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Inflammatory and immunological markers in preterm infants: correlation with disease

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SUMMARY

Newborn infants often suffer from bacterial and viral infections without presenting typical symptoms. Therefore, reliable methods for detecting and monitoring sepsis in the newborn would be beneficial. In older patients C-reactive protein (CRP) and neopterin have proved useful serum markers of infection and inflammation. Both of these markers are regulated by cytokines, and it has been proposed that cytokines themselves could be used to monitor immune activation and infection. This study has examined the levels of CRP, neopterin, soluble IL-2R, tumour necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ) in cord blood samples from both pre-mature and term neonates. Having established reference ranges for these analytes, serial measurements were made in babies requiring intensive care support. The results suggest that in preterm infants the simultaneous measurement of CRP and neopterin, and possibly soluble IL-2R, may provide an accurate early diagnosis of sepsis and may be of use in differentiating between bacterial and viral etiologies. In addition, serial measurement of these markers may help in the early diagnosis of necrotizing enterocolitis (NEC).

Keywords newborn sepsis cytokines enterocolitis

INTRODUCTION

There is an increased risk of sepsis in neonates, and in particular preterm infants, caused by incomplete maturity of the immune system. Premature infants often develop sepsis without typical signs such as fever. Reliable methods for detecting and monitoring sepsis in the newborn would therefore be beneficial in terms of patient management and treatment. In older children and adults, serum/plasma levels of C-reactive protein (CRP) and neopterin have proved useful indicators of infection and inflammation [1].

CRP is an acute-phase protein produced by the liver as part of the body's inflammatory response to bacterial infection and tissue injury. CRP is produced during fetal development, preceding the production of immunoglobulins [2]. The level of CRP is known to rise in both bacterial and fungal infections in children beyond the neonatal age, but remains low in viral infections [3]. CRP alone can be a good indicator of bacterial infection in preterm and term neonates. Serum CRP reference ranges have been established in neonates, but there is little or no information on the levels of other inflammatory and immune markers in the newborn. There is a lack of correlation between the levels of CRP in maternal blood with those found in cord blood, showing that there is no appreciable transfer of CRP across the placenta [4]. Neopterin is a pteridine bi-product of activated monocytes/macrophages [5], and raised systemic levels correlate with bacterial and viral sepsis and immune activation [6]. Inflammatory and immune responses are regulated by cytokines. Cytokines are a highly diverse group of potent intercellular messengers which act via specific receptors upon the surface of their target cells. They are produced by activated cells and include the interleukins, interferons, tumour necrosis factors, and haematopoietic growth factors. Bacterial infection and CRP production have been shown to be associated with the cytokines IL-1, IL-6 and tumour necrosis factor-alpha (TNF-α) [7]. Immune activation, viral infections and increased neopterin levels are associated with the production of the cytokine interferon-gamma (IFN-γ) synthesized by activated T cells [8]. The major cytokine involved in T cell activation is IL-2, which itself is produced by T cells [8]. A good marker of IL-2 stimulation is the production of the interleukin receptor (IL-2R), particularly the soluble form [9]. Neopterin and soluble IL-2R levels have been shown to be positively correlated with active Crohn's disease [10], but there have been no previous studies on neopterin in neonates. TNF-α is known to be an important mediator in the pathogenesis of Gram-negative shock [11] and, in experimental rats, it has been shown to be involved in the development of necrotizing enterocolitis (NEC) [12,13]. The production of TNF-α by preterm infants during infection has been documented [11], but, along with other cytokines (e. g. IFN-γ), it is readily taken

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up into cells [8]. It is possible that such molecules could be used as markers for detecting and monitoring bacterial sepsis and viral infections in the newborn.

Little is known about the levels of neopterin, soluble IL-2R, IFN-γ and TNF-α in newborn babies. Accordingly, the first objective of this study was to establish normal ranges in cord blood (serum) collected at birth from preterm and full term neonates. Having established normal ranges in cord blood serum samples, the study investigated their measurement in the diagnosis and management of infection in babies requiring intensive care support after birth.

SUBJECTS AND METHODS

Cord blood samples

With approval from Riverside Health Ethical Committee, 44 blood samples were obtained from the fetal side of the umbilical cord of premature ($n = 14$) and term infants ($n = 30$) approximately 5 min after delivery (Table 1). The samples were collected in non-heparinized syringes into plain specimen bottles with no additives. The blood was allowed to clot and the serum harvested within 4 h of collection. The serum was aliquoted and stored at -70°C.

