Results of a Phase I/II Trial of Recombinant Human Granulocyte-Macrophage Colony-Stimulating Factor in Very Low Birthweight Neonates: Significant Induction of Circulatory Neutrophils, Monocytes, Platelets, and Bone Marrow Neutrophils

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Neonates, especially those of very low birthweight (VLBW), have an increased risk of nosocomial infections secondary to deficiencies in development. We previously demonstrated that granulocyte-macrophage colony-stimulating factor (GM-CSF) production and mRNA expression from stimulated neonatal mononuclear cells are significantly less than that from adult cells. Recombinant murine GM-CSF administration to neonatal rats has resulted in neutrophilia, increased neutrophil production, and increased survival of pups during experimental Staphylococcus aureus sepsis. In the present study, we sought to determine the safety and biologic response of recombinant human (rhu) GM-CSF in VLBW neonates. Twenty VLBW neonates (500 to 1,500 g), aged <72 hours, were randomized to receive either placebo (n = 5) or rhuGM-CSF at 5.0 µg/kg once per day (n = 5), 5.0 µg/kg twice per day (n = 5), or 10 µg/kg once per day (n = 5) given via 2-hour intravenous infusion for 7 days. Complete blood counts, differential, and platelet counts were obtained, and tibial bone marrow aspirate was performed on day 8. Neutrophil C3bi receptor expression was measured at 0 and 24 hours. GM-CSF levels were measured by a sandwich enzyme-linked immunosorbent assay at 2, 4, 6, 12, and 24 hours after the first dose of rhuGM-CSF. At all doses, rhuGM-CSF was well tolerated, and there was no evidence of grade III or IV toxicity. Within 48 hours of administration, there was a significant increase in the circulating absolute neutrophil count (ANC) at 5.0 µg/kg twice per day and 10.0 µg/kg once per day, which continued for at least 24 hours after discontinuation of rhuGM-CSF. When the ANC was normalized for each patient's first ANC, there was a significant increase in the ANC on days 6 and 7 at each dose level. By day 7, all tested doses of rhuGM-CSF resulted in an increase in the absolute monocyte count (AMC) compared with placebo-treated neonates. In those receiving rhuGM-CSF 5.0 µg/kg twice per day, there was additionally a significant increase in the day 7 and platelet count. Tibial bone marrow aspirates demonstrated a significant increase in the bone marrow neutrophil storage pool (BM NSP) at 5.0 µg/kg twice per day and 10.0 µg/kg once per day. Neutrophil C3bi receptor expression was significantly increased 24 hours after the first dose of rhuGM-CSF at 5.0 µg/kg once per day. The elimination half-life (T1/2) of rhuGM-CSF was 1.4 ± 0.8 to 3.9 ± 2.0 hours. In summary, this phase I/II trial demonstrated that 7-day administration of rhuGM-CSF in VLBW neonates appeared to be safe and well tolerated and induced a significant increase in the ANC, AMC, platelet count, neutrophil C3bi receptor expression, and BM ASP. A multicenter, randomized, prospective, double-blinded, placebo-controlled phase III trial is currently under way to determine whether prophylactic administration of rhuGM-CSF will reduce nosocomial infections in high-risk VLBW neonates.

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HOST DEFENSE in the neonate is characterized by an immaturity of phagocytic, humoral, and cellular immunity. Developmental changes in hematopoiesis and phagocytic immunity predispose the infected neonate to neutropenia, neutrophil dysfunction, and overwhelming bacterial sepsis. In term infants, the risk of bacterial sepsis is approximately 1 to 10 per 1,000 live births, and the mortality rate varies from 15% to 75%. The risk of sepsis, especially nosocomial infection, is significantly higher in very low birthweight (VLBW) neonates, occurring in 25% or more of those weighing between 500 and 1,000 g, and in 14% of those weighing between 1,000 and 1,500 g. Indwelling catheters, invasive procedures, prolonged hospitalization, and exposure to resistant organisms additionally predispose the VLBW neonate to a high incidence of nosocomial infection.

Recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF) stimulates the proliferation and egress of committed myeloid progenitor cells and mature neutrophils from the bone marrow and induces functional activation of mature neutrophils. We have previously demonstrated a significant decrease in GM-CSF gene expression and protein production from activated mononuclear cells obtained from human term neonates compared with adults. A defect in neonatal mononuclear cell GM-CSF gene expression appears to be secondary to an alteration in GM-CSF mRNA posttranscriptional stability and may predispose the neonate to reduced committed myeloid progenitor pools, a significant decrease in the bone marrow neutrophil storage pool (BM NSP: polymorphonuclear leukocytes [PMN] + bands + metamyelocytes) and, therefore, an increased tendency to neutropenia during overwhelming bacterial sepsis. Although activated neonatal mononuclear cells have decreased GM-CSF gene expression and protein production, neonatal neutrophils express normal numbers of GM-CSF receptors and, by Scatchard analysis, have similar binding characteristics compared with adult neutrophils. In vitro administration of rhGM-CSF primes neonatal neutrophils for enhanced oxidative metabolism, chemotaxis, and improved...
biologic effects of rhuGM-CSF in VLBW neonates are.

Previously, we investigated the prophylactic effect of recombinant murine (rm) GM-CSF on hematopoiesis in the neonatal rat. Seven days of intraperitoneal administration of rmGM-CSF in neonatal rats resulted in a significant increase in peripheral neutrophils, an increase in BM NSPs, and an increase in the tritiated thymidine suicide rate of bone marrow granulocyte-macrophage colony-forming unit (CFU-GM). More recently, Wheeler and Givner demonstrated that rhuGM-CSF improved the outcome over that with antibiotics alone during experimental Group B streptococcal sepsis in neonatal rats. In pups treated with rhuGM-CSF, there was a 37% mortality rate compared with 67% in those treated with placebo (P < .003). Additionally, French et al demonstrated that intraperitoneal administration of rmGM-CSF to neonatal rats significantly increased survival during experimental S. aureus sepsis compared with animals administered saline (54% vs 10% survival; P < .001).

We hypothesize that rhuGM-CSF, when administered prophylactically, may reduce the morbidity and mortality rate associated with overwhelming bacterial sepsis and nosocomial infection in VLBW neonates. However, the toxicity and the biologic effects of rhuGM-CSF in VLBW neonates are unknown. Therefore, in this trial, we evaluated the safety, pharmacokinetics, and biologic effects of rhuGM-CSF in 20 VLBW neonates.

MATERIALS AND METHODS

Patients. From December 1992 to August 1994, neonates weighing between 500 and 1,500 g who were aged less than 72 hours, at Children’s Hospital of Orange County (Orange, CA), University of Utah Medical Center (Salt Lake City, UT), and Kosair Children’s Hospital, University of Louisville (Louisville, KY), were eligible for study entry. Neonates were excluded from study if they had evidence of a major congenital anomaly or genetic condition, prior or concurrent cytokine therapy, prior or concurrent leukocyte transduction, or known grade III to IV intracranial hemorrhage. This protocol was approved by the Human Subjects Review Committee at all three institutions, and written informed consent was obtained before study entry.

GM-CSF administration. This trial was an open-label, dose-ranging study. The rhuGM-CSF was supplied by Immunex (Seattle, WA; specific activity, 5.6 x 10^6 IU/mg protein) and was diluted with sterile water to a concentration of 10.0 μg/mL. Dilution to less than 10 μg/mL required the use of 0.1% human serum albumin. The rhuGM-CSF solution was infused by microinfusion pump over 2 hours once or twice daily for 7 consecutive days. Twenty neonates (five per group) were randomized to receive either placebo or rhuGM-CSF at 5.0 μg/kg once per day, 5.0 μg/kg twice per day, or 10.0 μg/kg once per day. An additional subject was enrolled at 10 μg/kg twice per day but is not presented here, because one patient does not constitute sufficient data on that dose level.

Blood and marrow studies. Blood was obtained by venipuncture or indwelling catheter for complete blood counts (CBCs) on day 1 at hours 0 and 6 and once daily on days 2 through 12. Hour 0 was defined as the time of study entry. CBCs were performed electronically, and differential leukocyte counts and platelet counts were performed manually on Wright-stained blood films. Serum chemistries (creatinine, blood urea nitrogen, glucose, electrolytes, total bilirubin, serum glutamic-pyruvic transaminase, and calcium) were drawn on days 1 and 8.

All patients underwent tibial bone marrow aspiration on day 8 (24 hours after the last dose of either placebo or rhuGM-CSF). Differential cell counts (250 to 500) were performed on Wright-stained marrow smears. The proportion of nucleated marrow cells identified as myeloblasts, promyelocytes, and myelocytes was termed the bone marrow neutrophil proliferative pool (NPP, %), and the proportion of nucleated marrow cells as metamyelocytes, bands, and neutrophils was termed the bone marrow neutrophil storage pool (NSP, %).

