0031-3998/00/4803-0369
PEDIATRIC RESEARCH
Vol. 48, No. 3, 2000
Printed in U.S.A.

Effect of Granulocyte and Granulocyte Macrophage Colony Stimulating Factors (G-CSF and GM-CSF) on Neonatal Neutrophil Functions

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ABSTRACT

Although there are many studies on the effect of granulocyte and granulocyte-macrophage colony stimulating factors (G/GM-CSF) on adult neutrophil functions, there is little information regarding their influence on neonatal cells. We studied the *in vitro* effect of G/GM-CSF on neutrophil chemotaxis, polarization, and superoxide anion generation in 47 neonates compared with 35 adults. We found that G-CSF and GM-CSF significantly enhanced the chemotaxis of newborn infants' neutrophils, normalizing their chemotactic defect [from 35 ± 7 cells/field (mean ± SE) to 49 ± 5 cells/field with G-CSF, $p < 0.05$ and to 55 ± 4 cells/field with GM-CSF, $p < 0.001$]. It is notable that the maximal neutrophil response to the cytokines was observed particularly in the newborn infants with severe impairment in their chemotactic activity. Statistical analysis of the data showed a significant inverse correlation, which supported this observation ($r = -0.6$, $p < 0.02$ for G-CSF; $r = -0.76$, $p < 0.001$ for GM-CSF). The reduced polarization of neonatal compared with adult cells [71 ± 5% *versus* 86 ± 2% (mean ± SE), $p < 0.05$], was corrected by CSF-priming (to 87 ± 4% with G-CSF and to 92 ± 2% with GM-CSF, $p < 0.05$). In addition, the neutrophil superoxide generation was significantly improved in both groups following the CSF-priming. GM-CSF and G-CSF gave comparable results in all functions studied except that GM-CSF improved superoxide release to a greater extent. This study shows a significant improvement of the neonatal neutrophil functions following *in vitro* CSF-priming and contributes to a better understanding of the neonatal neutrophil behavior when treated with G/GM-CSF. **(*Pediatr Res* 48: 369–373, 2000)**

Abbreviations

VLBW, very low birth weight
G-CSF, granulocyte colony stimulating factors
GM-CSF, granulocyte-macrophage colony stimulating factors
PMN, polymorphonuclear leukocytes
HBSS, Hanks' buffered salt solution
fMLP, N-formyl-methionyl-leucyl-phenylalanine
PVP, polyvinylpyrrolidone
GLM, general linear model
PMA, phorbol myristate acetate
NSP, neutrophil storage pool

Neonatal sepsis continues to be a major cause of morbidity and mortality in the nursery and the neonatal intensive care unit, particularly in the VLBW newborn infant (1, 2). Despite the aggressive supportive treatment and the appropriate use of antibiotics, the mortality rate remains high. New forms of adjunctive immunotherapy are needed. Neutropenia and depleted neutrophil storage pools have been found to be associated with neonatal bacterial sepsis and are predictive of a poor prognosis (3–6). Neutrophil functions were also reported to be impaired in newborn infants (2, 7–11).

Hematopoietic growth factors, G-CSF, and GM-CSF, are soluble glycoproteins that regulate the proliferation and differentiation of hematopoietic precursor cells (12–14). CSF have been found to exert functional effects on neonatal and adult neutrophils (15–23). Additionally, in adult neutrophils G-CSF and GM-CSF appear to have different profiles of activity (15, 19).

Hematopoietic CSF have been shown to improve the clinical condition of patients with neutropenias and to reduce the length of neutropenia following chemotherapy and bone marrow transplantation (24–29). In addition, because neonatal neutrophils have been found to produce less G-CSF than adults (30–33), supportive therapy with hematopoietic growth factors has been used in an effort to improve quantitative and qualitative defects of neonatal immunity (34–38).

There are few reports on the effects of G/GM-CSF on neonatal neutrophil functions (20–22, 34, 35). We studied the *in vitro* effects of CSF on neutrophil chemotaxis, polarization, and superoxide anion generation in isolated cells of neonates compared with adults.

Received June 8, 1999; accepted April 27, 2000.
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METHODS

The study enrolled 47 healthy full-term newborn infants from healthy nonsmoking mothers, appropriate for gestational age, after an uneventful pregnancy and delivery. The study also included 35 healthy adult volunteers. Five milliliters of venous peripheral blood were obtained from 2- to 5-d-old newborn infants and 10 mL were withdrawn from the adult group. Assays were performed simultaneously in neonates and adults. We tested the neutrophil random migration, chemotactic activity, polarization, and superoxide anion release before and after preincubation with colony stimulating factors. All experiments were performed in duplicates.

The Helsinki Committee at Meir General Hospital, Sapir Medical Center, Kfar Sava, Israel, approved the study, and parental signed consent was obtained.