Patients admitted to NICU

Serum samples were obtained, as described above, from 46 preterm infants born between 25 and 36 weeks of gestation. All had been admitted to the Neonatal Intensive Care Unit (NICU) at West London Hospital. The study was approved by the Riverside Ethical Approval Committee. The first samples were taken within 36 h of birth and subsequently at 2–3 day intervals. The babies were divided into three groups: Group A, 21 infants with no evidence of infection and from whom 43 samples were collected (Table 3); Group B, 19 preterm infants who had one or more episodes of proven or clinically suspected infection and from whom 75 samples were collected (Table 3); Group C; five babies with clinical and radiological features of NEC and from whom 30 samples were collected (Table 3).

Table 1. Cord blood sample population

		Preterm ($n = 14$)	Full term ($n = 30$)
Gestational age (weeks)		32 (Range 25–36)	39 (Range 37–40)
Baby weight range (g)		700–3005	2700–4600
Male/female ratio		9/5	15/15
Delivery:	SVD	11	24
	CS	2	3
	Breech	1	3
Apgar		10 elective intubation	29 (8^1; 9^5)
		4 (4^1; 6^5)	1 (2^1; 6^5)
Drug use for delivery		5 N_2O inhalation	6 N_2O inhalation
			10 epidural
		2 pethidine	7 pethidine
		7 none	7 none
Labour	<12 h	10	24
	>12 h	4	6

SVD, Spontaneous vaginal delivery; CS, caesarian section.
Apgar, Newborn health score at 1 min[1] and 5 min[5].

Assays

CRP was measured by rate nephelometry, using an array nephelometer (Beckman Instruments, High Wycombe, UK). Neopterin was measured by commercially produced radioimmunoassay kits (Henning Berlin, IDS, Tyne & Wear, UK). The lower limit of detection in these assays was 1 mg/*l* and 3 μmol/ml, respectively, with between batch coefficients of variation for both assays of <7%.

TNF-α and IFN-γ were measured by ELISA using paired monoclonal and polyclonal antibodies (a gift from Dr A. Meager, NIBSC, Potter's Bar, UK), and reference standards from NIBSC. The lower limits of detection were 7·5 and 0·5 U/ml, with between batch coefficients of variation of 9·4% and 3·2%, respectively.

Soluble IL-2R was measured using commercial ELISA kits (T Cell Diagnostics, Lab. Impex, London, UK). The between batch coefficient of variation was <5%.

Table 2. Cord blood levels of C-reactive protein (CRP), neopterin and cytokines compared with published normal levels

		Preterm ($n = 14$)	Term ($n = 30$)	Normal level child (>1 year)	Normal level adult
CRP	Range	2–4	2–4		
	Median	3·0 mg/*l*	3·0 mg/*l*	<10 mg/*l* [1]	<10 mg/*l* [1]
Neopterin	Range	8–22	12–38		
	Median	14 nmol/*l*	17 nmol/*l*	<10 nmol/*l* [1]	<10 nmol/*l* [1]
TNF-α	Range	<7·5–8·5	<7·5–9·3		
	Median	<7·5 U/ml	<7·5 U/ml	<7·5 U/ml*	<7·5 U/ml*
IFN-γ	Range	<0·5–3·4	<0·5–0·98		
	Median	<0·5 U/ml	<0·5 U/ml	<0·5 U/ml*	<0·5 U/ml*
Sol. IL-2R	Range	353–1469	543–1750	Not known	573–919
	Median	927 U/ml	707 U/ml		U/ml†
Upper limit of normal					

* A. Meager (NIBSC).
† Kit manufacturer's specification.

RESULTS

Cord blood samples

Cord blood samples were obtained from term ($n = 30$) and preterm ($n = 14$) babies (Table 1). Term was considered to be 37 or more weeks gestation. There were more male babies in the preterm group, but otherwise both groups were similar with respect to delivery, duration of labour and drug usage. One preterm baby was born with congenital toxoplasmosis, but none of the others or their mothers had culture-proven sepsis. All babies survived more than 72 h after birth, except for one preterm neonate born at 28 weeks gestation who died at 3 h of age from severe hyalline membrane disease.