Neutrophil activation. Because of the limited amount of blood that could be drawn in the VLBW babies (including the pharmacokinetic studies), we decided to investigate only C3bi expression as a measure of neutrophil activation. C3bi receptor expression was determined at one central laboratory on samples drawn on day 1 before administration of rhuGM-CSF and 24 hours after the first dose. Blood was collected in EDTA-containing tubes and immediately placed on ice. Next, it was diluted with Hanks’ Balanced Salt Solution without calcium or magnesium (Sigma, St Louis, MO), and erythrocytes were removed by density sedimentation using dextran-70 (Baxter Healthcare, Deerfield, IL). The leukocyte-enriched fraction was removed, and the cell count was performed. Cells (1.5 x 10^6) were washed in Dulbecco’s phosphate-buffered saline supplemented with azide (0.1%) and bovine serum albumin (2%). Cells were labeled for fluorescent-activated analysis with Leu 15 (C3bi receptor) fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody (Becton Dickinson, San Jose, CA). Null controls (unstained cells) and isotypic controls (goat antimouse IgG, conjugated to FITC; Becton Dickinson) were included at each time point. Samples were stained, washed, and preserved with 0.5% paraformaldehyde before analysis. Analysis was performed on a Becton Dickinson FacStar (Mountain View, CA) with gating on the granulocyte population using forward and side scatter parameters. C3bi receptor results are expressed as the percent of time 0 levels.

Pharmacokinetics. Serum for GM-CSF levels was drawn at 2, 4, 6, 12, and 24 hours after the first dose of rhuGM-CSF. The detection of GM-CSF was with a sandwich enzyme-linked immunosorbent assay (ELISA) in which an immobilized GM-CSF-specific monoclonal antibody was bound to GM-CSF in the serum sample. The serum was subsequently labeled by a sheep-derived GM-CSF-specific polyclonal antibody and a peroxidase-labeled ant sheep IgG conjugate. The ELISA was sensitive to concentrations as low as 78 pg/mL (156 pg/mL in small volume samples diluted 2:1) and did not crossreact with other known cytokines or growth factors. Area under the curve (AUC) was calculated using the standard trapezoidal rule. Half-lives were calculated using linear regression on the log of the concentrations. Maximum concentration (Cmax) and time to Cmax (tmax) were calculated using standard noncompartmental analyses.

Statistical analysis. Results are expressed as mean values plus or minus standard error of the mean (SEM). The probability of significant differences was determined using the one-sided, unpaired Student’s t test, while the probability of significant differences when examining multiple groups was determined by using the analysis of variance (ANOVA) followed by the Student-Newman-Keuls multiple range tests to define the unique subsets within the study. Student’s t tests and ANOVAs were all performed as values compared with baseline for specified days and times. Statistical analyses were performed using the InStat (GraphPad Software, San Diego, CA) program for the Macintosh computer (Apple Computers, Cupertino, CA). Values of P less than .05 are considered significant.

RESULTS

There were 20 patients entered and assessable in this trial: 11 males and nine females ranging in gestational age from
Fig 1. ANC response during intravenous rhuGM-CSF dose of 5.0 μg/kg twice per day or 10.0 μg/kg once per day in newborn infants with presumed sepsis (n = 5 per dose). CBC counts were performed electronically, and manual differentials were performed on Wright-stained blood films. *P < .05: 5.0 μg/kg twice per day or 10.0 μg/kg once per day versus placebo; **P < .01: 5.0 μg/kg twice per day or 10.0 μg/kg once per day versus placebo.

The rhuGM-CSF was well tolerated at all doses administered in this study. One patient receiving 5.0 μg/kg twice per day developed grade III disseminated intravascular coagulation secondary to necrotizing enterocolitis on day 8. There were no grade III or IV pulmonary, renal, cardiac, neurologic, gastrointestinal, hepatic, or hematologic toxicities. There were no deaths during the 30-day follow-up of this study.

