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IP-10 Is an Early Diagnostic Marker for Identification of Late-Onset Bacterial Infection in Preterm Infants

PAK C. NG, KAREN LI, KIT M. CHUI, TING F. LEUNG, RAYMOND P. O. WONG, WINNIE C. W. CHU, ERIC WONG, AND TAI F. FOK

Departments of Pediatrics [P.C.N., K.L., K.M.C., T.F.L., R.P.O.W., T.F.F.] and Diagnostic Radiology and Organ Imaging [W.C.W.C.], Center of Epidemiology and Biostatistics [E.W.], Prince of Wales Hospital, Chinese University of Hong Kong, Hong Kong, China

ABSTRACT: Very low birth weight (VLBW) infants with suspected late-onset infection requiring sepsis screening were enrolled in a prospective study to evaluate the diagnostic utilities of a comprehensive panel of key chemokines and cytokines, both individually and in combination, to identify diagnostic markers for early recognition of bacterial sepsis and necrotizing enterocolitis (NEC). Plasma chemokines interleukin (IL)-8, interferon-γ–inducible protein 10 (IP-10), monokine induced by interferon-γ (MIG), monocyte chemoattractant protein 1 (MCP-1), growth-related oncogene-α (GRO-α), and regulated upon activation of normal T cell expressed and secreted (RANTES) and cytokines IL-1β, IL-6, IL-10, IL-12p70, and tumor necrosis factor α (TNF-α) were measured at the onset of sepsis (0 h) and 24 h later. Of 155 suspected infection episodes, 44 were classified as infected. Concentrations of all studied inflammatory mediators (except IL-1β and RANTES) were significantly higher in the infected than in the noninfected group at 0 h, but the levels decreased precipitously by 24 h. IP-10 with a plasma cutoff concentration $\geq$1250 pg/mL could identify all septicemic and NEC cases and had the highest overall sensitivity (93%) and specificity (89%) at 0 h. We conclude that preterm infants have the ability to induce a robust chemokine and cytokine response during sepsis, and IP-10 is a sensitive early marker of infection. **(*Pediatr Res* 61: 93–98, 2007)**

Late-onset (>72 h of life or nosocomial) bacterial infection and NEC are important risk factors that predispose to increased morbidity and mortality in preterm VLBW infants (1–4). A recent multicenter survey suggested that 21% of VLBW infants who survived >72 h of age had at least one episode of septicemia (1). Preterm infants who developed NEC had threefold increased risk of mortality (4). As the immunologic defense of preterm infants is considered to be immature and/or deficient (5), this category of patients is particularly vulnerable to developing severe and opportunistic infections in the immediate postnatal period (1,3,4). However, early warning signs and symptoms of systemic infection and NEC are often nonspecific and inconspicuous and can easily be confused with noninfective causes such as apnea of prematurity, gastrointestinal dysmotility, and acute exacerbation of bronchopulmonary dysplasia (6–8). Thus, early identification of infants with bacterial sepsis and NEC has been recognized to be a major diagnostic challenge (7,8).

Exposure to microorganisms and their derived products triggers a rapid and coordinated sequence of host reactions resulting in recruitment of leukocytes into areas of inflammation or sites of microbial invasion (9–12). The regulation and trafficking of leukocytes into specific body tissues are principally controlled by chemoattractant cytokines or chemokines (10). The activation of leukocytes coupled with interaction of pro- and anti-inflammatory cytokines can influence the orchestration of the anti-infectious process and the magnitude of tissue damage and ultimately can determine the outcome of infection (13–16). Up-regulation (or down-regulation) of these mediators occurs very early in the inflammatory process, and, thus, an increase (or decrease) in circulating levels of these compounds can potentially be used as an early indicator or marker of systemic neonatal sepsis. Although circulating concentrations of many chemokines and cytokines are likely to be different in healthy and infected subjects, only a minority of these mediators are adequately sensitive and specific to be regarded as useful diagnostic markers of infection (7,8). Furthermore, the latest advances in flow cytometry enable a comprehensive panel of inflammatory mediators to be studied with only a minimal volume of plasma (50 μL), and this technology is most desirable for use in preterm newborns (17,18). This prospective study was, therefore, designed to use flow cytometry and enzyme-linked immunosorbent assay (ELISA) 1) to evaluate the diagnostic utilities of a panel of chemokines, including IL-8, IP-10, MIG, MCP-1, GRO-α, and RANTES, and the pro- and anti-inflammatory cytokines IL-1β, IL-6, IL-10, IL-12p70, and TNF-α, both individually and in combination, to identify useful infection markers for early recognition of late-onset bacterial sepsis and NEC and 2) to define the optimal cutoff value using the receiver operating characteristics (ROC) curve for each marker in identifying neonatal infection so that these cutoff values can be used as

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Correspondence: Pak C. Ng, M.D., Department of Pediatrics, Level 6, Clinical Sciences Building, Prince of Wales Hospital, Shatin, N.T., Hong Kong; e-mail: pakcheungng@cuhk.edu.hk

