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Decreased G-CSF and IL-3 Production and Gene Expression from Mononuclear Cells of Newborn Infants[1]

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ABSTRACT. Newborns are predisposed to neutropenia and thrombocytopenia during bacterial sepsis. The presence of peripheral cytopenias during overwhelming infection may be secondary to decreased hematopoietic growth factor production during states of increased demand. We therefore examined circulating levels of granulocyte-colony stimulating factor (G-CSF) and IL-3, production of G-CSF and IL-3 from unstimulated and stimulated mononuclear cells (MNC), expression of G-CSF and IL-3 genes during unstimulated and stimulated conditions, and equilibrium and binding of G-CSF receptors on mature effector peripheral blood cells of adults and neonates. Serum from cord and adult peripheral blood contained negligible amounts of both G-CSF (≤50 pg/mL) and IL-3 (≤5 pg/mL). Constitutive supernatant levels of G-CSF and IL-3 from cord and adult unstimulated MNC were also undetectable. However, there was a significant difference in G-CSF and IL-3 production from stimulated cord and adult MNC. Supernatants from stimulated adult MNC had significantly more G-CSF ($p < 0.007$) and IL-3 ($p < 0.02$). Additionally, Northern blot hybridization and densitometry of autoradiographs demonstrated significantly more G-CSF and IL-3 mRNA transcripts from adult than from cord MNC. Lastly, affinity, binding, and number of G-CSF receptors on cord and adult peripheral effector cells were equal. These data suggest that, during states of increased demand, cord MNC produce less G-CSF and IL-3 than do adult MNC and have an associated reduction in their respective mRNA transcripts. These findings may have implications in the pathogenesis of neonatal cytopenias during states of increased demand, such as sepsis. (*Pediatr Res* 31: 574–578, 1992)

Abbreviations

CSF, colony stimulating factor
G-CSF, granulocyte-colony stimulating factor
rhG-CSF, recombinant human granulocyte-colony stimulating factor
MNC, mononuclear cell
CFU-GM, colony forming unit-granulocyte macrophage
PMN, polymorphonuclear cell

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During states of increased demand for blood cells, such as during overwhelming bacterial sepsis, newborn infants tend to develop neutropenia because of reduced mature effector neutrophil storage pools, reduced myeloid progenitor pools (CFU-GM), and accelerated steady state myeloid progenitor proliferative rates (1–4). Additionally, neonatal mature effector neutrophils have qualitative deficiencies, especially with respect to chemotaxis, phagocytosis, C3bi receptor expression, and bacterial killing compared with adult neutrophils (1, 5). During experimental group B streptococcal sepsis, newborn rats develop profound neutropenia secondary to depletion of bone marrow neutrophil storage pool reserves and reduced proliferation of CFU-GM progenitor pools compared with those of adult animals (4, 6, 7).

Regulation of hematopoiesis is developmentally immature in the newborn (8, 9). The control of hematopoiesis is in part regulated by CSF and IL. G-CSF, a lineage-specific hematopoietic growth factor, stimulates myeloid progenitor proliferation, induces egress of mature neutrophil storage pool cells from the bone marrow into the peripheral blood, and enhances mature neutrophil effector function (10–12). IL-3 is less lineage-specific and stimulates hematopoiesis at an earlier progenitor cell stage, resulting in the formation of multiple lineages, including colony forming unit-granulocyte, erythroid, monocyte, megakaryocyte; CFU-GM; colony forming unit-megakaryocyte; and burst forming unit-erythroid (13, 14).

We previously demonstrated that single-pulse administration of rhG-CSF to neonatal rats results in neutrophilia, induces neutrophil proliferative pools, and acts prophylactically or simultaneously with antibiotics to reduce mortality during experimental group B streptococcal infection (15, 16). Prolonged administration of rhG-CSF to neonatal rats modulates neonatal myelopoiesis, resulting in neutrophilia, increases marrow neutrophil storage pools, and reduces mortality during experimental group B streptococcal infection (17). Recently, we demonstrated that prolonged administration of recombinant murine IL-3 to newborn rats does not induce neutrophilia, but does increase bone marrow myeloid progenitor pools (18). The present study was undertaken to determine the difference, if any, between human G-CSF and IL-3 production and gene expression from human neonatal and adult peripheral MNC.

