Circulating Concentrations of Chemokines in Cord Blood, Neonates, and Adults

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ABSTRACT

Chemokines are critical for the movement of leukocytes. Chemotaxis is deficient in neonates, particularly those delivered prematurely, and this likely contributes to their increased vulnerability to sepsis. The concentrations of circulating chemokines in neonates have not been reported, nor is it known whether low chemokine concentrations contribute to their defective chemotaxis. We hypothesized that serum concentrations of chemokines 1) would be lower in preterm than term neonates, and 2) would be lower in preterm and term neonates than adults. Samples were obtained from preterm and term neonates with normal neutrophil and eosinophil counts, umbilical cord blood samples from pregnancies without clinical evidence of intra-amniotic infection, and healthy adult volunteers. The concentrations of epithelial neutrophil activating peptide-78, growth-related oncogene-α, eotaxin, RANTES (regulated upon activation, normal T cell expressed and secreted), and macrophage inflammatory protein-1α were measured using specific ELISA. Serum concentrations from preterm infants were either similar to or higher than those measured in term neonates and adults. We conclude that the chemotactic defect observed in premature neonates is not the result of diminished circulating concentrations of any of the specific chemokines we measured. (*Pediatr Res* 51: 653–657, 2002)

Abbreviations:
ENA-78, epithelial neutrophil activating peptide-78
GRO-α, growth-related oncogene-α
MIP-1α, macrophage inflammatory protein-1α
RANTES, regulated upon activation, normal T cell expressed and secreted

Neonatal sepsis is a life-threatening event affecting 3–5 neonates per 1000 live births (1). The susceptibility of neonates to serious bacterial and fungal infections is related, in part, to quantitative and qualitative neutrophil deficiencies (2). Neutrophil migration, or chemotaxis, involves the recruitment of neutrophils from the circulation, their adherence to vascular endothelial cells, and their migration through the vessel wall to the site of microbial invasion (3). Important elements in this process are the chemotactic cytokines collectively known as chemokines. These are members of a family of homologous proteins with molecular weights in the range of 8–12 kD (4, 5).

IL-8 is the most extensively studied chemokine in neonates (6), but no information exists regarding the circulating concentrations of other leukocyte-specific chemokines during the neonatal period. Because preliminary *in vitro* studies suggest that perturbations in the concentration of chemokines are, in part, responsible for deficiencies in leukocyte chemotaxis (7), we quantified the circulating concentrations of specific leukocyte chemokines in cord blood sera/plasma and in the sera/plasma of uninfected preterm and term neonates. On the basis of the chemotactic deficiencies of neonates, we hypothesized that the serum concentrations of leukocyte chemokines would 1) be lower in preterm neonates than in term neonates and 2) be lower in term neonates than in adults. Using specific ELISA, we measured the concentrations of neutrophil-specific α-chemokines—ENA-78 and GRO-α—as well as eosinophil-, basophil-, and monocyte-specific β-chemokines—RANTES, eotaxin, and MIP-1α.

MATERIALS AND METHODS

Subjects

Preterm (*n* = 50) and term neonates (*n* = 50) without evidence of infection, who had neutrophil and eosinophil counts within normal limits (8, 9), were studied during their

first 3 d of life. Cord blood sera from preterm ($n = 50$) and term pregnancies ($n = 50$) without clinical evidence of intra-amniotic infection and healthy adults were also studied.

Sample Preparation

ENA-78, GRO-α, and RANTES. Samples of serum or plasma (citrate, EDTA, or heparin as the anticoagulant) were collected after centrifuging blood samples for 10 min at 1000 $\times g$. Serum was immediately separated into aliquots and stored at $-20°C$ until assay. When plasma was used, an additional centrifugation step was performed at $10,000 \times g$ for 10 min to insure removal of platelets. All samples for ENA-78 were then diluted at least 2-fold using Calibrator Diluent RD6−1 (R & D Systems, Minneapolis, MN, U.S.A.). All samples for RANTES were diluted at least 50-fold using Calibrator Diluent RD5H (R & D Systems).

Eotaxin and MIP-1α. Samples of serum or plasma (citrate or EDTA as the anticoagulant) were collected after centrifuging for 10 min at 1000 $\times g$. The samples were then separated into aliquots and stored at $-20°C$ until assay.

All samples were thawed on ice at the time of assay. No repeated freezing and thawing of samples occurred. Informed consent was obtained from all women who donated cord blood and from all adult volunteers. In regard to the samples from term and preterm neonates, the only information obtained about the infants was gestational age and postnatal age; no information was collected about the clinical characteristics. All specimens from neonates were the residual blood from samples collected for laboratory tests, after completion of these tests. Therefore, informed consent did not need to be obtained according to the discarded specimen policy at the University of Florida.