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Abbreviations: CRP, C-reactive protein; **GRO-α,** growth-related oncogene-α; **IP-10,** interferon-γ–inducible protein 10; **MCP-1,** monocyte chemoattractant protein 1; **MIG,** monokine induced by interferon-γ; **NEC,** necrotizing enterocolitis; **RANTES,** regulated upon activation of normal T cell expressed and secreted; **ROC,** receiver operating characteristics; **VLBW,** very low birth weight

references for comparison in future studies and in clinical applications. These specific chemokines were selected because they represented chemoattractant factors of major leukocyte subsets involved in the infection cascade (10,19,20), and most of these chemokines (except IL-8) have not been systematically evaluated as diagnostic markers in preterm infants during sepsis. A set of key pro- and anti-inflammatory cytokines were also chosen, as the fine balance between these two types of mediators has been shown to greatly influence the prognosis and outcome of infection (13,14,17).

SUBJECTS AND METHODS

Patients. Preterm infants with 1) a birth weight <1500 g, 2) a postnatal age >72 h, 3) signs and symptoms suggestive of systemic infection or NEC and requiring full sepsis evaluation and antibiotic treatment, and 4) parental consent in the neonatal unit at Prince of Wales Hospital, Hong Kong, were eligible for enrollment in the study. Patients who were already receiving parenteral antibiotic at the time of sepsis evaluation or had severe congenital anomalies, chromosomal abnormalities, or inborn errors of metabolism were excluded (6,21). Recruitment of suspected infection episodes was conducted prospectively over a 29-mo period.

Methods. Signs and symptoms suggestive of infection and NEC were described in detail in our previous studies (6,21). In brief, these features included unstable temperature, gastrointestinal disturbances (abdominal distention or bloody stool), respiratory dysfunction (increase in ventilator settings or oxygen requirement and apneic spells), cardiovascular dysfunction (poor peripheral circulation and hypotension), and unexplained abnormal biochemical and hematologic parameters (persistent metabolic acidosis, thrombocytopenia, leukopenia, or leukocytosis) (6,21).

All infants were recruited at the time of clinical presentation during the initial sepsis evaluation (0 h). In each episode, a full sepsis screen was performed that included cultures of bacteria and fungi from cerebrospinal fluid (CSF), blood, urine, stool, and endotracheal aspirate (infants on ventilator); indwelling central lines or catheters; and specific infected sites or surgical specimens such as peritoneal fluid, abscess, and biopsy specimen. Chest radiograph was routinely performed during the initial screening procedure, and an abdominal radiograph was requested when patients presented with signs suggestive of intra-abdominal pathology. Hematologic and biochemical investigations, including clotting studies, differential white cell and platelet counts, arterial blood gas, serum glucose concentration, and serial C-reactive protein (CRP) measurements, were also performed (6,21). Blood specimens were obtained for evaluation of chemokines and pro- and anti-inflammatory cytokines at 0 h and 24 h after the onset. This schedule of blood sampling coincided exactly with our unit policy for routine blood count and CRP measurements after a suspected infection episode was identified. Antibiotics (i.v.) were started immediately after the sepsis screening and collection of the first set of blood samples (6,21).

Two categories of infective episodes were prospectively defined. Group 1, the infected group, consisted of infants who had been confirmed as septicemic from positive blood cultures when the same organism was isolated from both blood culture bottles. Other microbiologically confirmed bacterial infections, including peritonitis and meningitis, and NEC (stage II or above in Bell's classification with or without positive blood culture) (22), were also included in this group. Pneumonia was diagnosed based on changes in the quality and an increase in the quantity of pulmonary secretions plus chest radiographic abnormalities compatible with pneumonia. Endotracheal cultures for bacteria and fungi were routinely performed in intubated patients and isolation of a predominant organism from at least two bronchopulmonary lavage specimens was required for confirming the diagnosis. However, the diagnosis of pneumonia in nonventilated infants mainly depended on clinical findings plus abnormal chest radiographic changes, and elevated serial serum CRP concentrations >10 mg/L (23). The images were stored digitally in a computed radiographic system (Mobilette Plus; Siemens, Erlangen, Germany) so that the brightness and contrast of these pictures could be optimized to highlight the abnormal lesions. The digital technology greatly enhanced the chance of picking up subtle features. Further, extreme care was exercised in interpreting the radiographic findings. All cases were reviewed by two independent investigators, an experienced neonatologist (P.C.N.) and a pediatric radiologist (W.C.W.C.), who were blinded to the patient's identity and results of chemokine and cytokine measurement. The investigators specifically looked for air space consolidation, pleural effusion, and peribronchial and perivascular interstitial infiltration. These abnormal images should resolve (or partially resolved) after a course of antibiotic treatment. Any difference in opinion between the investigators was subjected to a further review, and the final interpretation of radiographic signs was based on a consensus. Group 2, the noninfected group, consisted of patients who met the initial screening criteria for suspected clinical infection but were subsequently found not to have positive bacterial cultures in blood and CSF plus no radiographic evidence suggestive of pneumonia or NEC and they continued to improve after antibiotics were stopped between 24 and 96 h after initiation of treatment (6,21).