MATERIALS AND METHODS

Isolation of human MNC and PMN. Heparinized venous blood was drawn from normal volunteers in accordance with the

principles of the Declaration of Helsinki. Venous cord blood was drawn from the umbilical vessels of placentas of normal, full-term, nonstressed infants immediately after vaginal delivery (10%) or scheduled cesarean section without labor (90%) (19, 20). Six percent Dextran-70 in 0.9% saline (McGaw Laboratories, Irvine, CA) was added to both the cord and donor whole blood samples, and their red blood cells were allowed to sediment. The leukocyte-enriched plasma was collected, layered on a Ficoll-Hypaque gradient ($p = 1.077$), and centrifuged for 30 min. MNC were collected from the Ficoll interface, and PMN cells were recovered in the cell pellet. Any remaining erythrocytes were hemolyzed by hypotonic lysis. Isolated MNC and PMN were then suspended in Dulbecco's (D-PBS), pH 7.4, or PBS, pH 7.4, with 5 mM glucose (PBS-G) at various concentrations depending on particular assay requirements. PMN were 98% pure in cord and adult preparations by flow cytometry and microscopy. The MNC distribution was similar in both cord and adult preparations (cord: 82 ± 8.0% lymphocytes and 8.8 ± 4.0% monocytes; adult: 86 ± 4.0% lymphocytes and 7.2 ± 3.0% monocytes). MNC (1×10^6) from both cord and adults were then stimulated with phorbol 12-myristate 13-acetate (20 ng/mL) and *Phaseolus vulgaris* phytohemagglutinin (2 μg/mL) to determine G-CSF production and phorbol 12-myristate 13-acetate (3 ng/mL) ± 0.5 μM A23187 (Sigma Chemical Co., St. Louis, MO) to determine IL-3 production. These stimulants were chosen because they were demonstrated to be the optimal agonists under the described conditions (data not shown). Supernatants were collected at 0, 6, 24, 48, and 72 h and frozen at -70°C for future studies.

ELISA for detecting IL-3. IL-3 levels from serum and from stimulated and unstimulated MNC supernatants were measured with a sandwich ELISA (Immunex, Seattle, WA). Microtiter plates were coated with a mouse anti-rhuIL-3 MAb overnight at 4°C and blocked with nonfat dried milk (Carnation, Los Angeles, CA) in PBS. Samples and standards (Genzyme, Boston, MA) were diluted 1:1 with 10% goat serum in PBS, added to the plates, and incubated for 2 h at room temperature. Plates were washed with 0.05% Tween-20 (Sigma Chemical Co.) in PBS, and rabbit-anti-mouse rhIL-3 was added and incubated for an additional hour. Plates were again washed, developed with 3,3′,5,5′-tetramethylbenzidine (BioRad Laboratories, Richmond, CA) for 30 min, and read at an OD of 690 nm. All samples were run in triplicate, and the data presented as mean ± SEM of eight to 10 separate supernate samples per group.

ELISA for detecting human G-CSF. G-CSF levels from serum and from stimulated and unstimulated MNC culture supernatants were measured by a sandwich ELISA assay (Amgen, Thousand Oaks, CA) Briefly, microtiter plates were coated with polyclonal rabbit anti-rhG-CSF Ig, and rhG-CSF standards and test samples were added and incubated at 37°C overnight. Horseradish peroxidase-conjugated mouse monoclonal anti-G-GSF antibody was added and incubated for 2 h at 37°C. Plates were washed and tetramethylbenzidine was added as the substrate. The reaction was stopped after 30 min by the addition of sulfuric acid (0.5 N). OD of the samples was measured at 450 nm with a BioRad EIA reader. All samples were run in triplicate and the data is presented as the mean ± SEM ($n = 7$).

RNA isolation and Northern blotting. Total RNA was extracted from stimulated and unstimulated cells by the method of Chomczynski *et al.* (21). Cells were collected in a buffer containing guanidinium thiocyanate, sodium citrate, sarcosyl, and mercaptoethanol. Sodium acetate and phenolchloroform were added to the buffer to extract RNA from the DNA and proteins. Samples were then precipitated with isopropanol followed by reprecipitation with ethanol, and contamination of ribonuclease was minimized by dissolving the RNA pellet in 0.5% SDS. Samples were then added to a loading buffer containing orange G and Ficoll (type 400), heated for 15 min at 65°C, and rapidly cooled before the addition of 1 μg/mL ethidium bromide. Samples were run on 1.0% agarose gel in MOPS buffer ± formaldehyde at 50 V overnight. Gels were transferred to nitrocellulose membranes. Filters were baked at 80°C for 2 h and prehybridized in hybridization buffer (50% formamide, 5% × SSC, 2 × Denhardt's, 50 mM sodium phosphate, pH 6.5, 50 μg/mL denatured single-strand salmon sperm DNA, and 0.1% SDS). Filters were hybridized for 18 h in HB ± 10% dextran sulfate to ^{32}P-labeled probes: IL-3 = pHycIL-3–2 (American Type Culture Collection, Rockville, MD); G-CSF = human G-CSF cDNA (about 1.8 kb) at the *Xho*I site of the vector pXM (amp resistant).