CRP levels. The lower limit of detection for CRP was 1 mg/*l*. Cord blood samples, including that from the baby with congenital toxoplasmosis, had CRP levels between 2 and 4 mg/*l*, giving a median value of 3 mg/*l* (Table 2), and there was no difference in levels between samples from premature and full-term babies.

Neopterin levels. In the full-term group, the highest neopterin level (38 nmol/*l*) was found in a cord blood sample from a baby born after a prolonged labour of 24 h and with meconium-stained liquor. In the remaining 29 cord blood samples from term babies, neopterin levels ranged from 12 to 38 nmol/*l*, giving a mean value of 17 nmol/*l*. In the preterm group, the baby with congenital toxoplasmosis had a neopterin level of 46 nmol/*l*. In the remaining 13, the neopterin levels ranged from 8 to 22 nmol/*l*, giving a median of 14 nmol/*l* (Table 2).

TNF-α levels. The lower limit of detection for TNF-α was 7·5 U/ml. Only six samples from the term babies had levels above this and all were below 9·3 U/ml, giving a median value of $<$7·5 U/ml. In the premature baby group only one sample had a detectable level of 8·5 U/ml (Table 2).

IFN-γ levels. IFN-γ was below the limit of detection (0·5 U/ml) in all but six cord blood samples from term babies. The highest level detected was 0·98 U/ml. All six were delivered after a short labour, through normal vaginal delivery, and with no medication. In the preterm group, one baby who was born at 28 weeks gestation, and who died at 3 h of age from severe hyalline membrane disease, had a raised level (3·4 U/ml) of IFN-γ in cord blood. In all the other preterm infants, IFN-γ was at or below the level of detection (Table 2).

Soluble IL-2R levels. Soluble IL-2R levels ranged between 543 and 1750 U/ml in cord blood samples from the term group and, with the exception of the preterm infant born with congenital toxoplasmosis who had a level of 3798 U/ml, between 353 and 1469 U/ml in the preterm baby group, giving a median value of 927 U/ml (Table 2).

Patients admitted to NICU

Three groups of patients (Table 3), Groups A and B with similar characteristics including number of babies, gestation, birth weight, diagnosis, resusitative manoeuvres, and Group C, a smaller third group of babies with NEC, were investigated. All the babies had their surface swabs and blood culture samples taken on day 1 of admission to the NICU, and they were commenced on i.v. penicillin and gentamycin. All babies received 10% dextrose infusion for the first 48 h of life, and were sedated with i.v. morphine infusion. The analysis of serum levels of CRP, neopterin, TNF-α, IFN-γ and soluble IL-2R from the three groups A, B and C is shown in Table 4.

Group A: infants with no evidence of sepsis. CRP, TNF-α and IFN-γ levels were within normal ranges. However the levels of neopterin and soluble IL-2R were higher than those from preterm cord blood samples (Table 4).

Table 3. Characteristics of babies admitted to paediatric intensive care unit (PICU)

	Group A: infants with no evidence of sepsis	Group B: infants with clinical sepsis	Group C: infants with NEC
Number of infants	21	19	5
Gestation (weeks)	25–35	25–36	28–36
Weight (kg)	0·8–1·84	0·83–2	0·53–3·4
Diagnosis (*n*)	HMD (20), TTN (1)	HMD (14), IUGR (5)	HMD (4) Birth asphyxia (1)
Delivery (*n*)	NVD (17), LSCS (4)	NVD (15), LSCS (4)	NVD (3), LSCS (2)
Management (*n*)	IPPV (19), CPAP (2), Exosurf (8), Morphine (17), Pancuronium (7)	IPPV (13), H/B O_2 (5) Exosurf (7), Morphine (10), Pancuronium (9)	IPPV (5) Exosurf (1) Pencillin and Gentamycin (5)
Outcome (*n*)	Alive (19) Dead (2)	Alive (15) Dead (4)	Alive (4) Dead (1)
Infective episodes (*n*)	Proven (0) Suspected (0)	Proven (16) Suspected (5) Proven episodes: *Staph. epidermidis* (8) *Staph. aureus* (2) Group B streptoccocal (2) Rotavirus enteritis (4)	Proven (0) Suspected (5) Onset of NEC: 3–27 days, median 8 days Onset of gut perforation: 4–30 days, median 9 days

HMD, Hyalline membrane disease; TTN, transient tachypnoeia of the newborn; IUGR, intra uterine growth retardation; NVD, normal vaginal delivery; LSCS, lower segment caesarian section; IPPV, intermitten positive pressure ventillation; CPAP, continuous positive airway pressure; H/B O_2, head box oxygen.