Measurement of chemokines and cytokines. Blood samples collected from indwelling arterial lines or venipunctures were immersed in ice and immediately transported to the laboratory for processing. Plasma was separated by centrifugation ($1900 \times g$ for 5 min) at 4°C and stored in 200 μL aliquots at -80°C until analysis. A panel of cytokines (IL-1β, IL-6, IL-10, IL-12p70, and TNF-α) and chemokines (IL-8, IP-10, MIG, MCP-1, and RANTES) was simultaneously quantified by the Cytometric Bead Array Kits (BD Biosciences Pharmingen, San Diego, CA) using flow cytometry as described previously (6,24). The assay sensitivities for IL-1β, IL-6, IL-10, IL-12p70, and TNF-α were 7.2, 2.5, 3.3, 1.9, and 3.7 pg/mL, respectively, and those for IL-8, IP-10, MIG, MCP-1, and RANTES were 0.2, 2.8, 2.5, 2.7, and 10.0 pg/mL, respectively. Fifty microliters of plasma or the provided standard cytokines were added to an equal volume of premix microbeads. After the addition of 50 μL of detecting reagent, the mixture was incubated for 3 h in the dark at room temperature. This mixture was washed with 1 mL of washing buffer and centrifuged at $200 \times g$ for 5 min. The pellet was resuspended in 300 μL of washing buffer and 3000 events were acquired for each sample by the FACS Calibur flow cytometer (BD Biosciences Pharmingen). The quantities of individual cytokines were computed using the standard reference curve by the CellQuest and CBA Software (BD Biosciences Pharmingen). Human GRO-α was measured by ELISA as recommended by the manufacturer (R&D System Inc., Minneapolis, MN). The assay sensitivity of GRO-α was 10 pg/mL.

Statistical analysis. The demographic data of infants and plasma concentrations of different inflammatory mediators in infected (group 1) and noninfected (group 2) groups were compared using the Mann-Whitney U test and χ^2 test where appropriate. As there were no well-defined diagnostic cutoff values for these inflammatory mediators in VLBW infants, an ROC curve was constructed for each sampling time point for each marker. The optimal cutoff value for individual markers was then determined on the graph by minimizing the number of misclassified episodes. As the diagnostic marker should ideally identify all genuinely infected episodes (*i.e.* 100% sensitivity) and at the same time would not misclassify too many noninfected cases (*i.e.* a high specificity), the optimal cutoff value was, therefore, chosen with the sensitivity approaching 100% and specificity $>85\%$ (7,8). However, if the diagnostic marker was unable to satisfy the above criteria, the optimal cutoff value would be adjusted so that both the sensitivity and specificity approached 75%. The calculated optimal cutoff values enable us to work out the diagnostic utilities (sensitivity, specificity, and positive and negative predictive values of these markers) and also to select the best marker or combination of markers at the most appropriate sampling time for diagnosing infection and NEC in VLBW infants. A combination of tests was considered positive if any one of the selected markers exceeded their respective cutoff values. All statistical tests were performed by SPSS for Windows (Release 13; SPSS Inc., Chicago, IL). The level of significance was set at 5% in all comparisons.

Ethical approval. The study was approved by the Research Ethics Committee of The Chinese University of Hong Kong. Written informed consent was obtained from the parents for all preterm infants.

RESULTS

There was no significant difference in clinical characteristics between infected (group 1) and noninfected (group 2) infants (Table 1). Of 155 suspected infection episodes investigated, 44 were classified to be infected. Table 2 summarizes details of the clinical pathologies and causative organisms of infected cases, and Table 3 describes the final diagnosis of noninfected cases. The most common group of organisms responsible for late-onset neonatal infection was coagulase-negative staphylococci (Table 2). The majority of cases in the noninfected group were due to respiratory etiologies secondary to either acute exacerbation of bronchopulmonary dysplasia or apnea due to various causes (Table 3).

Table 1. *The clinical characteristics of the infected (group 1) and noninfected (group 2) groups*

	Group 1 (n = 44 cases)	Group 2 (n = 111 cases)
No. of infants	36	50
Gestational age, wk	28.5 (26.3–30.5)	28.8 (25.8–30.8)
Birth weight, g	1058 (923–1303)	1033 (893–1273)
Male-to-female ratio	20:16	24:26
Apgar scores		
1 min	6 (5–8)	6 (5–8)
5 min	8 (7–9)	8 (8–9)
Arterial cord blood		
pH	7.29 (7.21–7.35)	7.30 (7.22–7.36)
Base excess, mmol/L	−4.2 (−2.0 to −6.3)	−4.4 (−2.3 to −6.9)
Age after birth preforming sepsis screening, d)	28 (16–45)	24 (14 – 44)

There was no significant difference in clinical characteristics between the infected (group 1) and noninfected (group 2) group. Twenty-eight infants had one and eight infants had two episodes of confirmed infection in group 1.

