinised blood. A value >10 mg/l was defined as increased. For cytokine determinations, aliquots (200 μl) of blood were collected in EDTA-coated plastic tubes and immediately centrifuged. Plasma was stored at -70 °C prior to cytokine assay. Plasma IL-6 and

IL-10 levels were measured by means of enzyme-linked immunosorbent assays (Quantikine, R&D Systems, Minneapolis, USA), according to the manufacturer's procedure. Detection limits for tests were 0.70 pg/ml and 1.5 pg/ml for IL-6 and IL-10 respectively. Double determinations were carried out under blind conditions for each plasma cytokine measurement. In neonates with sepsis, pneumonia and NEC, the first blood sample was taken 1 h before beginning antibiotic treatment and subsequent samples were taken every 12 h over 24 h.

Statistical evaluations were performed with the Mann-Whitney U test, Friedman test and Spearman correlation test. The chosen level of statistical significance was $P < 0.05$.

Results

A total of 45 preterm neonates with suspected sepsis were admitted to the study. Prior to Group classification, six neonates were excluded because of insufficient quantity or number of blood samples for adequate interleukin analysis. Of the remaining 39, 25 met the criteria of Group 1; seven that of Group 2; and seven of Group 3. Twenty healthy, preterm neonates were included as a control group (Group 4). Characteristics of neonates are given in Table 1.

In Group 1, the most common signs of sepsis in order of frequency included hypotonia (80%), lethargy (80%), poor peripheral circulation (72%), apnoea (68%), increased ventilator assistance (68%) and temperature instability (40%). Twenty two neonates had gram-positive infections and three had gram-negative infections. Isolated organisms included coagulase negative Staphylococci – *Staphylococcus epidermidis* ($n = 12$) and *Staphylococcus hominis* ($n = 3$), *Staphylococcus aureus* ($n = 2$), *group B Streptococcus* ($n = 2$), *Listeria monocytogenes* ($n = 1$), *Enterococcus spp.* ($n = 1$), *Micrococcus* ($n = 1$), *Escherichia coli* ($n = 2$) and *Enterobacter spp.* ($n = 1$). Thirteen neonates (52%) required a central venous catheter and 12 (48%) needed mechanical ventilation.

All neonates of Group 2 with pneumonia exhibited clinical signs of respiratory distress and required increased mechanical ventilation at the time of enrolment. These patients showed positive results on BALF culture: *Staphylococcus aureus* ($n = 3$), *group B Streptococcus* ($n = 1$), *Chlamydia trachomatis* ($n = 2$), *Ureaplasma urealyticum* ($n = 1$), and characteristic chest radiography for pneumonia, but negative blood cultures. Two neonates (28.6%) required a central venous catheter.

Group 3 neonates with NEC were classified in accordance with the criteria of Bell et al. [1] and treatment requirements. Stage 1 patients ($n = 2$) were treated with antibiotic therapy and enteral nutrition was withheld for 5 days; stage 2 patients ($n = 3$) were treated with antibiotic therapy and enteral nutrition was withheld for 6–10 days; stage 3 patients ($n = 2$) required surgery for intestinal perforation. Three neonates (42.8%) required a central venous catheter and three (42.8%) needed mechanical ventilation.

The control group (Group 4) consisted of 20 healthy preterm neonates who had no signs or symptoms of infection. Six neonates (30%) required a central venous catheter and six (30%) needed mechanical ventilation.

The presence of coagulase negative staphylococci in blood culture [11, 23] was considered as pathogenic in critically ill neonates who showed a clinical improvement after antibiotic therapy. No sepsis was catheter related. Gestational age and birth weight were equally represented in the four groups. Neonates with sepsis and NEC were older at the onset of illness than those with pneumonia ($P < 0.05$). No significant differences were found when haematological values were compared among the groups. Of the 39 neonates, 5 (12.8%) died (two in Group 1, two in Group 2 and one in Group 3).