ELISA Determinations

Serum concentrations of each chemokine were quantified by specific ELISA using a double-antibody sandwich technique, following the manufacturer's instructions (Quantikine ENA-78, GRO-α, RANTES, eoxtaxin, and MIP-1α, R & D Systems). The volume of sample required and the upper and lower limits of detection are shown in Table 1.

Data Analysis

Descriptive statistics are given by the median and quartiles. Overall group comparisons between preterm neonates, term neonates, preterm cord blood, term cord blood, and adults were carried out for each chemokine using the Kruskal-Wallis rank-based tests. If the overall p value was ≤ 0.05, pairwise comparisons were carried out using the Bonferroni correction. Values above the upper limit of detection were truncated to the limit of detection for the rank analysis.

RESULTS

The concentrations of each chemokine are shown in Figure 1. Box plots, a five-number summary including the minimum, the lower quartile (25th percentile), median, upper quartile (75th percentile), and maximum values, were used to display the data.

ENA-78. Preterm cord blood and term cord blood concentrations of ENA-78 were higher than preterm neonatal and adult concentrations ($p < 0.005$ for each). There was no difference when preterm concentrations were compared with term ($p = 0.37$), or when either preterm or term concentrations were compared with adult values ($p = 0.72$ and 0.58, respectively).

GRO-α. Preterm cord blood concentrations were higher than concentrations in adults ($p < 0.005$) but similar to concentrations in term cord blood. There was no difference between preterm cord blood concentrations and preterm neonatal concentrations ($p = 0.08$). Whereas GRO-α concentrations in preterm neonates were higher than adult concentrations ($p < 0.005$), there was no difference between preterm and term neonatal concentrations ($p = 0.18$) or between term neonatal concentrations and adult concentrations ($p = 0.08$).

RANTES. Concentrations of RANTES in preterm cord blood were lower than those in term cord blood ($p < 0.005$). Preterm and term neonatal concentrations were similar to each other ($p = 0.06$) and similar to adult RANTES concentrations ($p = 0.40$ and 0.12, respectively).

Eotaxin. Concentrations of eotaxin in preterm cord blood, term cord blood, and term neonates were lower than concentrations in adults ($p < 0.005$ for each). However, there were no significant differences between eotaxin concentrations in preterm neonates when compared with term neonates ($p = 0.16$) or adults ($p = 0.26$).

MIP-1α. All cord blood and neonatal concentrations of MIP-1α were higher than concentrations in adults ($p < 0.005$). Whereas term neonatal concentrations were higher than term cord blood MIP-1α concentrations ($p < 0.005$), preterm neonatal and preterm cord blood concentrations were similar ($p = 0.25$). Preterm neonatal concentrations of MIP-1α were similar to term neonatal concentrations ($p = 0.03$), and preterm cord blood concentrations were similar to term cord blood concentrations ($p = 0.01$).

DISCUSSION

Each of the leukocyte chemokines we studied was measurable in the serum or plasma of preterm and term neonates. Concentrations of these α-chemokines in preterm neonates were either higher than or the same as those in term neonates or adults. Therefore, the chemotactic defect of neonatal neutrophils is not the result of a deficiency in the circulating concentrations of the α-chemokines we evaluated. Because ELISA for granulocyte chemotactic peptide-2, neutrophil acti-