The comparison of plasma chemokine and cytokine concentrations between the two groups is summarized in Table 4. All inflammatory mediators at 0 h, with the exceptions of IL-1β and RANTES, were significantly higher in the infected (group 1) than in the noninfected (group 2) group. Plasma IL-1β concentrations did not differ significantly between the groups, whereas plasma RANTES concentrations were significantly lower in infected cases. Likewise, a similar trend was observed at 24 h, but at this stage, more inflammatory mediators including IL-1β, IL-12p70, TNF-α, and MCP-1 did not show a significant difference between the groups. In addition, there was a significant decrease in chemokine [IP-10 ($p < 0.001$), MCP-1 ($p = 0.008$), and GRO-α ($p = 0.007$)] and cytokine [IL-1β ($p = 0.006$), IL-6 ($p < 0.001$), IL-10 ($p < 0.001$), and IL-12p70 ($p = 0.007$)] levels at 24 h compared with their corresponding values at 0 h in infected infants (group 1). None of the inflammatory mediator levels showed a significant reduction from 0 h to 24 h in the noninfected group ($p > 0.10$).

Table 3. *Clinical diagnosis of noninfected episodes (n = 111)*

Clinical diagnosis	No. (%) of cases (n = 111)
Respiratory causes	
Acute exacerbation of bronchopulmonary dysplasia	59 (53)
Apnea	
Apnea of prematurity	12 (11)
Gastroesophageal reflux	5 (4)
Increased intracranial pressure	2 (2)
Parenteral nutrition fluid extravasation in hemithorax	1 (1)
Gastrointestinal causes	
Gastrointestinal dysmotility	11 (10)
Ileal perforation (indomethacin induced)	2 (2)
Others	
Left heart failure (patent ductus arteriosus)	9 (8)
Unstable body temperature (environmentally induced)	6 (5)
Anemia	3 (3)
Transient adrenocortical insufficiency of prematurity	1 (1)

Table 5 summarizes 1) the calculated optimal cutoff values derived by minimizing the number of misclassified episodes over all possible cutoff values for 0 h and 24 h, 2) the diagnostic utilities (sensitivity, specificity, positive and negative predictive values), and 3) the area under the ROC curve of the studied markers. Of the inflammatory mediators studied, IP-10 (93%), MIG (80%), GRO-α (82%), RANTES (86%), IL-6 (82%), IL-8 (80%), and IL-10 (84%) had sensitivity $\geq$80%, but only IP-10 (89%) and IL-6 (82%) had specificity $\geq$80% at 0 h (Table 5). Further, the diagnostic utilities of all studied mediators at 24 h were inferior to their corresponding values at 0 h, confirming early increase (or decrease) of circulating concentration of these mediators during sepsis. Thus, the overall assessment of individual markers indicated that IP-10 has the best diagnostic utilities with the highest sensitivity and negative predictive values at 0 h and 24 h. In addition, a comparison of diagnostic utilities of a

Table 2. *The characteristics of clinical pathologies and organisms of infected episodes (n = 34)*

Organisms	No. of episodes	Comments
Gram-positive organisms: n = 17 (50%)		
Coagulase-negative staphylococci	12	Septicemia (x9); septicemia with meningitis (x2); septicemia with liver abscess (x1)
Staphylococcus aureus	1	Septicemia (x1)
Streptococcus bovis	1	Septicemia (x1)
Enterococcus sp.	3	Septicemia (x1); NEC (x1); pneumonia (x1)
Gram-negative organisms: n = 14 (41%)		
Klebsiella sp.	4	Septicemia (x1); septicemia with meningitis (x1); NEC (x2)
Enterobacter sp.	4	Septicemia (x)
Escherichia coli	2	Septicemia (x1); pneumonia (x1)
Pseudomonas sp.	1	Pneumonia (x1)
Stenotrophomonas sp.	2	Pneumonia (x2)
Acinetobacter sp.	1	Pneumonia (x)
Mixed organisms: n = 3 (9%)		
Coagulase-negative staphylococci and *Enterococcus* sp.	1	Septicemia (x1)
Klebsiella sp. and *Candida parasilosis*	1	Septicemia (x1)
E. coli and *Enterococcus* sp.	1	NEC (x1)

There were also six patients with proven NEC (stage 2 or higher) of whom two had small bowel perforation and histologic confirmed NEC and four had clinical pneumonia with definitive radiographic changes and increased serial serum CRP concentrations (>10 mg/L), but no organism was identified.