The values of plasma IL-6, IL-10 and CRP concentrations are shown in Table 2. Neonates with sepsis, pneumonia and NEC had higher median IL-6 levels than those of the control group at the time of the first blood samples: sepsis (median IL-6 1500 pg/ml, range 487–10000 pg/ml); pneumonia (median IL-6 1500 pg/ml, range 747–8000 pg/ml); NEC (median IL-6 6650 pg/ml, range 1595–7950 pg/ml) and control group (median IL-6 208 pg/ml range 198–349 pg/ml) and of the second blood samples: sepsis (median IL-6 810 pg/ml, range 161–8000 pg/ml); pneumonia (median IL-6 547 pg/ml, range 48–6250 pg/ml); NEC (median IL-6 4750 pg/ml, range 1065–7375 pg/ml) and control group (median IL-6 75 pg/ml, range 45–150 pg/ml) ($P < 0.01$ for Group 1 and $P < 0.05$ for Groups 2 and 3). Plasma IL-6 concentrations did not significantly correlate with post-natal age in any of the four Groups. Furthermore, IL-6 levels in preterm neonates with sepsis were higher at onset of infection than 24 h later; a significant difference was found when comparing the second sample with the third (2nd sample versus 3rd sample, $P < 0.05$).

Table 1 Characteristics of study groups

	Group 1 sepsis ($n = 25$)	Group 2 pneumonia ($n = 7$)	Group 3 NEC ($n = 7$)	Group 4 controls ($n = 20$)
[a]Gestational age (weeks)	29 (2.3; 25–34)	28 (1.5; 27–31)	28 (2.5; 25–32)	30 (2.1; 28–35)
[a]Birth weight (g)	1114 (476; 570–2320)	1073 (249; 640–1410)	1330 (559; 820–2180)	1378 (390; 900–2110)
[b]Day of admission	17 (7–55)*	3 (2.5–13.5)	15 (11–32.5)*	13 (1–22.5)

[a] Data shown as mean (SD; range)
[b] Data shown as median (25th–75th percentiles)
*$P < 0.05$, Groups 1–3 versus Group 2

Table 2 Plasma concentrations of IL-6, IL10 and CRP at study times. Data shown as median (25th–75th percentiles)

	Group 1 sepsis	Group 2 pneumonia	Group 3 NEC	Group 4 controls
IL-6 (pg/ml)				
On admission	1500 (487–10000)**	1500 (747–8000)*	6650 (1595–7950)*	208 (198–349)
12 h After	810 (161–8000)**	547 (48–6250)*	4750 (1065–7375)*	75 (45–150)
24 h After	235 (75–470)	575 (415–2450)	8000 (4000–8000)	
IL-10 (pg/ml)				
On admission	113 (70–196)**	84 (76–92)*	80 (61–147)*	36 (19–50)
12 h After	146 (62–248)**	112 (42–186)*	75 (70–600)*	40 (28–60)
24 h After	85 (47–446)	82 (75–100)	23 (16–76)	
CRP (mg/l)				
On admission	22 (4–80)***	10 (8–33)***	3 (2.8–8)****	< 2
24 h After	46 (7–143)	73 (11–134)	5 (4–33)	

*$P < 0.05$ Groups 2–3 versus Group 4
**$P < 0.01$ Group 1 versus Group 4
***$P < 0.001$ Groups 1–2 versus Group 4
****$P = 0.006$ Group 3 versus Group 4

In neonates with sepsis, pneumonia and NEC, median IL-10 levels were significantly higher as compared to controls on admission (sepsis: 113 pg/ml, range 70–196 pg/ml; pneumonia: 84 pg/ml, range 76–92 pg/ml; NEC: 80 pg/ml, range 61–147 pg/ml; control group 36 pg/ml, range 19–50 pg/ml) and 12 h later (sepsis: 146 pg/ml, range 62–248 pg/ml; pneumonia: 112 pg/ml, range 42–186 pg/ml; NEC: 75 pg/ml, range 70–600 pg/ml; control group: 40 pg/ml, range 28–60 pg/ml) ($P < 0.01$ for Group 1 and $P < 0.05$ for Groups 2 and 3). No significant correlation was found between IL-10 levels and post-natal age in any of the four Groups. Plasma IL-10 concentrations did not change significantly during the study period but the second sample was higher than the first. Plasma Il-6 concentrations correlated significantly with IL-10 levels only in newborn babies with sepsis at the time of the second sample ($r = 0.65$; $P = 0.04$).