Table 1. *Relevant information for each chemokine ELISA*

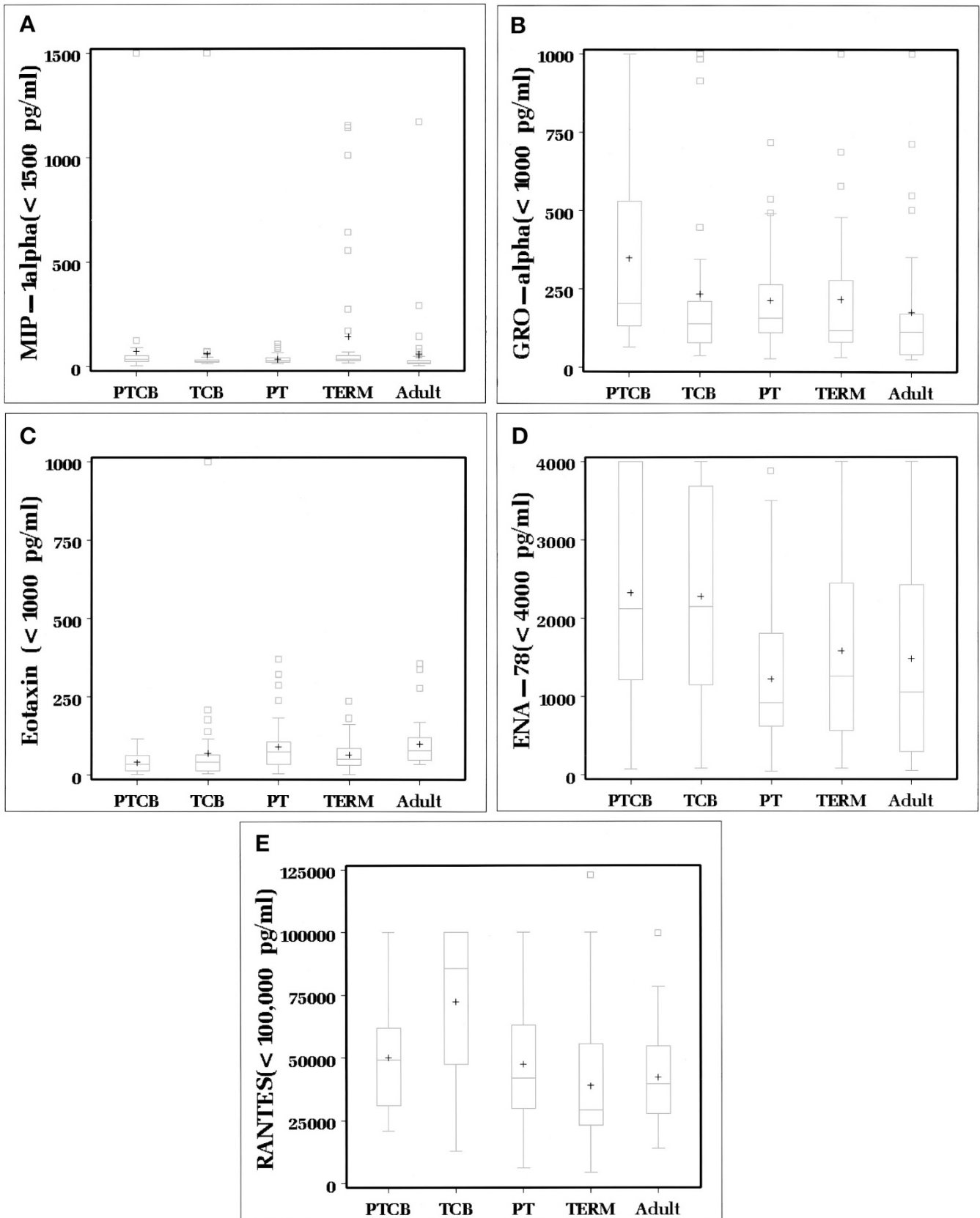| Chemokine	Volume (μL) of serum required	Lower limit of detection (pg/mL)	Upper limit of detection (pg/mL)
ENA-78	50 (requires 2-fold dilution)	62.4	4000
GRO-α	200	31.2	1000
Eotaxin	50	15.6	1000
MIP-1α	200	46.9	1500
RANTES	100 (requires 50-fold dilution)	156	100 000

Figure 1. Concentrations of MIP-1α, GRO-α, eotaxin, ENA-78, and RANTES in preterm cord blood (*PTCB*), term cord blood (*TCB*), preterm serum/plasma (*PT*), term serum/plasma (*TERM*), and adult serum/plasma (*Adult*) presented as box plots. The means are marked by asterisks (*), and the medians are designated by *horizontal lines* through the central box. The upper and lower hinges (*H*) comprise the edges of the central box representing the interquartile range. The H spread is the absolute value of the differences between the values of the two hinges. The fences ($\perp$) show the range of the values that fall within 1.5 H spreads of the hinges. Values outside the inner fences ($\pm$1.5 H spread) are plotted as open boxes ($\square$).

vating peptide-2, and lipopolysaccharide (LPS)-induced CXC (cysteine-x-cysteine) chemokine were not commercially available at the time of this study, we cannot rule out that deficiencies in other α-chemokines (4) might be responsible for the chemotactic defect of neonatal neutrophils. Another potential explanation for α-chemokine concentrations in preterm infants not differing from those in term infants and adults could be related to the process of preterm delivery. Cytokine concentrations in umbilical cord blood can be higher than in maternal blood (10).