Table 4. *Levels of chemokines and cytokines at 0 h and 24 h in the infected and noninfected groups*

	Infected (group1): 44 episodes	Noninfected (group 2): 111 episodes	*p*-value
0 h			
Chemokines (pg/mL)			
IL-8	236 (66–667)	50 (36–97)	<0.001
IP-10	3555 (2077–17733)	804 (550–1073)	<0.001
MIG	1930 (710–4529)	420 (243–632)	<0.001
MCP-1	924 (279–4520)	224 (152–406)	<0.001
GRO-α	143 (60–654)	45 (29–72)	<0.001
RANTES	5738 (2089–9850)	9999 (4325–16945)	0.001
Cytokines (pg/mL)			
IL-1β	2.7 (0.0–31.7)	0.0 (0.0–17.8)	0.12
IL-6	157.5 (31.8–4006.2)	10.3 (5.3–19.9)	<0.001
IL-10	60.8 (25.6–313.5)	4.0 (2.4–6.4)	<0.001
IL-12p70	4.7 (0.0–10.3)	2.4 (0.0–5.6)	0.019
TNF-α	1.8 (0.0–3.3)	0.0 (0.0–1.9)	0.005
24 h			
Chemokines (pg/mL)			
IL-8	87 (51–357)	57 (38–107)	0.012
IP-10	2695 (1499–4539)	792 (570–1127)	<0.001
MIG	1448 (695–3317)	385 (224–566)	<0.001
MCP-1	289 (147–725)	218 (149–378)	0.055
GRO-α	55 (41–194)	42 (28–66)	0.001
RANTES	4938 (1527–8500)	8106 (3446–18471)	0.021
Cytokines (pg/mL)			
IL-1β	0.0 (0.0–14.3)	0.0 (0.0–21.4)	0.29
IL-6	25.8 (9.2–449.7)	9.4 (5.2–21.2)	<0.001
IL-10	16.0 (5.6–39.3)	3.5 (2.3–5.4)	<0.001
IL-12p70	2.1 (0.0–4.4)	3.0 (0.0–4.8)	0.56
TNF-α	0.0 (0.0–2.1)	0.0 (0.0–1.7)	0.44

Results are median (interquartile range).

combination of favorable markers (*i.e.* the best six markers) suggested that the use of multiple markers only marginally improved the sensitivity of IP-10 by 0% to 7%, but adversely affected the specificity by 13%–50% (Table 6).

DISCUSSION

This study evaluated a comprehensive panel of key chemokines and cytokines in preterm infants with systemic infection. To our knowledge, only two studies have so far reported on these relatively new chemokines (IP-10, MIG, MCP-1, and GRO-α) in healthy preterm infants (25) and newborns with perinatal infection (26), but none has evaluated them as diagnostic markers of neonatal infection. The latter study focused on term and preterm infants with perinatal asphyxia and described only a relatively small number of patients with infection ($n = 10$) (26). Thus, the current trial represented a much larger and homogeneous cohort and specifically targeted evaluation of these inflammatory mediators as diagnostic markers in late-onset infection. Our findings revealed that four of the studied markers (IP-10, MIG, IL-6, and IL-10) could achieve a sensitivity >80% and specificity >75% (Table 5). IP-10 with a calculated optimal cutoff value ≥1250 pg/mL

Table 5. *Comparison of sensitivity, specificity, positive and negative predictive values, and area under the ROC curve between different diagnostic markers at 0 h and 24 h*

Diagnostic markers	Cutoff values (pg/mL)	0 h					24 h				
		Sen	Sp	PPV	NPV	AUC	Sen	Sp	PPV	NPV	AUC
Chemokines											
IL-8	≥62	0.80	0.60	0.44	0.88	0.78	0.66	0.54	0.35	0.81	0.63
IP-10	≥1250	0.93	0.89	0.77	0.97	0.95	0.88	0.83	0.66	0.95	0.90
MIG	≥650	0.80	0.78	0.59	0.91	0.84	0.78	0.79	0.58	0.91	0.83
MCP-1	≥357	0.68	0.68	0.46	0.84	0.78	0.46	0.74	0.40	0.79	0.60
GRO-α	≥55	0.82	0.60	0.45	0.89	0.81	0.48	0.67	0.35	0.77	0.68
RANTES	≤11,800	0.86	0.45	0.38	0.89	0.67	0.81	0.33	0.31	0.82	0.62
Cytokines											
IL-1β	≥3.7	0.50	0.63	0.35	0.76	0.57	0.29	0.60	0.21	0.69	0.44
IL-6	≥26.1	0.82	0.82	0.64	0.92	0.88	0.48	0.82	0.50	0.81	0.69
IL-10	≥7.6	0.84	0.84	0.67	0.93	0.90	0.69	0.84	0.62	0.88	0.81
IL-12p70	≥2.7	0.64	0.56	0.36	0.80	0.62	0.43	0.44	0.23	0.67	0.45
TNF-α	≥0.6	0.57	0.61	0.37	0.78	0.63	0.38	0.64	0.29	0.73	0.54

Sen, sensitivity; Sp, specificity; PPV, positive predictive value; NPV, negative predictive value; AUC, area under the curve.