Binding of ^{125}I-G-CSF to PMN. Binding assays were performed by a phthalate oil separation method (22). However, isolated PMN were suspended in a binding medium of RPMI-1640 with 2% BSA, 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid buffer, and 0.2% sodium azide (Sigma Chemical Co.), pH 7.2. Cells were placed in 96-well microtiter plates and incubated with ^{125}I-G-CSF (kindly provided by Linda Park, Immunex) in binding medium for 1 h at 37°C. Replicate aliquots of cells were then transferred to a precooled phthalate oil mixture of 1.5 parts dibutylphthalate (Kodak, Rochester, NY) and 1 part bis (2-ethylhexyl) phthalate (Kodak), and cell-bound ^{125}I-cytokines were separated from unbound ^{125}I-cytokine by centrifugation. Radioactivity was measured on an LKB Universal Gamma Counter (Pharmacia, Milwaukee, WI). Nonspecific binding was measured using a 100-fold molar excess of unlabeled G-CSF mixed with radiolabeled G-CSF before the addition of cells. Specific binding is defined as the amount of binding blocked by competition with excess unlabeled cytokines. Data was analyzed by weighted nonlinear least squares curve-fitting developed by Munson and Rodbard (23).

Statistical analysis. Results are expressed as mean values ± SEM of triplicate tests of three to 10 samples. The probability of significant differences when comparing two groups was determined with the use of the unpaired *t* test, and the probability of significant differences when examining multiple groups was determined by using the analysis of variance followed by the Student-Newman-Keuls multiple range tests to define the unique subsets within the study. Statistical analyses were performed using the Biostat I statistical program (Sigma Soft, Placentia, CA) for the IBM personal computer. p values < 0.05 were considered significant.

RESULTS

Serum G-CSF and IL-3 concentrations from both cord and adult peripheral blood ($n = 6$) were below the limit of our detectability (<50 pg/mL for G-CSF and <5 pg/mL for IL-3). Positive G-CSF values included serum from 12 patients with neutropenia after bone marrow transplantation (1000–2500 pg/mL) and additionally were obtained by using commercial rhG-CSF and recombinant human IL-3 incubated control serum. Constitutive supernatant levels of G-CSF and IL-3 from cord and adult unstimulated MNC were undetectable. There was a significant difference, however, in G-CSF and IL-3 levels obtained from stimulated cord and adult MNC. Maximal stimulation occurred between 48 and 72 h. There was significantly less G-CSF at 48 ($p < 0.02$) and 72 h ($p < 0.007$) from cord MNC than from adult cells (Fig. 1). Similarly, there was significantly less IL-3 at 24 h ($p < 0.000\ 1$), 48 h ($p < 0.003$), and 72 h ($p < 0.02$) from stimulated cord MNC than from adult cells (Fig. 2).

Unstimulated (at h 0) cord and adult MNC G-CSF mRNA transcripts were undetectable. However, after stimulation, fewer G-CSF mRNA transcripts were detected from cord MNC than from adult cells (6–24 h) ($n = 3$) (Fig. 3). Similarly, unstimulated (at h 0) cord and adult MNC IL-3 mRNA were undetectable, but, upon stimulation, decreased IL-3 mRNA transcripts were detected from stimulated cord *versus* adult MNC throughout the 24 h of stimulation ($n = 3$) (Fig. 4).