Table 4. Serum levels of C-reactive protein (CRP), neopterin, tumour necrosis factor-alpha (TNF-α), IFN-γ and soluble IL-2R in serum from babies in Groups A, B and C

Group (*n*)	CRP mg/*l*	Neopterin, μmol/*l*	TNF-α, U/m*l*	IFN-γ, U/m*l*	Soluble IL-2R, U/m*l*
Cord blood (median)	2–4 (3)	8–22 (14)	<7·5–8·5	<0·5–3·4	353–1469 (927)
Group A (21)	2–4	4–56	<7·5	<0·5	328–1972
Group B (19)	3–175	9–131	<7·5	<0·5	543–3705
Group C (5)	27–82	58–212	<7·5	<0·5	2427–3375

Group B: infants with clinical sepsis. Babies infected with *Staphylococcus epidermidis* or *Staph. aureus* had an eight-fold rise in CRP level compared with babies in Group A (Table 4). However, CRP levels were normal in babies with streptococcal, viral and clinically suspected infection. High neopterin and soluble IL-2R levels were found in infants with viral, streptococcal and staphylococcal infection. Infants with clinically suspected but not microbiologically proven infection had normal levels of CRP, neopterin, and soluble IL-2R. TNF-α and IFN-γ remained normal in all infants throughout their illnesses. Figure 1 shows the typical pattern of response for a baby with staphylococcal sepsis.

Group C: infants with NEC. In all infants with NEC (Table 4) there was a sudden rise of CRP, neopterin and soluble IL-2R at the time of diagnosis, and further rises occurred at gut perforation. TNF-α and IFN-γ levels remained normal throughout. Figure 2 shows the pattern of response for a baby believed to be developing NEC at 18 days old and with gut perforation on day 38 of life.

DISCUSSION

Early diagnosis of neonatal infections and related conditions, in particular NEC, is important in reducing overall morbidity and mortality in the newborn. In this study, markers of inflammation and immune activation, namely neopterin, soluble IL-2R, TNF-α and IFN-γ, have been measured in cord blood samples to establish normal reference ranges applicable to the newborn, and in serial blood samples from preterm babies requiring neonatal intensive care. CRP levels were also monitored.

Cord blood levels of CRP, TNF-α and IFN-γ in samples obtained from healthy preterm and term babies were within the normal reference ranges for children (>1 year old) and adults (Table 2). The ranges for neopterin and soluble IL-2R were marginally greater than those established for children over 1 year and adults (Table 2).

Our results suggest that the combined use of some of these markers may enable early detection of infection in neonates. The infants in Group A showed normal CRP, TNF-α and IFN-γ levels. All of these infants had raised (almost double) neopterin and soluble IL-2R levels compared with cord blood values. The mechanism for this is unclear, but adult patients in intensive care have been shown to have raised neopterin levels [14], suggesting that endotracheal intubation may induce a degree of macrophage stimulation, resulting in the production of neopterin. CRP levels do not appear to be influenced by the presence of an arterial catheter, endotracheal tube and intraventricular haemorrhage [15]. In Group B, infants with suspected infection had normal CRP and neopterin levels. In cases of proven staphylococcal sepsis, high levels of CRP were found in addition to significantly raised levels of neopterin and soluble IL-2R (Fig. 1). In other documented infections, notably streptococcal and viral infections, CRP levels remained normal but neopterin and soluble IL-2R increased.

Both TNF-α and IFN-γ remained undetected in all infants in this study, even in those with proven infection and NEC. In this latter group of five infants (Group C), all had sudden rises in CRP, neopterin and soluble IL-2R at the first clinical suggestion of the diagnosis. The levels rose further at the time of gut perforation (Fig. 2).