The rhuGM-CSF induced a significant increase in the absolute neutrophil count (ANC). Within 48 hours of administration, there was a significant increase in the circulating ANC at all doses, which continued for at least 24 hours after discontinuation of rhuGM-CSF (Fig 1). When the ANC was normalized (% baseline ANC) for each patient’s first ANC, there was a significant increase in the day 6 and 7 ANC at each dose level (Fig 2). Relative neutrophilia was sustained at 120 hours after the last dose of rhuGM-CSF at all dose levels.

At a dose level of 10.0 μg/kg once per day, rhuGM-CSF induced a significant increase in the absolute monocyte count (AMC) on days 1, 4, and 6 through 9, which was sustained.
48 hours after the last dose of rhuGM-CSF (Fig 3). By day 7, all dose levels of rhuGM-CSF induced a significant increase in the AMC compared with placebo-treated neonates. There were no significant changes in the hematocrit or absolute eosinophil count at any dose level of rhuGM-CSF compared with placebo. However, in neonates receiving 5.0 µg/kg twice per day of rhuGM-CSF, there was a significant increase in the day 7 and 8 platelet count compared with placebo-treated controls (Fig 4).

At two doses of rhuGM-CSF, there was a significant increase in the BM NSP compared with placebo (5.0 µg/kg twice per day, P < .05; 10.0 µg/kg once per day, P < .01; Fig 5). There was, however, no significant difference in the absolute bone marrow NPP when compared with placebo.

Neutrophil C3bi receptor expression was determined 24 hours after the first dose of rhuGM-CSF. At the 5.0 µg/kg once per day dose of rhuGM-CSF, there was a significant increase in PMN C3bi receptor expression (156% ± 55.4% vs 77% ± 0%, P < .05; Fig 6).

Serum GM-CSF levels by ELISA were determined at 2, 4, 6, 12, and 24 hours after the first dose of rhuGM-CSF. Patients receiving placebo had undetectable levels of rhuGM-CSF in the serum. Table 1 lists the mean AUC, C_max, and elimination half-life for each dose level. Peak levels occurred 2 hours postinfusion and were dose-dependent. By 24 hours after administration of the first dose of rhuGM-CSF, levels were virtually nondetectable.

Three patients had a positive blood culture during the study. One patient treated with placebo developed a positive blood culture for *Staphylococcus epidermidis* on day 8, and two patients treated with rhuGM-CSF had positive blood cultures, one for *S epidermidis* on day 11 and one for *Staphylococcus hemolyticus* on day 6. There were no other patients who developed positive blood cultures during the 10-day observation period of this study.

DISCUSSION

An immaturity in neonatal phagocytic immunity predisposes neonates to the development of neutropenia during overwhelming bacterial sepsis and is associated with an increased mortality rate.\textsuperscript{17-20} Nosocomial infections still account for a major cause of morbidity and mortality in VLBW neonates (≥1.5 kg).\textsuperscript{7} Recently, we have shown that adult donor neutrophil transfusions have improved the survival
There has been limited experience with the use of rhuGM-CSF in children and no experience reported to date of the use of rhuGM-CSF in VLBW neonates. Recombinant human GM-CSF has been used successfully in the treatment of children with aplastic anemia and Fanconi's anemia to ameliorate neutropenia after standard and high-dose chemotherapy and to enhance neutrophil reconstitution after bone marrow transplantation.

We have previously shown that activated mononuclear cells from neonates express and produce decreased amounts of GM-CSF compared with adults. Basal GM-CSF expression and circulating serum levels of GM-CSF are negligible in the neonate. Decreased GM-CSF expression and production during activated conditions in the neonate may predispose the neonate to decreased committed myeloid progenitor pool expansion, reduced NSPs, and a tendency to develop neutropenia.

In the present study, we assessed the safety and biologic efficacy of rhuGM-CSF in VLBW neonates. The use of rhuGM-CSF in VLBW neonates was safe and well-tolerated. Peak serum concentrations occurred at the end of a 2-hour intravenous infusion, were dose-dependent, and were undetectable by 24 hours. The mean elimination half-life ranged between 1.0 ± 0.5 and 3.9 ± 2.8 hours. The elimination half-life after 2 hours' administration in older children receiving chemotherapy at doses ranging between 215 μg/m² and 1,500 μg/m² was similar, ranging between 1.6 and 2.8 hours with a median of 2 hours. In adults receiving 10.0 μg/kg subcutaneously, peak serum GM-CSF levels occurred later (6.3 hours), and the Cmax was less (16.2 ng/mL) compared with the 2-hour intravenous infusion of 10.0 μg/kg/d in VLBW neonates.
Table 1. Pharmacokinetics of rhuGM-CSF After Two-Hour Intravenous Administration in VLBW (500 to 1,500 g) Neonates