Table 6. *Comparison of sensitivity, specificity, positive and negative predictive values of different combinations of markers at the onset of sepsis evaluation (0 h)*

	0 h			
Diagnostic markers	Sen	Sp	PPV	NPV
IP-10 or IL-6	0.98	0.72	0.58	0.99
IP-10 or IL-6 or IL-10	0.98	0.61	0.50	0.99
IP-10 or IL-10	0.98	0.76	0.61	0.99
IP-10 or GRO-α	0.96	0.58	0.47	0.97
IP-10 or GRO-α or IL-8	1.00	0.39	0.39	1.00
IP-10 or IL-8	0.98	0.53	0.45	0.98
IP-10 or IL-8 or MIG	0.98	0.44	0.41	0.98
IP-10 or MIG	0.93	0.76	0.60	0.97
IP-10 or GRO-α or IL-10	0.98	0.51	0.44	0.98
IP-10 or GRO-α or MIG	0.96	0.47	0.42	0.96

exhibited the best diagnostic utilities both at 0 h and 24 h. Using this cutoff value, IP-10 was able to diagnose all septicemic and NEC episodes but missed three pneumonia cases; two of the latter cases grew *Stenotrophomonas* sp. and one grew *Pseudomonas* sp. from the endotracheal aspirate specimens. The pneumonia cases tended to have a modest increase in circulating IP-10 levels [median (interquartile range): 1962 (1119–4342) pg/mL] compared with NEC [2810 (1473–8324) pg/mL] and septicemic cases [7805 (2422–22,379) pg/mL]. Three infants died of sepsis in this cohort, and all had markedly elevated plasma IP-10 concentrations (range: 23,817–34,415 pg/mL). We speculate that the severity of infection might be associated with the magnitude of upregulation of circulating IP-10. Such a phenomenon has also been documented in other Th1-associated inflammatory diseases in which levels of IP-10 correlated with the tissue infiltration of T lymphocytes and the severity of illness (9,27). In addition, we explored the possibility of using multiple markers for the diagnosis of late-onset infection. IP-10 in combination with IL-6, IL-8, IL-10, MIG, or GRO-α could only marginally improve the sensitivity and negative predictive value but adversely affected the specificity and positive predictive value of the test (Table 6). Theoretically, CRP should not be assessed as an infection marker in this study because serial CRP concentrations were used as one of the indicators for classification of sepsis. Nonetheless, using the conventional cutoff value >10 mg/L, CRP correctly diagnosed 29 of 44 infected cases at 0 h, and thus confirmed our suspicion that it was not a very sensitive early marker of infection (6). The overall findings suggested that IP-10 was the best marker for identifying late-onset infection and NEC in VLBW infants at the onset of sepsis (0 h). Measurement of plasma IP-10 concentration can be efficiently performed on an *ad hoc* basis using a minimal volume of plasma (50 μL), and the result can be made assessable within 4 h after blood sampling (7,8,21). The cost of the reagents for measurement of IP-10 was estimated to be about US$6.55 per test in our study.

Our findings indicated that plasma concentrations of all mediators, with the exceptions of IL-1β and RANTES, were substantially increased at a very early stage of infection. These elevated levels, however, fell precipitously within 24 h following antibiotic treatment (Table 4). In contrast, RANTES behaved differently, with plasma levels decreasing significantly during infection. Such a pattern of response was in accordance with the results observed in animal studies (28,29) and adult patients with severe sepsis (9,10,30). The early and marked increase (or decrease) of these inflammatory mediators and the narrow window of opportunity ($<$24 h) for obtaining a blood specimen implied that only the initial sample collected at the onset of sepsis presentation (0 h) would likely be of value for diagnostic purposes. These inflammatory mediators should, therefore, be considered as early warning markers of neonatal sepsis (6–8).

IP-10 acts *via* the chemokine receptor CXCR3 and plays a vital role in the generation and delivery of an effector T-cell response (27). A recent study in newborn infants suggested that serum levels of IL-8, IP-10, and macrophage inflammatory protein-1α (MIP-1α) were significantly increased in birth asphyxiated (IL-8 and IP-10) and perinatally infected neonates (26). Conversely, serum MCP-1 was not found to be significantly elevated (26). The discrepancy of results compared with our observations is probably related to their relatively small cohort of infected patients studied ($n = 10$) (26). The current data provide strong evidence that preterm VLBW infants are capable of eliciting a robust chemokine response to bacterial infection and NEC.

An intrinsic limitation of assessing new infection markers in preterm infants is the accuracy in classification of pneumonia cases (23,31). First, unlike adult patients with lower respiratory tract infection in whom chest radiographic features are usually well defined and microbiologic culture of secretion can be easily obtainable by induction of sputum production, the radiographic appearance of pneumonia in preterm infants is often difficult to be differentiated from bronchopulmonary dysplasia or complications associated with chronic mechanical ventilation. Further, secretion from the lower respiratory tract can only be successfully obtained in intubated infants. Even microorganisms retrieved from endotracheal specimens may be considered as contamination or colonization. Second, focal lung consolidation is a localized infection and may, therefore, reflect in the circulation as a milder inflammatory reaction (32) than systemic infection such as septicemia with or without disseminated intravascular coagulation. In this study and as well as in our previous trials (6,21,23), we took elaborate precautions to minimize misclassification of these cases by 1) providing a predetermined and stringent definition of pneumonia, 2) blinding investigators to the identity of patients and results of the studied infection markers during the classification procedure, 3) employing the latest digital technology in enhancing the radiographic image quality, and 4) having two independent assessors for interpreting the radiographic changes (23). Thus, the classification process has been designed to be as stringent and objective as possible. In the current study, an intermediate increase in inflammatory mediator levels in pneumonia cases supported a mild/moderate systemic immunologic response rather than misclassification of cases, as otherwise one would expect plasma inflammatory mediator concentrations to be distributed at the two extreme levels (*i.e.* markedly elevated concentrations representing gen-