In conclusion, simultaneous estimation of CRP, neopterin and possibly soluble IL-2R in preterm infants at risk of infection may

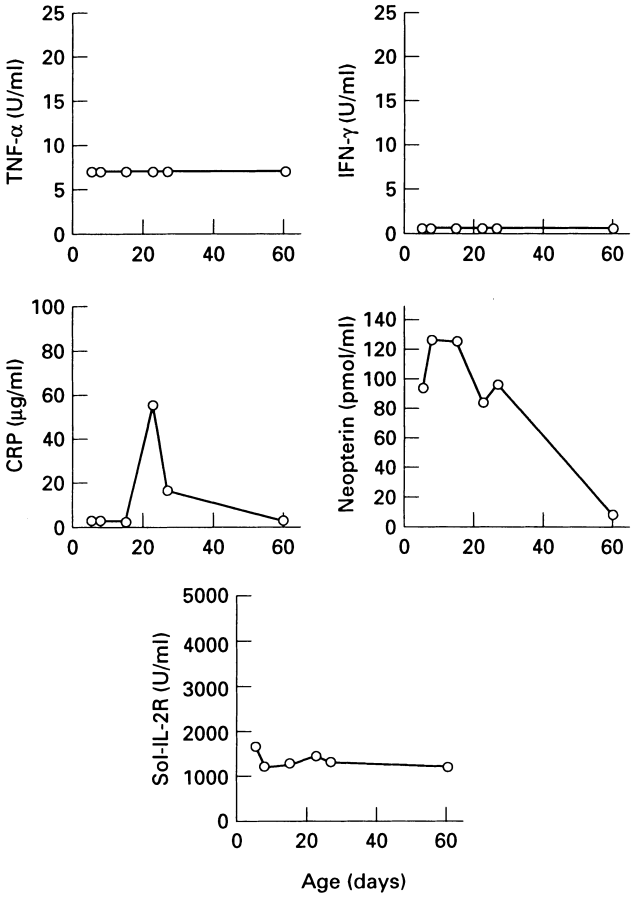

Fig. 1. Production of inflammatory and immunological markers in a baby with staphylococcal sepsis.

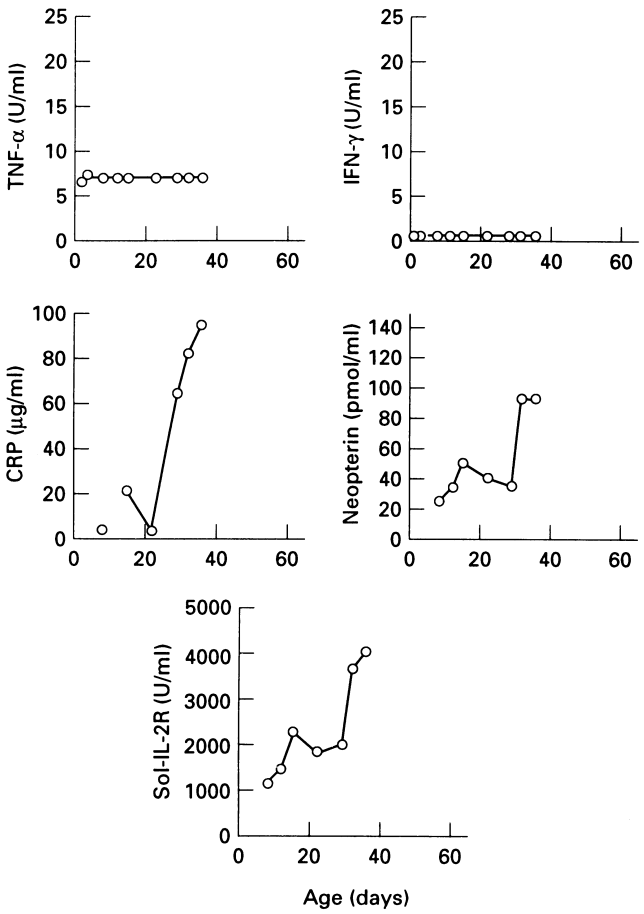

Fig. 2. Production of inflammatory and immunological markers in a baby who developed necrotizing enterocolitis. First clinical signs occurred on day 18, with gut perforation on day 38.

provide an accurate early diagnosis of sepsis, and may be of use in differentiating between bacterial and viral etiologies. Serial measurements of these markers, particularly when high levels are detected, may help in establishing a diagnosis of NEC in susceptible patients.

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