Affinity, binding, and G-CSF receptor numbers on cord and adult peripheral effector cells were measured (Fig. 5). The binding curve and Scatchard analysis of cord and adult neutrophil G-

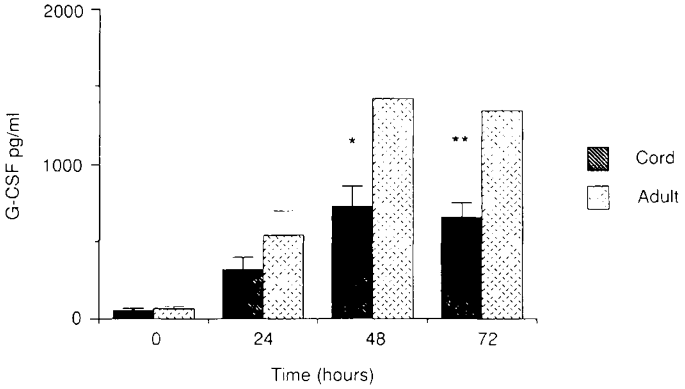

Fig. 1. G-CSF supernatant levels (ELISA) produced from adult and cord MNC (1×10^6) stimulated for 0, 24, 48 and 72 h with phorbol myristate acetate (20 ng/mL) and phytohemagglutinin (2 μg/mL). Data represents mean ± SEM of eight to 10 supernatant samples. *, $p < 0.02$; **, $p < 0.007$ (cord *vs* adult).

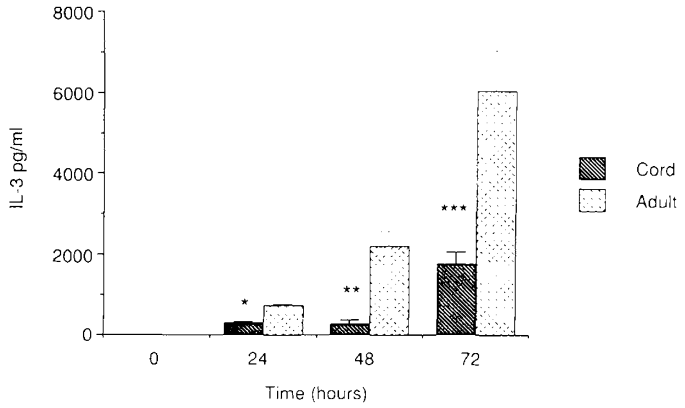

Fig. 2. IL-3 supernatant levels (ELISA) produced from adult and cord MNC (1×10^6) stimulated with phorbol myristate acetate (3 ng/mL) and A23187 (0.5 μM). Data represents mean ± SEM of seven supernatant samples. *, $p < 0.0001$; **, $p < 0.003$; ***, $p < 0.02$ (cord *vs* adult).

CSF receptors revealed similar affinity and equilibrium curves (k_d: 402 ± 65 *versus* 637 ± 52 pM, cord *versus* adult) (p = NS). The number of G-CSF receptors was also similar on cord and adult neutrophils (805 ± 68 *versus* 981 ± 105 sites/cell, cord *versus* adult) (p = NS).

DISCUSSION

Peripheral neutropenia and thrombocytopenia are characteristic findings during overwhelming bacterial sepsis in the newborn infant and may predict a poor outcome (24). During states of increased demand, such as bacterial sepsis, CSF and IL appear to be the major regulators of increased myeloid and megakaryocyte proliferation and maturation.

Recently, rhG-CSF therapy has been demonstrated to correct the neutropenia in infants and children with congenital agranulocytosis (Kostmann syndrome), reverse cyclic neutropenia in both children and adults, and shorten the period of neutropenia after high-dose multiagent chemotherapy (25–27). IL-3 also induces significant changes in hematopoiesis, both in primates and adults. Recombinant human IL-3 administered in primates induced a significant increase in the circulating white blood cell count, platelet count, and reticulocyte count (28). Recently, IL-3 therapy in patients with either advanced malignancy or aplastic anemia has been demonstrated to induce an increase in circulating neutrophils, eosinophils, and platelets (29, 30).

Laver *et al.* (31), using a bioassay, recently reported increased circulating GM-CSF and G-CSF serum levels during steady state