uine pneumonia cases and low serum concentrations indicating falsely labeled episodes). The exclusion of pneumonia cases in the analysis would definitely improve the diagnostic utilities and sensitivity of the markers. However, it would defeat the objective of this study because recruitment and analysis of all cases should be consecutive and nonselective (31). Furthermore, none of the plasma inflammatory mediator concentrations showed a significant reduction from 0 h to 24 h in the noninfected group, indicating that the majority of noninfected cases had not been misclassified.

Our results suggest that preterm infants have the ability to induce a robust chemokine and cytokine response during sepsis. We have provided evidence that IP-10 is a valuable infection marker for predicting late-onset neonatal infection. Plasma IP-10 with an optimal cutoff value $\geq$1250 pg/mL measured at clinical presentation could diagnose all cases of septicemia and NEC. As these mediators are regulated very early in the infection cascade, only the first blood sample taken at the onset of clinical presentation (0 h) is likely to be of value for diagnostic purposes. At this stage, despite the high sensitivity and negative predictive value of IP-10, most frontline neonatologists would be reluctant to withhold antibiotic treatment for sick infants at the onset of sepsis presentation. However, low plasma IP-10 concentration could be used as an indicator to confidently discontinue antimicrobial treatment at a very early phase in noninfected patients. In the next step, we plan to perform a randomized, controlled study to assess the clinical usefulness of a combination of promising markers that we had previously assessed, including IL-6 (6,24), IL-10 (24), neutrophil CD64 (21,23), and IP-10, for the diagnosis of infection and prediction of prognosis in VLBW infants with severe sepsis. The flow cytometric technique for quantitative measurement of inflammatory mediators is a powerful tool that might eventually develop into a routine screening test for neonatal sepsis once the procedure has been validated across laboratories.