Isolation of polymorphonuclear leukocytes

Human purified PMN (98%) were isolated from heparinized venous blood. After sedimentation with 3% dextran (MW 250.000; Sigma Chemical Co., St. Louis, MO, U.S.A.), the leukocyte enriched-plasma was layered on to a Ficoll-Hypaque gradient (Lymphoprep; Nycomed Pharma AS, Oslo, Norway) and centrifuged at 400 g for 30 min, as previously described (39). The supernatant was discarded and the pellet was subjected to hypotonic lysis for 20 s to free the PMN from contaminating red blood cells. The PMN were resuspended for chemotaxis in M199 medium (Earle's salt with L-glutamine; Biologic Industries, Kibutz Beth Haemek, Israel) and for superoxide anion release and neutrophil morphology in HBSS (Sigma Chemical Co.) with 10 nM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; Sigma Chemical Co.).

Priming neutrophils with G-CSF and GM-CSF

Optimal dosage and time exposure of neutrophil CSF priming were previously evaluated in our laboratory for each assay and results obtained are in accordance with previous reports (19, 40, 41). Priming for chemotaxis was done with 0.3 ng/mL G-CSF and 0.4 ng/mL GM-CSF, at 37°C for 15 min. For polarization, priming was done with 3 ng/mL G-CSF and 1.5 ng/mL GM-CSF, at 37°C for 15 min. Priming for superoxide production was done with 10 ng/mL G-CSF and 1 ng/mL GM-CSF, at 37°C in CO_2 environment, for 2 h. As basal level we used medium without the addition of G/GM-CSF.

The reported therapeutic serum levels that were achieved in patients treated with G/GM-CSF are in the range of those we used *in vitro* for neutrophil priming (37, 42).

Chemotaxis assay

A 48-well chemotaxis-microchamber (Neuro Probe, Inc., Bethesda, MD, U.S.A.) was used to determine random migration and chemotaxis (43). The chemoattractant (fMLP; Sigma Chemical Co.) was suspended in the medium M199 at a concentration of 1 μM. Either the medium alone or the chemoattractant was added to the bottom wells. A polycarbonate membrane filter, PVP-free, with 3-μm pores (Nucleopore Corp., Pleasanton, CA, U.S.A.), was placed on top of the wells in the bottom plate. The gasket and the top plate were affixed, and 50 μL of 10^6 PMN/mL were added to the upper wells. The assembly was incubated for 60 min in humidified air. After incubation the filter was wiped off and stained with May-Grunwald-Giemsa dye. The average number of cells in nine fields was counted under light microscopy with a 20× objective and an optical grid at 10× magnification. Net chemotaxis was calculated by subtracting the random migration from the chemotactic activity.

Neutrophil morphology

Assessment of neutrophil polarization was done according to our previous studies (7). Following priming with or without G/GM-CSF, cells of neonates and adults were stimulated by adding 100 nM fMLP solution (final concentration). Cell morphology was captured by the addition of equal volume of ice-cold 10% formaldehyde in HBSS, supplemented with 10 nM HEPES, pH 7.4. The rate of polarized cells was calculated using a phase-contrast microscope.

Superoxide anion release

This assay was performed as previously reported (18). PMN (10^6) were suspended in HBSS with 60 μM of ferricytochrome C (Sigma Chemical Co.), with (control) or without 214 U of superoxide dismutase (Sigma Chemical Co.). The rate of superoxide anion release was measured by adding 100 nM of fMLP (Sigma Chemical Co.) for 5 min, at 37°C, in a UV-260 Shimadzu spectrophotometer (Kyoto, Japan), at 550 nm. The superoxide anion release was calculated using the Massey extinction coefficient of 2.1×10^4 M^{-1} cm-1.

Statistical analysis

Mann-Whitney nonparametric test, the two-tailed *t* test (paired or unpaired), and the GLM repeated measures procedure were used for data analysis of all leukocyte functions, where appropriate.

RESULTS

Effect of G-CSF and GM-CSF on neutrophil net chemotaxis

The results of net chemotaxis are shown in Table 1. The net neonatal chemotaxis was significantly lower than that found in adult neutrophils ($p < 0.001$). GM-CSF priming induced significant increase of net neutrophil chemotaxis of neonates ($p < 0.001$) and adults ($p < 0.01$). A similar trend in the neutrophil

Table 1. *Priming effect of growth factors on FMLP-stimulated neutrophil net chemotaxis (cells/field)*

	Medium	G-CSF (0.3 ng/mL)	GM-CSF (0.4 ng/mL)
Adults	61 ± 4	101 ± 11*‡	79 ± 5*‡
(*n*)	(13)	(8)	(10)
Neonates	35 ± 7§†	49 ± 5†‡	55 ± 4†‡
(*n*)	(27)	(16)	(21)