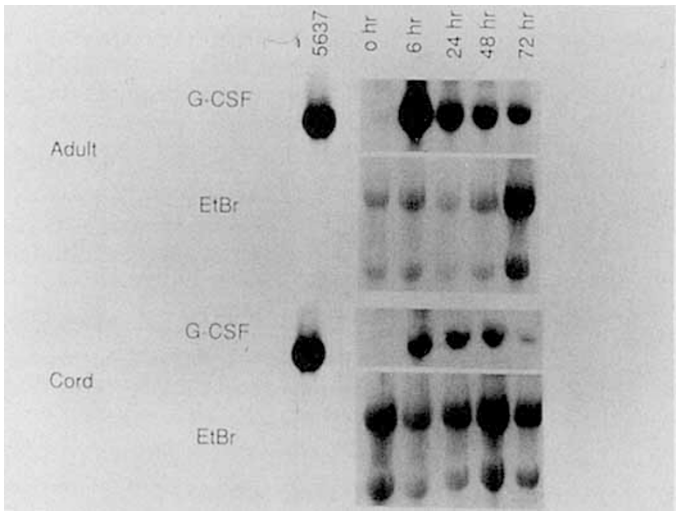

Fig. 3. G-CSF mRNA from unstimulated (at h 0) and stimulated MNC from cord and adult blood. Cells were isolated, stimulated with phorbol myristate acetate (20 ng/mL) and PHA (2 μg/mL), and RNA was extracted. The Northern blot was hybridized with ^{32}P-labeled G-CSF probe (G-CSF cDNA). 5637 is a human bladder carcinoma cell line serving as a positive control for G-CSF (n = 3). Ethidium bromide (*EtBr*) stain was used to determine that the amount of RNA deposited on the gel was equal throughout.

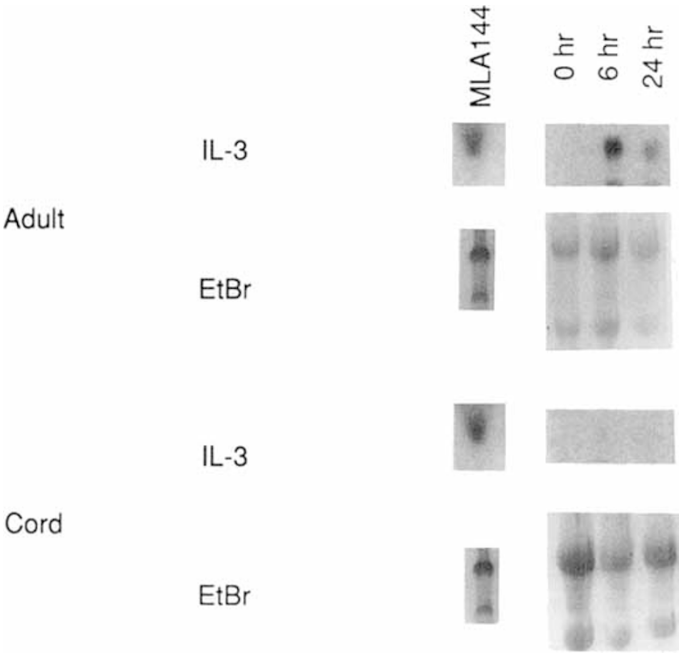

Fig. 4. IL-3 mRNA from unstimulated (at h 0) and stimulated MNC from cord and adult blood. Cells were isolated, stimulated with phorbol myristate acetate (3 ng/mL) and A23 187 (10^{-8} M), and RNA was extracted. The Northern blot was hybridized with ^{32}P-labeled IL-3 probe (pHuclL33–2). *MLA144* is a gibbon lymphoma cell line serving as a positive control for IL-3 (n = 3). Ethidium bromide (*EtBr*) stain was used to determine that the amount of RNA deposited on the gel was equal throughout.

conditions from cord compared with adult peripheral blood. When they used an anti-G-CSF MAb, a 28% residual activity remained, suggesting that other growth factors may be influencing the results of their bioassay. Serum levels of G-CSF and IL-3 measured by an immunologic assay, however, may not correlate completely with biologic activity. Our results, using a specific ELISA assay, demonstrated negligible circulating G-CSF levels