REFERENCES

1. Stoll BJ, Hansen N, Fanaroff AA, Wright LL, Carlo WA, Ehrenkranz RA, Lemons JA, Donovan EF, Stark AR, Tyson JE, Oh W, Bauer CR, Korones SB, Shankaren S, Laptook AR, Stevenson DK, Papile LA, Poole WK 2002 Late-onset sepsis in very low birth weight neonates: the experience of the NICHD Neonatal Research Network. Pediatrics 110:285–291
2. Stoll BJ, Hansen NI, Adams-Chapman I, Fanaroff AA, Hintz SR, Vohr B, Higgins RD National Institute of Child Health and Human Development Neonatal Research Network 2004 Neurodevelopmental and growth impairment among extremely low birth-weight infants with neonatal infection. JAMA 292:2357–2365
3. Ng PC, Wong HL, Lyon DJ, So KW, Liu F, Lam RK, Wong E, Cheng AF, Fok TF 2004 Combined use of alcohol hand rub and gloves reduces the incidence of late onset infection in very low birthweight infants. Arch Dis Child Fetal Neonatal Ed 89:F336–F340
4. Siu YK, Ng PC, Fung SC, Lee CH, Wong MY, Fok TF, So KW, Cheung KL, Wong E, Cheng AF 1998 Double blind, randomised, placebo controlled study of oral vancomycin in prevention of necrotising enterocolitis in preterm, very low birthweight infants. Arch Dis Child Fetal Neonatal Ed 79:F105–F109
5. Schultz C, Temming P, Bucsky P, Göpel W, Strunk T, Härtal C 2004 Immature anti-inflammatory response in neonates. Clin Exp Immunol 135:130–136
6. Ng PC, Cheng SH, Chui KM, Fok TF, Wong MY, Wong W, Wong RP, Cheung KL 1997 Diagnosis of late onset neonatal sepsis with cytokines, adhesion molecules, and C-reactive protein in preterm very low birthweight infants. Arch Dis Child Fetal Neonatal Ed 77:F221–F227
7. Ng PC 2004 Diagnostic markers of infection in neonates. Arch Dis Child Fetal Neonatal Ed 89:F229–F235
8. Ng PC, Lam HS 2006 Diagnostic markers for neonatal sepsis. Curr Opin Pediatr 18:125–131
9. Cavaillon JM, Adib-Conquy M, Fitting C, Adrie C, Payen D 2003 Cytokine cascade in sepsis. Scand J Infect Dis 35:535–544
10. Luster AD 1998 Chemokine-chemotactic cytokines that mediate inflammation. N Engl J Med 338:436–445
11. Maghazachi AA 2000 Intracellular signaling events at the leading edge of migrating cells. Int J Biochem Cell Biol 32:931–943
12. Ghosh S, Latimer RD, Gray BM, Harwood RJ, Oduro A 1993 Endotoxin-induced organ injury. Crit Care Med 21:S19–S24
13. Marchant A, Alegre ML, Hakim A, Pierard G, Marecaux G, Friedman G, De Groote D, Kahn RJ, Vincent JL, Goldman M 1995 Clinical and biological significance of interleukin-10 plasma levels in patients with septic shock. J Clin Immunol 15:266–273
14. van Dissel JT, van Langevelde P, Westendorp RG, Kwappenberg K, Frolich M 1998 Anti-inflammatory cytokine profile and mortality in febrile patients. Lancet 351:950–953
15. Miller EJ, Cohen AB, Nagao S, Griffith D, Maunder RJ, Martin TR, Weiner-Kronish JP, Sticherling M, Christophers E, Matthay MA 1992 Elevated levels of NAP-1/interleukin-8 are present in the airspaces of patients with the adult respiratory distress syndrome and are associated with increased mortality. Am Rev Respir Dis 146:427–432
16. Gogos CA, Drosou E, Bassaris HP, Skoutelis A 2000 Pro- versus anti-inflammatory cytokine profile in patients with severe sepsis: a marker for prognosis and future therapeutic options. J Infect Dis 181:176–180
17. Ng PC, Li K, Wong RP, Chui K, Wong E, Li G, Fok TF 2003 Proinflammatory and anti-inflammatory cytokine responses in preterm infants with systemic infections. Arch Dis Child Fetal Neonatal Ed 88:F209–F213
18. Hodge G, Hodge S, Haslam R, McPhee A, Sepulveda H, Morgan E, Nicholson I, Zola H 2004 Rapid simultaneous measurement of multiple cytokines using 100 μL sample volumes-association with neonatal sepsis. Clin Exp Immunol 137:402–407
19. Rossi D, Zlotnik A 2000 The biology of chemokines and their receptors. Annu Rev Immunol 18:217–242
20. Alam R 1998 Chemokines in the biology of cell movement and inflammation. In: Middleton E Jr, Reed CE, Ellis EF, Adkinson NF Jr, Yunginger JW, Busse WW (eds) Allergy: Principles and Practice. Mosby, St. Louis, pp 124–136
21. Ng PC, Li K, Wong RP, Chui KM, Wong E, Fok TF 2002 Neutrophil CD64 expression: a sensitive diagnostic marker of late-onset nosocomial infection in very low birthweight infants. Pediatr Res 51:296–303
22. Walsh MC, Kliegman RM, Fanaroff AA 1988 Necrotizing enterocolitis: a practitioner's prospective. Pediatr Rev 9:219–226
23. Ng PC, Li G, Chui KM, Chu WC, Li K, Wong RP, Chik KW, Wong E, Fok TF 2004 Neutrophil CD64 is a sensitive diagnostic marker for early-onset neonatal infection. Pediatr Res 56:796–803
24. Ng PC, Li K, Leung TF, Wong RP, Li G, Chui KM, Wong E, Cheng FW, Fok TF 2006 Early prediction of sepsis-induced disseminated intravascular coagulation with interleukin-10, interleukin-6, and RANTES in preterm infants. Clin Chem 52:1181–1189
25. Sullivan SE, Staba SL, Gersting JA, Hutson AD, Theriaque D, Christensen RD, Calhoun DA 2002 Circulating concentrations of chemokines in cord blood, neonates, and adults. Pediatr Res 51:653–657
26. Fotopoulos S, Mouchtouri A, Xanthou G, Lipsou N, Petrakou E, Xanthou M 2005 Inflammatory chemokine expression in the peripheral blood of neonates with perinatal asphyxia and perinatal or nosocomial infections. Acta Paediatr 94:800–806
27. Dufour JH, Dziejman M, Liu MT, Leung JH, Lane TE, Luster AD 2002 IFN-γ-inducible protein 10 (IP-10; CXCL10)–deficient mice reveal a role for IP-10 in effector T cell generation and trafficking. J Immunol 168:3195–3204
28. Kopydlowski KM, Salkowski CA, Cody MJ, van Rooijen N, Major J, Hamilton TA, Vogel SN 1999 Regulation of macrophage chemokine expression by lipopolysaccharide in vitro and in vivo. J Immunol 163:1537–1544
29. Frangogiannis NG, Mendoza LH, Smith CW, Michael LH, Entman ML 2000 Induction of the synthesis of the C-X-C chemokine interferon-γ-inducible protein-10 in experimental canine endotoxemia. Cell Tissue Res 302:365–376
30. Møller AS, Bjerre A, Brusletto B, Joø GB, Brandtzaeg P, Kierulf P 2005 Chemokine patterns in meningococcal disease. J Infect Dis 191:768–775
31. Ng PC 2005 Clinical trials for evaluating diagnostic markers of infection in neonates. Biol Neonate 87:111–112
32. Ng PC, Lam CW, Li AM, Wong CK, Cheng FW, Leung TF, Hon EK, Chan IH, Li CK, Fung KS, Fok TF 2004 Inflammatory cytokine profile in children with severe acute respiratory syndrome. Pediatrics 113:e7–e14