Values are mean ± standard error.
* $P < 0.02$; † $P < 0.001$.
‡ CSF *vs* medium.
§ Neonates *vs* adults.

chemotactic activity was observed in neonates ($p < 0.001$) and adults ($p < 0.02$) following neutrophil G-CSF priming. Although neonatal chemotaxis after GM-CSF priming achieved levels similar to those found in the adult neutrophil (mean ± SE: 55 ± 4 cells/field *versus* 61 ± 4 cells/field in neonates and adults, respectively, $p = 0.19$), priming with G-CSF only reached partial correction (49 ± 5 cells/field *versus* 61 ± 4 cells/field in neonates and adults, respectively, $p < 0.05$).

It is notable that the maximal neutrophil response to cytokines was particularly observed in those newborn infants with severe impairment in their chemotactic activity. Statistical analysis of the data showed a significant inverse correlation between the basal chemotactic level and the result following CSF priming, which supported this observation ($r = -0.6, p < 0.02$ for G-CSF; $r = -0.76$; $p < 0.001$ for GM-CSF).

The random migration was not affected by the addition of CSF. GM-CSF seems to be more potent than G-CSF in neonatal cell chemotaxis, inasmuch as only the former brought about a complete recovery of the reduced neutrophil chemotaxis.

Morphologic response

Neonatal neutrophils were significantly less responsive than adult cells to optimal concentrations of 100 nM fMLP (Table 2) ($p < 0.05$). CSF significantly increased the fMLP-stimulated polarization of both neonatal and adult cells ($p < 0.05$, $p < 0.005$, respectively), blunting the differences between the basal neonatal and adult neutrophil response. CSF by themselves had a significant stimulating effect on neonatal and adult neutrophils ($p < 0.05$ and $p < 0.005$, respectively).

Effects of GM-CSF and G-CSF on neutrophil superoxide anion release

Neonatal neutrophils had significantly higher fMLP-induced superoxide generation ($p < 0.02$) than adult cells, as previously reported (7).

Preexposure of neutrophils to the cytokines for 2 h enhanced significantly the fMLP- stimulated superoxide anion release of 14 newborn infants and 16 adult volunteers (Table 3). The enhancement was significantly higher using GM-CSF than G-CSF, on both adult ($p < 0.001$) and neonatal cells ($p < 0.05$). For neonatal neutrophils, GM-CSF brought about an increase of 89% of superoxide production, whereas G-CSF enhanced the superoxide release by only 52%. In the adult neutrophils, the effect of the CSF was more evident; the superoxide production by neutrophils increased by 207% and 56% with GM-CSF and G-CSF, respectively. GM-CSF was more potent than G-CSF in the superoxide generation, in both neonatal and adult neutrophils, a finding already reported in adult neutrophils (19, 41).

Table 2. *Priming effect of growth factors on neutrophil polarization (%)*

fMLP	Medium	G-CSF (3 ng/mL)	GM-CSF (1.5 ng/mL)
Adults ($n = 9$)			
+	86 ± 2	95 ± 1†‡	95 ± 1†‡
−	4 ± 1	45 ± 6†‡	53 ± 5†‡
Neonates ($n = 6$)			
+	71 ± 5*§	87 ± 4*‡	92 ± 2*‡
−	4 ± 1	33 ± 10†‡	26 ± 9*‡§

Values are mean ± standard error.
* $P < 0.05$; † $P < 0.005$.
‡ CSF *vs* medium.
§ Neonates *vs* adults.

Table 3. *Priming effect of growth factors on FMLP-stimulated neutrophil superoxide production (nmol $O_2^-/10^6$ PMN/min)*

	Medium	G-CSF (10 ng/mL)	GM-CSF (1 ng/mL)	*P* value (G *vs* GM-CSF)
Adults	1.47 ± 0.15	2.29 ± 0.24†‡	4.52 ± 0.23†‡	<0.001
(*n*)	(16)	(7)	(7)	
Neonates	2.09 ± 0.19*§	3.18 ± 0.2*‡	3.95 ± 0.0.36*‡	<0.05
	(14)	(9)	(5)	

Values are mean ± standard error.
* $P < 0.01$; † $P < 0.001$.
‡ CSF *vs* medium.
§ Neonates *vs* adults.

DISCUSSION

CSF were shown to affect *in vitro* functions of adult neutrophils (15–19, 40, 41, 44–48), but little information is available regarding their effect on the neonatal cells (20–22). The mechanisms by which hematopoietic CSF alter PMN function are not well defined, but it has been proposed that they modulate cytoskeletal structures as well as the number and affinity of neutrophil receptors (16, 44–47), particularly fMLP receptors (40, 47, 48), which mediate the functions studied here.