<table>
<thead>
<tr>
<th>Dose Level of rhuGM-CSF</th>
<th>AUC</th>
<th>t1/2 (ng/mL)</th>
<th>t1/2 (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 μg/kg once per day</td>
<td>19,267</td>
<td>7.7 ± 6.0</td>
<td>3.9 ± 2.8</td>
</tr>
<tr>
<td>5 μg/kg twice per day</td>
<td>18,972</td>
<td>11.6 ± 11.8</td>
<td>1.0 ± 0.5</td>
</tr>
<tr>
<td>10 μg/kg once per day</td>
<td>61,163</td>
<td>22 ± 2.2</td>
<td>1.4 ± 0.8</td>
</tr>
</tbody>
</table>

We also determined that rhuGM-CSF induced a significant increase in the circulating ANC as early as day 2 at all dose levels tested and continued to increase throughout the 7 days of administration compared with placebo-treated VLBW neonates. After discontinuation of rhuGM-CSF, there was a relative sustained neutrophilia for 120 hours. Tibial bone marrow aspirates 1 day after the 7-day administration of rhuGM-CSF (5.0 μg/kg twice per day and 10.0 μg/kg once per day) demonstrated a significant (71%) increase in the BM NSP compared with placebo-controlled neonates. These results were similar to those demonstrated in our studies using rmGM-CSF in neonatal rats.14 Similar to our neonatal rat study, we also saw no significant increase in the bone marrow NPP after rmGM-CSF in VLBW neonates. However, the present study demonstrated at the higher dose levels a significant increase in the circulating absolute monocyte count after 4 days of rhuGM-CSF, which had not been previously shown in our neonatal rat studies.

Our previous studies using rhuG-CSF in human neonates with presumed sepsis did not demonstrate an increase in the circulating absolute monocyte or platelet count.24 In the present study, there was a significant increase in the circulating platelet count after 7 days of rhuGM-CSF (5.0 μg/kg twice per day). Although GM-CSF has not previously been demonstrated to be a potent cytokine for induction of megakaryocytopoiesis, previous in vivo studies using rmGM-CSF in mice and rhuGM-CSF in humans have suggested a possible indirect stimulatory effect on megakaryocytopoiesis.35,36 Intriguingly, children treated with higher doses of rhuGM-CSF (750 to 1,500 μg/m²/d) appeared to have earlier platelet reconstitution after myelosuppressive chemotherapy.32 The findings in our present studies suggest that the use of rhG-CSF in VLBW neonates may have a broad range of hematopoietic effects, including neutrophil maturation and production, monocyte production, and platelet production.

Besides altering the kinetics of neutrophil production in VLBW neonates, this study also demonstrated that at 5.0 μg/kg once per day, rhuGM-CSF enhanced neutrophil C3bi receptor expression and thereby possibly induced functional activation of mature effector neutrophils. Limited blood drawing prevented further analysis of the implications of this activation of neutrophils. These results are similar to those that previously demonstrated that rhuGM-CSF induces neutrophil C3bi receptor expression in adult patients.37 Increased PMN C3bi expression might enhance PMN adhesion, aggregation, and phagocytosis, either leading to increased antimicrobial activity or increased neutrophil sequestration, vascular viscosity, and local thrombotic complications.

In summary, the present study demonstrated that 7 days of administration of rhuGM-CSF appeared to be safe and well tolerated in VLBW neonates. Administration of rhuGM-CSF to this high-risk group of neonates appears to induce significant circulating and bone marrow neutrophilia and, at higher doses, induces a significant increase in the absolute monocyte and platelet counts. At higher doses, rhuGM-CSF also appears to induce the expression of surface receptors that are markers of activation. Two-hour intravenous administration of rhuGM-CSF in VLBW neonates appears to have a similar elimination half-life as in older children and induces peak serum levels at the end of the 2-hour infusion. Because GM-CSF expression and production during activated conditions are significantly decreased in cord versus adult mononuclear cells, we hypothesize that future strategies to reduce nosocomial infection in VLBW neonates may benefit from the prophylactic use of rhuGM-CSF. A multicenter, randomized, prospective, double-blind, placebo-controlled phase III trial is currently under way to test this hypothesis.

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REFERENCES


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