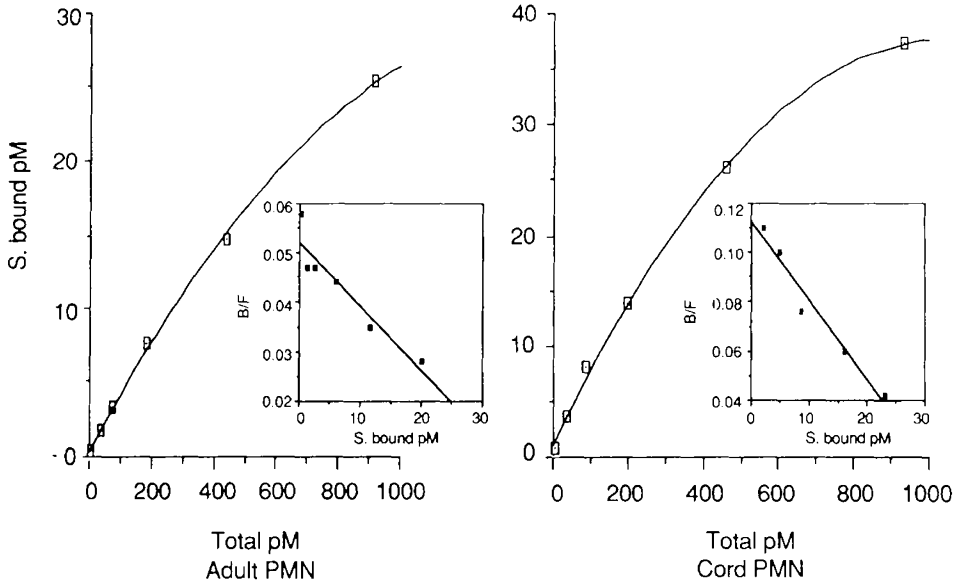

Fig. 5. Specific binding of ^{125}I-labeled G-CSF to PMN from cord and adult peripheral blood. The amount of nonspecific binding not blocked by competition with a 100-fold excess of unlabeled G-CSF was substrated from the total binding. *Insert*, Scatchard analysis of the equilibrium binding data.

in both cord and adult serum. Additionally, during states of inactivation, supernatants from peripheral MNC from both cord and adult had negligible amounts of G-CSF. Similarly, our study has shown negligible amounts of circulating IL-3 in the serum of cord and adult peripheral blood; constitutive supernatant levels of IL-3 were also undetectable. However, during specific agonist stimulation, there was a significant difference in both G-CSF and IL-3 production by cord and adult MNC.

Although the present study demonstrated decreased G-CSF and IL-3 production from stimulated cord *versus* adult MNC, total neonatal G-CSF and IL-3 production may be somewhat different because of our inability to measure these cytokines from other sources, such as endothelial cells and fibroblasts. Additionally, our study did not determine whether the decrease in G-CSF or IL-3 production from cord MNC was secondary to a decrease per MNC or a decrease in the specific subpopulation of G-CSF and IL-3 producing MNC. These results are consistent with our previous findings demonstrating negligible amounts of cord serum GM-CSF and decreased GM-CSF production after stimulation of cord MNC (32).

Analysis of G-CSF and IL-3 mRNA expression from cord MNC by Northern blot hybridization confirms negligible G-CSF and IL-3 mRNA expression during constitutive or resting conditions. However, upon stimulation of cord MNC, there was a significant reduction in G-CSF mRNA transcript expression during the entire 24-h period of stimulation and barely detectable IL-3 mRNA transcript expression during a similar time period. This reduced CSF mRNA transcript expression is similar to that in our previous studies analyzing the regulation of GM-CSF mRNA between cord and adult MNC (32).

We have previously analyzed the mechanisms associated with decreased GM-CSF mRNA expression from stimulated cord MNC. Nuclear run-on transcriptional events and actinomycin D half-life studies (posttranscriptional events) have suggested reduced stabilization of GM-CSF mRNA and reduced GM-CSF mRNA half-life (32). The decrease in G-CSF and IL-3 protein production and mRNA expression from cord MNC may be secondary to a similar mechanism such as the previously described dysregulation of cord MNC GM-CSF, but further studies are needed to corroborate this. Reduced G-CSF and IL-3 mRNA message during states of activation may lead to decreased cytokine production during overwhelming stress, precipitating peripheral neutropenia and a subsequent increase in morbidity. Further studies are needed to test this hypothesis.

We conclude that serum and constitutive supernatant levels of G-CSF and IL-3 are negligible and undetectable in cord and adult peripheral blood. However, during states of increased demand (*i.e.* after agonist stimulation), there is significantly less G-CSF and IL-3 production by neonatal than adult MNC. The clinical significance of this reduction of G-CSF and IL-3 remains to be determined. The affinity and the number of G-CSF receptors on cord and adult peripheral effector cells, however, were similar. Reduced G-CSF and IL-3 production during states of increased demand may play a role in the pathogenesis of neutropenia and thrombocytopenia that occurs during bacterial infection in the newborn. Further studies are needed to assess the relationship of the present findings with the abnormalities observed during overwhelming bacterial sepsis.

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