Upon admission, neonates with sepsis, pneumonia and NEC had higher median CRP levels than those of the control group (sepsis: 22 mg/l, range 4–80 mg/l; pneumonia: 10 mg/l, range 8–33 mg/l; NEC: 3 mg/l range 2.8–8 mg/l); control group < 2 mg/l) ($P < 0.001$ for Groups 1 and 2 and $P = 0.006$ for Group 3) (Table 2). No significant correlation was found between CRP levels and gestational or post-natal age in any of the four Groups. IL-6 and IL-10 levels correlated significantly with CRP levels on admission in all three groups (IL-6: $r = 0.45$, $P = 0.026$; IL-10: $r = 0.42$, $P = 0.033$).

We also assessed the plasma IL-6, IL-10 and CRP concentrations in surviving and non-surviving neonates. Due to the small numbers of non-survivors in each study Group, we compared results obtained in the total non-survivors ($n = 5$) with results obtained in survivors ($n = 34$). Median IL-10 levels were significantly higher in non-survivors than in survivors at the time of onset non-survivors: 507 pg/ml, range 422–753 pg/ml; survivors: 76 pg/ml, range 61–143 pg/ml) ($P = 0.01$), 12 h after admission (non-survivors: 600 pg/ml range 538–800 pg/ml; survivors: 74 pg/ml, range 53–161 pg/ml) ($P = 0.01$) and 24 h later (non-survivors: 610 pg/ml, range 416–804 pg/ml; survivors: 68 pg/ml, range 33–90 pg/ml) ($P < 0.05$). Plasma CRP levels were significantly higher in non-survivors than in survivors at the time of onset (non-survivors: 123 mg/l, range 20–219 mg/l; survivors: 8 mg/l, range 3–46 mg/l) ($P = 0.03$) and 24 h later (non-survivors: 204 mg/l, range 123–282 mg/l; survivors: 22 mg/l, range 5–129 mg/l) ($P = 0.03$). Plasma IL-6 levels were higher in non-surviving neonates than in survivors, but the difference did not reach statistical significance (Table 3).

Table 3 Plasma concentrations of IL-6, IL-10 and CRP in surviving and non-surviving babies. Data shown as median (25th–75th percentiles). (*NS* not significant)

	Surviving babies ($n = 34$)	Non-surviving babies ($n = 5$)	*P*
IL-6 (pg/ml)			
On admission	1500 (295–8000)	7500 (6300–8000)	NS
12 h After	1000 (130–8000)	6000 (4047–8000)	NS
24 h After	337 (65–1650)	5500 (4250–6750)	NS
IL-10 (pg/ml)			
On admission	76 (61–143)	507 (422–753)	0.01
12 h After	74 (53–161)	600 (538–800)	0.01
24 h After	68 (33–90)	610 (416–804)	< 0.05
CRP (mg/l)			
On admission	8 (3–46)	123 (20–219)	0.03
24 h After	22 (5–129)	204 (123–282)	0.03

Discussion

Results of this study contribute to understanding the role of IL-10 and its relationship to IL-6 in preterm neonates with sepsis, pneumonia and NEC. The Groups were similar with regard to gestational age and birth weight but neonates with sepsis and NEC were older than those with pneumonia. We found that plasma IL-6, IL-10 and CRP levels were independent of post-natal age; therefore differences in post-natal age do not explain the modified cytokine levels. Both Harris et al. [14]

and Sullivan et al. [24] found higher plasma IL-6 levels in non-surviving babies, but in our study this did not reach statistical significance.