For normal chemotaxis the biochemical and biophysical integrity of the cytoskeleton and the cell membrane are essential (8, 47–50). We evaluated the effect of CSF on neonatal neutrophil chemotaxis and polarization. Our results revealed significant enhancement of the net chemotaxis of neonatal and adult neutrophils. In parallel, the PMN polarization significantly increased after CSF priming, emphasizing the close relationship between proper cytoskeleton function and optimal cell motility.

There are conflicting reports on the effect of G/GM-CSF on the adult *in vitro* and *in vivo* neutrophil chemotaxis (15); whereas some investigators reported decreased neutrophil chemotaxis following neutrophil priming with CSF *in vitro* (23, 28, 47), or *in vivo* (17, 51), others have shown significant enhancement of the chemotactic activity (19, 40, 52). In neonates few reports are available; Hill *et al.* reported no effect of G/GM-CSF on the neutrophil chemotaxis of three babies (20), whereas Frenck *et al.* found a dose-related response—significant enhancement of the neonatal neutrophil locomotion at low GM-CSF concentrations but inhibition at high concentrations (21). The discrepancy between the studies done in neonates and adults could reflect different priming conditions, distinct CSF dosage, and time exposure (19, 21, 40), which were found to be critical for achieving the optimal responses (15, 40, 41). Another aspect in neonates is the presence of neutrophil subpopulations, which could reflect different stages of maturation and thus functional responses (53–55). Supporting this neonatal cell heterogeneity, we and others found a remarkably wide range of neutrophil functions in neonates

compared with adults (2, 7, 8, 53). Furthermore, whereas some newborns disclosed severely impaired cell chemotaxis, others showed normal activity. Hematopoietic CSF have been shown to enhance the myeloid series maturation and function (12–14) thus improving the cells' functional capability. The current study shows a significant inverse correlation between the basal chemotactic activity of neonatal neutrophils and their response to G/GM-CSF priming, possibly reflecting maturation and homogenization of the neutrophil population.

Another finding of this study is the significant enhancement of superoxide release by priming neonatal and adult neutrophils with G-CSF and GM-CSF. This positive effect of CSF on the neutrophil superoxide release is in accordance with studies done in newborn infants *in vivo* (34, 35) and *in vitro* (21, 34). In contrast in adults, although the *in vitro* studies showed a consistent significant enhancement of fMLP-superoxide generation following G/GM-CSF priming (16, 40, 41, 48, 56, 57), the *in vivo* superoxide production showed controversial results. Reevaluating the reports we found that although G-CSF administration to adult volunteers showed no effect of PMA and opsonized zymosan on superoxide release (17, 51), an increased neutrophil superoxide generation was achieved by fMLP stimulation (51). The improvement is probably related to the reported increased fMLP receptors' affinity (40, 48). Prematurity, sepsis, and stress were shown to significantly depress the respiratory burst activity of neonatal neutrophils (58); this fact emphasizes the possible advantage of G/GM-CSF administration by enhancing the respiratory oxidative burst.

Clinically, *in vivo* studies performed in the pediatric population (34–38, 59) showed that the administration of recombinant G-GM/CSF brought about a significant increase in neutrophil counts and NSP. Further, a significant reduction (61%) in the incidence of nosocomial infections in VLBW was reported (34). Exceptionally, Schibler *et al.* (60) reported no effect of G-CSF administration on neutrophil counts and maturation and in the outcome of preterm and full-term septic babies. In contrast to others, these investigators pooled together preterm with full-term neonates in the analysis of the data. Moreover, while they treated septic babies with G-CSF only for 3 d, Goldman *et al.* and Cairo *et al.* (34, 38) administered GM-CSF prophylaxis to VLBW infants for 28 consecutive days. Another explanation might be derived from differences in mechanisms of action of G-CSF and GM-CSF.

We found that GM-CSF was more potent than G-CSF in enhancing the neonatal and adult neutrophil functions. Other investigators also reported similar results in adults (19, 41, 57). These cytokines differences seem to be related to different mechanisms of action (41, 57) and were reported to have important clinical implications (13, 19).

It is well known that newborn infants have an increased susceptibility to bacterial infections. The reason for that seems to be multifactorial (2, 7, 8, 10, 11). G/GM-CSF, which play a major role in the regulation of the granulopoiesis, were found to be decreased in the neonate (27, 30–32). There is consensus about the fact that administration of hematopoietic CSF induces peripheral blood neutrophilia in several conditions (24–27, 36–38). This study also shows a significant improvement of the neonatal neutrophil functions following *in vitro* CSF priming and contributes to a better understanding of the neonatal neutrophil behavior when treated with G/GM-CSF.

Acknowledgments. We thank Dr. Jacob Nusbacher for valuable discussions and advice during the preparation of this manuscript and to Navah Jelin, M.A., for the statistical analysis of the data.

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