Concentrations of the β-chemokines, RANTES and eotaxin (specific for eosinophils, basophils, and monocytes), were noted to be less in certain comparisons. RANTES concentrations in preterm cord blood were significantly less than concentrations in term cord blood, and eotaxin concentrations in preterm and term cord blood and term neonatal samples were all significantly less than adult concentrations.

Little is known regarding what directs the migration of leukocytes under unperturbed conditions, during stress, or during infection. Even less is known about circulating concentrations, or production, of chemokines during the neonatal period. Dammann *et al.* (11) measured MIP-1α and RANTES along with 14 other markers of inflammation in cord blood and postnatal serum from extremely low gestational age newborn infants. Using recycling immunoaffinity chromatography, they found that MIP-1α and RANTES were considerably higher in preterm samples than published values in term newborns (8.2 and 11.4 pg/mL *versus* 5.0 and 5.0 pg/mL, for preterm and term MIP-1α and RANTES, respectively) (12). These results differ from our results in that we found no difference between preterm and term neonatal MIP-1α concentrations or RANTES concentrations. Several potential explanations exist. First, these differences might reflect differences in the assays used to determine chemokine concentrations in each situation. Recycling immunoaffinity chromatography utilizes whole blood samples, whereas the ELISA in this study used serum or plasma samples. Second, Dammann and colleagues also chose a more select group of preterm neonates (<28 wk), whereas our study population of preterm neonates included all neonates <37 wk. Finally, we excluded any samples from women with known intra-amniotic infection. The influence of these discrete variables merits further investigation.

The production of RANTES and MIP-1α *in vitro* by cord blood mononuclear cells was examined by Hariharan *et al.* (7). They found a decreased ability of mononuclear cells in term cord blood to secrete RANTES when stimulated with LPS when compared with adult mononuclear cells. RANTES is predominantly produced by a subset of lymphocytes (CD8$^+$/ CD45RO$^+$) that are fewer in term cord blood than in adult blood. Hariharan and colleagues hypothesized that the decreased number of these cells might explain the poor secretion of RANTES in term cord blood samples. In addition, they did not observe a difference in the production of MIP-1α when LPS-stimulated mononuclear cells from term cord blood samples were compared with adult samples. The findings by Hariharan and colleagues appear inconsistent with our reported circulating concentrations of RANTES and MIP-1α. One possible explanation for this is that we measured concentrations of chemokines in noninfected subjects, and they measured the production of chemokines in LPS-stimulated cells.

Another potential explanation for the differences between our study and that of Hariharan *et al.* (7) is that, as with other cytokines, *in vitro* stimulation of mononuclear cells as a measure of the ability of cells to produce cytokines might not correlate with *in vivo* cytokine production. Schibler *et al.* (13) found that *in vitro* production of granulocyte colony-stimulating factor by monocytes stimulated with LPS was lower using cells from preterm neonates compared with term neonates and adults. They speculated that this defect in production of granulocyte colony-stimulating factor might contribute to a defective up-regulation of neutrophil production and function during infection. However, when granulocyte colony-stimulating factor concentrations were measured in the serum of neonates with neutropenia and confirmed bacterial sepsis, they were significantly higher than those of neonates who were neutropenic and uninfected (14).

Studies of circulating concentrations of chemokines in adults during sepsis suggest that perturbations in chemokine concentrations occur. Cummings *et al.* (15) evaluated the plasma concentrations of IL-8, GRO-α, and ENA-78 in adults during sepsis. The median IL-8 concentration in patients with sepsis was 157 pg/mL, whereas levels were undetectable in noninfected subjects ($p < 0.005$). The median concentration of GRO-α was 170 pg/mL in the plasma of patients with sepsis compared with 30 pg/mL in controls ($p = 0.07$). In contrast, the median concentration of ENA-78 did not differ in patients with and without sepsis. Knapp *et al.* (16) evaluated the prognostic value of MIP-1α, transforming growth factor-β2, soluble endothelial leukocyte adhesion molecule-1, and soluble vascular adhesion molecule-1 in patients with Gram-positive sepsis. Concentrations of MIP-1α were significantly higher than concentrations in healthy control patients (>50 pg/mL *versus* 25 pg/mL). Much remains to be learned about the expression of chemokines in adults and neonates during infection.

Chemotaxis is a complicated process involving activated cells, intact functional receptors, and corresponding ligands. The influence of circulating chemokines on leukocyte chemotaxis is only one element of this process. The present study demonstrates that chemokines are measurable in cord blood serum and neonatal serum and that concentrations are generally similar to those found in adults.

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