We observed that preterm neonates are able to produce IL-10 during sepsis as well as during pneumonia and NEC. IL-10 has only recently been evaluated in humans [19]. Studies suggest an important role for endogenous IL-10 as an anti-inflammatory cytokine and it has also been suggested that IL-10 could play an auto-regulatory role [8]. Our data indicate that high IL-10 concentrations persist longer than high IL-6 concentrations. An inverse relationship between IL-6 and IL-10 could be related to a suppressive effect of IL-10 or even to totally different phenomena. In neonates with sepsis, the high IL-6 values found upon admission decreased rapidly over 12 h while IL-10 levels tended to increase. However, in Group 3, IL-10 levels were lower with persistently higher IL-6 levels in respect to the sepsis Group. Apart from circulating cytokines, enterocytes are exposed to local concentrations of IL-6, IL-10, IL-1 and TNF-α as well as other cytokines secreted by endothelial cells, fibroblasts, and enterocytes themselves. IL-6 and its receptors are expressed by intestinal epithelial cells and bowel smooth muscle cells. During sepsis there is an increase in enterocyte secreted IL-6, augmented by invasive bacteria, endotoxin and IL-1. IL-10 is expressed by intestinal epithelial cells in culture and production is increased by endotoxin and TNF-α. When macrophages or lymphocytes are present there is further enhancement of the already increased IL-10 expression [18]. In our opinion, lower IL-10 levels in the NEC Group could also be influenced by local intestinal factors, which are difficult to study in vivo.

Our data on preterm neonates with sepsis showed that IL-10 peaks 12 h after IL-6 peaks. This suggests an initial acute phase characterised by a predominance of pro-inflammatory cytokines, followed by a secondary phase with anti-inflammatory cytokine predominance. Groeneveld et al. [13] also reported this trend in adults with sepsis. These data appear to be in conflict with observations by Derkx et al. [7] who, in children with fulminant meningococcal septic shock, demonstrated that higher IL-10 levels are released during the initial phase of infection. Suspicion of sepsis occurs earlier in preterm neonates than in older children; therefore, the different trend in IL-10 observed in our investigation may be related to this earlier recognition of the pathology, different sampling times and different bacteria.

Increased IL-10 values have been found in patients with meningococcal septic shock and high concentrations have been associated with disease severity [7, 25]. The current study demonstrates significantly higher IL-10 concentrations in non-surviving preterm neonates, as previously observed in meningococcal disease [22]. An important factor influencing the anti-inflammatory activity of IL-10 could be the time of its synthesis. Recently, in a neonatal mouse model of lethal group B streptococcal sepsis, it was found that administration of recombinant IL-10 at 20 to 4 h before challenge, but not later, actually improved survival [5]. This suggests the importance of IL-10 production time in modulating synthesis of pro-inflammatory cytokines. Even if CRP levels in non-survivors were significantly higher than in survivors, an overlap of relevant data did not allow correlation of CRP levels with mortality.

The correlation between concentrations of IL-6 and IL-10 resulting from our investigation could indicate that the degree of pro- and anti-inflammatory response is related and probably determined by similar factors. The positive correlation between CRP and IL-6 and CRP and IL-10 levels observed at onset, could imply that plasma IL-10 concentrations follow the pattern of other common acute-phase reactants like CRP. While CRP dosage would be easier to perform, further investigation is needed to fully define the role of IL-10, which may not be simply anti-inflammatory [9, 17].

Our study shows that preterm neonates with sepsis, pneumonia or NEC are capable of producing both IL-6 and IL-10. In the presence of sepsis, neonates even show the same immune response as adults. Moreover, the very high plasma IL-10 levels found in non-surviving preterm neonates warrants further investigation to clarify the real role of this cytokine which may even be an early indicator of prognosis.

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